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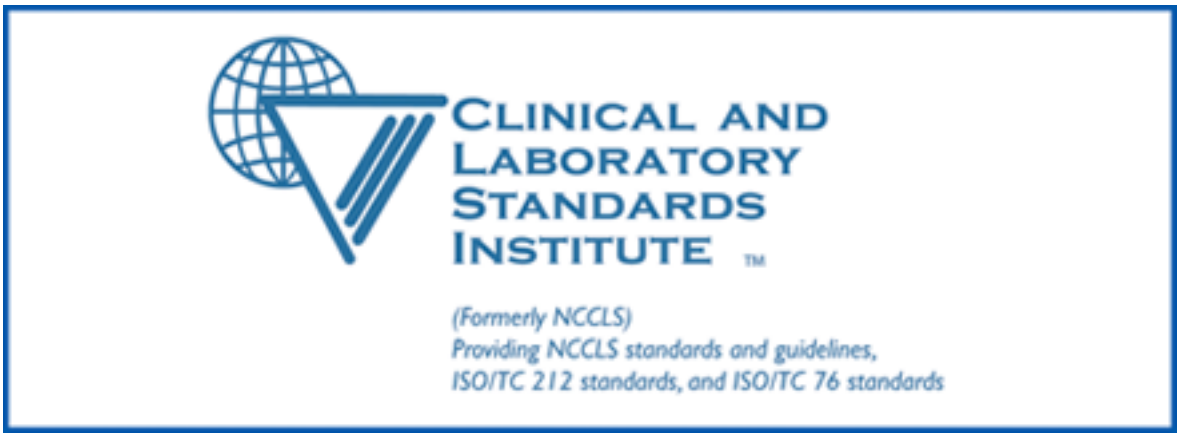
# Apolipoprotein Immunoassays: Development and Recommended Performance Characteristics; Approved Guideline




This document provides guidance for the characterization and preparation of immunogens, antibodies, samples, and methods, as well as for immunochemical testing of apolipoproteins.

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I/LA15-A
THIS NCCLS DOCUMENT HAS BEEN
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# Apolipoprotein Immunoassays: Development and Recommended Performance Characteristics; Approved Guideline

## Abstract

*Apolipoprotein Immunoassays: Development and Recommended Performance Characteristics; Approved Guideline* (NCCLS document I/LA15-A) addresses the characterization and preparation of immunogens and antibodies, immunochemical methods, and reference preparations. It is intended to improve the quantification of apolipoproteins by immunochemical means, and ultimately improve the diagnosis and management of many aspects of cardiovascular and peripheral vascular disease. The purpose of this guideline is to orient and assist the laboratorian, and the laboratory industry in general, with the needs and problems that exist in this rapidly growing area of laboratory medicine.

[NCCLS. *Apolipoprotein Immunoassays: Development and Recommended Performance Characteristics; Approved Guideline*. NCCLS document I/LA15-A (ISBN 1-56238-331-0). NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087 USA, 1997.]

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# Apolipoprotein Immunoassays: Development and Recommended Performance Characteristics; Approved Guideline

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## Contents

## Foreword

This guideline is intended for use by all clinical and reference laboratories performing immunochemical analyses of apolipoproteins and by the manufacturers of commercial kits.

Apolipoprotein is the protein component of lipoprotein and is comprised of a heterogeneous group of approximately 16 classes of apolipoproteins with distinct chemical, physical, and immunological characteristics. The unique lipoprotein, Lp(a), contains not only apoB-100 but also the polypeptide known as apo(a). Use of this guideline will promote a better understanding of the requirements, capabilities, and limitations of these extremely important diagnostic tests. Interlaboratory comparability and performance will be further enhanced.

Most people in developed countries are at increased risk for premature atherosclerosis as the result of varying combinations of inherited traits, inappropriate diet, smoking of tobacco, sedentary style of life, obesity, hypertension, diabetes, and other risk factors. Most of these factors have been shown to affect lipoprotein levels adversely and therefore to increase the risk of coronary artery disease (CAD) through the mechanism of disturbed lipid/lipoprotein metabolism. Quantitative analysis of human serum or plasma apolipoproteins represents a major research and clinical laboratory diagnostic effort to assess the risk factors associated with risk for CAD. This document sets forth accepted guidelines to assist the industry in reaching an optimal level of performance for the quantification of these analytes.

Document I/LA15-A directly addresses the measurement of the following apolipoproteins whose analyses have clinical utility:

- Apolipoprotein A-I (apoA-I)
- Apolipoprotein B-100 (apoB)
- Apolipoprotein (a) (Lp(a)).

**NOTE**: Although ApoB-100 and ApoB-48 are products of the same gene, for the purpose of this document, only apo-B-100 specificity will be considered. The term apoB-48 refers to the 2,515 amino acid polypeptides synthesized by the intestine,[1,2] by post-translational modification of ApoB-100 mRNA, and to the identical N-terminal (NH2) portion of apoB-100 synthesized by the hepatocyte. ApoB-100 is the crucial structural component of VLDL, LDL, and IDL particles and is found primarily in the circulation. It is recognized that an analytical distinction between apoB-48 and apoB-100 will eventually be desired; however, at this time the practical and clinical requirements focus attention on only the apoB-100 form.

Lp(a) is the lipoprotein containing the apolipoprotein apo(a) chemically bound to apoB. Apo(a) is the plasminogen-like apoprotein chemically bound to apoB to form the apolipoprotein of lipoprotein (a).

The additional analytes listed below are of interest and must be kept in perspective in this document; however, these apolipoprotein assays have not yet become a standard, integral part of clinical evaluation of the dyslipoproteinemias:

- Apolipoprotein A-II (apoA-II)
- Apolipoprotein A-IV (apoA-IV)
- Apolipoprotein C-II (apoC-II)
- Apolipoprotein C-III (apoC-III)
- Apolipoprotein E (apoE)
- Apolipoprotein B-48 (apoB-48).

The lipoproteins of mammalian serum and plasma have been convincingly shown to be associated with the development of atheromata, the complex process of inter- and extracellular lipid accumulation, fibrous tissue deposition and cellular proliferation, in close proximity to the lining of large, medium, and small arteries. Over the life span of an individual, accumulation of these deposits gradually narrows vessel diameter until a critical level is reached. This results in the clinical manifestations of angina pectoris, myocardial infarction, stroke, peripheral vascular insufficiency, and intermittent claudication. In some instances, roughening of the normally smooth endothelial lining may foster the attachment of platelets and clot formation. As a result of either or both of these processes, blood flow beyond the narrowing may be sufficiently decreased to induce tissue hypoxia or to halt blood flow altogether. A wide variety of other circumstances may contribute to the event which leads to hypoxia and possible necrosis of tissue beyond the occlusion. Examples include increased demand for blood flow due to exercise or stress or spasm resulting from exposure to stimulants, pollutants (such as are found in cigarette smoke), certain drugs, adrenergic stimuli, and perhaps even certain allergens.

Atherosclerotic cardiovascular heart disease is responsible for more than 40 percent of the deaths in the United States and in other Westernized countries. Often, the first clinical manifestation of coronary atherosclerosis is sudden death, precluding any opportunity to intervene therapeutically. Identifying patients at high risk prior to the onset of clinically apparent disease is thus the only practical approach to controlling this continuing epidemic. Numerous epidemiologic studies have shown that the following have predictive value for accelerated (early) atherosclerosis: high serum levels of total cholesterol,[3] high serum levels of LDL cholesterol, low serum levels of HDL cholesterol, hypoapolipoproteinemia A-I, hyperbetalipoproteinemia, hypertension, male gender, obesity, sedentary life style, cigarette smoking, diabetes mellitus, family history of early CAD, genetic traits manifesting as identifiable protein variants (apo(a), hyper apoB, apoE variants, etc.) and EKG abnormalities, particularly evidence of left ventricular hypertrophy.

Prospective, randomized clinical intervention trials have convincingly shown that reduction of hypercholesterolemia, cessation of cigarette smoking, and control of hypertension can slow or even reverse atherosclerosis in some individuals.[4] In general, the younger the individual exhibiting CAD the higher the incidence of detectable risk factors, especially evidence of familial lipid disorders. However, many patients with accelerated atherosclerosis do not exhibit traditional risk factors. Thus, there remains a pressing need to identify additional parameters which may select individuals at risk for early CAD. Several studies now indicate that measurement of serum levels of apoB-100 and apoA-I, respectively, may be better indicators of the presence or risk of CAD than is the quantification of total cholesterol, HDL cholesterol, or LDL cholesterol.[5,6,7,8,9] More recent evidence indicates that Lp(a) (concentration and/or phenotype) is another independent risk factor that can be examined practically.[10]

Several recent case-controlled studies suggest that serum apolipoprotein levels have higher predictive value than conventional lipid and lipoprotein measurements for the presence of CAD and are independent of serum lipid and lipoprotein levels.[11] In some case-controlled studies, the most common abnormality of lipid metabolism in survivors of myocardial infarction is a low level of apoA-I.[12] Elevated levels of apoB are also common in normolipidemic patients with early CAD even when total and LDL cholesterol levels are normal.[9,13] Hyperapolipoproteinemia B, with normal LDL cholesterol, has been noted in 50 percent of patients with angiographically documented CAD and in 80 percent of survivors of myocardial infarction.[13] Hyperapolipoproteinemia B is associated with small, dense LDL particles which are believed to result from excess synthesis of VLDL apoB in the liver; it may occur in patients with obesity, diabetes, or familial-combined hyperlipidemia, or as an isolated finding.[14,15] Thus, in addition to predicting CAD risk, measurement of apolipoproteins can identify patients with certain inheritable abnormalities of lipoprotein metabolism not identified by any other means.[16] Measurement of apolipoprotein B may be more relevant to the identification of CAD risk since the amount of apoB is relatively constant per LDL particle whereas the amount of cholesterol is variable.

Recent work strongly suggests that the genetically-controlled phenotypes and blood levels of Lp(a) correlate highly with the occurrence and severity of CAD in some ethnic groups.[17] In addition, from a qualitative point of view, determination of apoE phenotypes is helpful in establishing the diagnosis of type

III hyperlipoproteinemia (broad-beta-disease), which is associated with accelerated coronary and peripheral vascular disease.

While such inherited predispositions to disease are relatively uncommon, the predictive value of apolipoprotein levels is broad and could be used to screen large numbers of individuals. Thus, measurement of apolipoproteins is assuming an increasingly important role in identifying persons at risk for premature CAD [18]

Readily available automated instruments and reagent systems for quantification of apolipoproteins are continually improving in speed, precision, ease of operation, and cost effectiveness. For laboratory analysis to be adaptable to widespread use and eventually to population screening, this mode of analysis will have to reach a level of acceptance by clinicians and laboratorians akin to that for serum cholesterol testing. Now that instrument performance and reagent quality have reached acceptable levels, the gap remaining can be bridged by consensus guidelines and education.[3]

## Universal Precautions

Because it is often impossible to know which might be infectious, all patient blood specimens are to be treated with universal precautions. Guidelines for specimen handling are available from the U.S. Centers for Disease Control and Prevention [*MMWR* 1987;36(suppl 2S):2S–18S]. NCCLS document M29, *Protection of Laboratory Workers from Infectious Disease Transmitted by Blood, Body Fluids, and Tissue,* deals specifically with this issue.

## Key Words

Antibody, immunochemical specificity, apolipoprotein, lipoprotein, reference material, atherosclerosis, coronary artery disease (CAD), screening, genetic testing, immunoassay.

# Apolipoprotein Immunoassays: Development and Recommended Performance Characteristics; Approved Guideline

## 1 Scope

This document will address several issues which require particular attention. These include the following:

(1) Qualification and assessment of immunogen— Section 3.

(2) Polymorphism— Section 3.1.1.

(3) Antiserum specificity and titer— Sections 4, and 5.

(4) Storage conditions for patient samples— Section 6.3.

(5) Detergent requirements and effects—Section 6.5.

(6) Patient preparation— Section 7.

(7) Immunochemical methods— Section 8.

(8) Particle size effects— Section 8.1, and Section 8.3.

(9) Comparability of assay results among methods— Section 8.8.

(10) Storage conditions for reference materials— Section 9.

(11) Value assignment and transfer to reference materials— Section 9.

(12) Data management— Section 10 through Section 10.6.

(13) Interpretation of values— Section 12.

## 2 Definitions

These terms are specifically directed toward the subject of NCCLS document I/LA15-P.

**Absorb,** *n* - To remove one reactant by the addition of the other soluble reactant (termed neutralization). For example, the activity of an antibody may be neutralized by the addition of soluble antigen. See **Adsorb**.

**Accuracy,** *n* - The nearness of a measurement to its true value.

**Adsorb,** *n* - To collect on a surface, usually by a monomolecular layer of reactant. Reactants coated on the surface of materials such as plastic, glass, or particles (latex, bentonite, cellulose, etc.) are used for the removal of undesired antibodies or antigens.

**Affinity,** *n* - The term expresses the intrinsic binding strength of a receptor-ligand pair. In an immunoassay, the receptor is the antibody and the ligand is the analyte. Strictly speaking, the term only applies to homogeneous receptors and their ligands. Typically, however, polyclonal antibody preparations, which are heterogeneous in their affinity for homo-geneous ligands, are used and some ligands are not available in homogeneous form.

The association constant, Ka, for the immunochemical reaction:

Antibody + Antigen $K_a$ Antibody - Antigen

can be expressed mathematically as:

$$K_a = \frac{[\text{Antobody - Antigen}]}{[\text{Antibody}]\ [\text{Antigen}]}$$

Average Affinity Constant = $K_a$ = The average binding constant of a population of antibody molecules.

The affinity constant, therefore, is usually expressed as the equilibrium constant for the receptor-ligand reaction under defined conditions for defined reactants. See **Avidity**.

**Allele,** *n* - Interspecies variance at a particular gene locus.

**Allotype,** *n* - The genetically determined antigenic differences of serum or cellular proteins, varying in different members of the same species.

**Analyte,** *n* - The substance, set of substances, activity, or "factor" to be assayed.

**Antibody,** *n* - Proteins that are the functional components of antiserum, often referred to collectively as a population of molecules, each member of which is capable of reacting with a specific antigenic determinant. A single antibody molecule is, by definition, monospecific but might also be idiospecific, heterospecific, polyspecific, or of unwanted specificity. Antibodies are immunoglobulins and bind by means of specific binding sites to a specific antigenic determinant.

**Antigen,** *n* - Classically, a substance that will elicit the formation of antibodies in a suitable host (see *Immunogen)*. Alternatively, a substance that will combine with antibody through its antibody binding sites.

**Antigenic determinant,** *n* - That part of the structure of an antigen molecule that is responsible for specific interaction with antibody molecules evoked by the same or a similar antigen. A determinant can be determined either by primary composition or conformational relationship.

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

**Apolipoproteins,** *n* - The intrinsic protein components of lipoproteins; several different apolipoproteins have been identified, differing in structure, function, and genetic control.

**Atherosclerosis,** *n* - The clinical disorder during which lipid, complex carbohydrates, blood proteins, fibrous tissue and calcium are deposited in the intima of large vessels with resulting narrowing of the lumen.

**Avidity,** *n* - The combined intensity of reactivities of an antiserum or antibody population. In effect, it represents the net affinity of all binding sites of all antibodies in the antiserum, under specified physical- chemical reaction conditions. The avidity is a function of the affinities of the antibody- combining sites on all antibodies present in an antiserum and all the antigenic determinants of available macromolecules. Sometimes avidity can be expressed as an effective affinity constant. See **Affinity**.

**Bias,** *n* - A quantitative measure of inaccuracy or systematic departure from accuracy.

**Binding capacity,** *n* - The capacity of a receptor such as an antibody to bind a ligand such as an antigen, expressed in operational units, unlike the quantitative molar units of the affinity constant.

**Calibrator,** *n* - A material of identical reactivity to unknowns, used to establish a dose response curve. See **Reference Material**.

**Cholesterol,** *n* - 1) A sterol of the formula $C_{27}Hy_{46}0_7$, found in blood plasma bound to lipoproteins as well as in other body tissues; 2) ***HDL-Cholesterol*** (HDLC), The cholesterol content of the high-density lipoprotein fraction; 3) ***LDL-Cholesterol*** (LDLC),The cholesterol content of the low-density lipoprotein fraction.

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

**Consensus,** *n* - A cooperative process resulting in a value or statement that is reached by the majority of the participants. In practice, the value is the result of various degrees of selection and statistical manipulation. The value bears no relationship to accuracy or precision.

**Cross-Reactivity,** *n* - The reaction of an antibody with an antigen other than that which elicited the formation of the antibody, due to the presence of antigenic determinants similar to but not identical with the immunogen.

**Deamination,** *n* - The process of removal of amino groups (NH2) from amino acid residues.

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

**Determinant,** *n* - The definition of a specific characteristic, e.g., antigenic determinant. See **Epitope**.

**End-point immunoassay,** *n* - An immunoassay in which the signal is measured when the antigen antibody reaction has reached effective equilibrium, and in which any nonspecific component of the signal is assumed to be small and constant.

**ELISA,** *n* - (Enzyme-Linked Immunosorbent Assay). A heterogeneous enzyme immunoassay method in which an antigen or antibody is firmly attached to a solid support and the presence of the analyte is detected with an antibody or antigen-enzyme conjugate.

**Enzyme conjugate,** *n* - A material, antigen, or antibody that is bound covalently to an enzyme.

**Epitope,** *n* - The minimum molecular structure that will react with an antibody; may be only a portion of an antigenic determinant.

**Fluorescence,** *n* - The property of emitting electromagnetic radiation as a result of absorption of radiation from an outside source. The radiation, emitted in all directions, persists only very briefly (approximately $10^{-8}$ seconds) after the outside light source is removed. Fluorescent radiation generally has a longer wavelength than the absorbed radiation.

**Fluorescence immunoassay,** *n* - (FIA). A generic term for an immunoassay in which the analyte content of the sample is measured by the amount of fluorescence from bound antibody or antigen. Immunoassays using a fluorogenic enzyme substrate (e.g., methyl-umbelliferone phosphate) can also be classified as fluorescence immunoassays.

**Genotype,** *n* - The specific allelic composition of a gene or set of genes established at the DNA level. See **Phenotype.**

**Heterogeneous immunoassay,** *n* - 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.

**Homogeneous immunoassay,** *n* - An immunoassay in which no separation step is performed. The specific activity of the label is modulated according to the analyte content of the sample.

**Hybridoma,** *n* - A cell line derived from the fusion between a B cell and a plasmacytoma cell.

**Hydrophilic,** *n* - Having a high affinity for water (e.g., sodium chloride).

**Hydrophobic,** *n* - Having a low affinity for water (e.g., triglyceride).

**Hypercholesterolemia,** *n* - The condition in which plasma/serum levels of cholesterol are elevated beyond a defined level.

**Immunoassay,** *n* - A ligand-binding assay that uses a specific antigen or antibody capable of binding to the analyte.

**Immunogen,** *n* - A substance that elicits a cellular/humoral immune response and the production of antibody. See **Antigen.**

**Immunoprecipitin analysis,** *n* - Any procedure that relies on formation of a precipitate of antibody and antigen. Immunoprecipitin analysis includes single radial immunodiffusion, double diffusion (Ouchterlony), immuno-electrophoresis, immunofixation, two-dimensional-immunoelectrophoresis, the multiple forms of electroimmunodiffusion, end-point and rate nephelometry, and turbidimetry, as well as the direct stoichiometric analysis of the precipitate formed.

**Imprecision,** *n* - The random dispersion of a set of replicate measurements and/or values expressed, quantitatively by a statistic, such as standard deviation or coefficient of variation.

**Isoform,** *n* - One of several forms of a protein having the same antigenic structure. Three-dimensional structure can affect how isotypes are viewed by monoclonal antibodies.

**Label,** *n* - An easily detected substance that is attached to a reagent in an immunoassay. The assay signal is either a measurable property of the label or is produced by the label. In EIA the label is the enzyme; in FIA the label is the fluorophore; in RIA the label is the radionuclide.

**Ligand,** *n* - A substance that can be bound by a receptor. See **Affinity.**

**Lipoprotein,** *n* - A family of particles combining water-insoluble lipid (cholesterol, cholesterol ester, triglyceride, phospholipid) and protein which allows their dissolution in plasma. Lipid content may vary in terms of concentration and composition between particles within a specific group (e.g., LDL) over time, within an individual, and between individuals.

**Matrix,** *n* - The milieux of the sample (e.g., serum, containing the analyte). The matrix can influence the behavior of an immunoassay due to specific (direct) and nonspecific (indirect) interferences.

**Monoclonal,** *n* - Arising from a single clone of cells; in the case of immunoglobulin, the term refers to the cell of origin. The monoclonal antibody is of a single immunoglobulin class containing only one light chain type of either the kappa or lambda variety. All molecules have identical physical-chemical characteristics and antibody specificity. Monoclonal anti-bodies have very restricted structural diversity and are homogeneous, unlike polyclonal antibodies.

**Monospecificity,** *n* - Functionally defined, the immunoreactivity of an antiserum with only its designated antigen (e.g., antihuman IgG, antihuman IgG Fc piece, antihuman IgG3 Fc piece, etc). In practice, true monospecificity to naturally occurring antigens is not seen in antisera produced by the immunization of an intact animal.

**Nonspecificity,** *n* - The quality of an antiserum that possesses reactivity (not necessarily immunological) different from and/or in addition to that which is desired (i.e., unwanted specificity).

**Phenotype,** *n* - The expression of genetic makeup as applied to a single characteristic. The outward manifestation of genotype, with only the expressed characteristics designated.

**Polyclonal,** *n* - Antibodies arising from different clones of B cells, and having affinity for different epitopes on the same antigen and/or different antigens. A typical antiserum obtained from conventional immunization of an intact animal is polyclonal.

**Potency,** *n* - The characteristic of an antibody representing the concentration (titer) of antibody and the avidity for a given substrate (antigen) in a defined method. Also, the characteristic of an antigen solution (calibrator) representing the concentration of the antigen in a defined method.

**Precision,** *n* - The extent to which replicate analyses of a sample agree with each other; usually expressed as imprecision (cf. the co-efficient of variation (CV) of a population of values to the standard deviation divided by the mean).

**Primary matrix reference material,** *n* - A material whose values have been assigned by value transfer from primary purified reference materials, and which may be used to assign values to secondary matrix reference materials.

**Primary purified reference material,** *n* - A single moiety purified according to the latest scientific knowledge and methods. The extent of contamination and denaturation should be described.

**Rate immunoassay,** *n* - An immunoassay in which the rate of change in signal is monitored by taking at least two measurements within a defined period.

**Reactivity,** *n* - The qualitative characteristic of an antigen or antibody describing its binding with another substance.

**Recombinant proteins,** *n* - Proteins produced by cells combining genetic materials from dissimilar lines.

**Recovery,** *n* - The fraction of added analyte measured by the assay (does not imply physical recovery of the material that constitutes the analyte).

**Reference cutoff,** *n* - A value from a given reference distribution, which represents a clinical decision point (e.g., below the 10th percentile or above the 95th percentile).

**Reference distribution,** *n* - A quantitative description of measurements for a reference population, often expressed as a relative frequency distribution of observed values.

**Reference interval,** *n* - A lower and upper value from a given reference distribution, between whicha specified proportion of the reference population is found. The 95th percentile prediction limits (2.5 to 97.5 percentiles) are often used.

**Reference material,** *n* - A substance used to assign values to materials of lower order.

**Reference population,** *n* - A group of N individuals in a described state of health or disease.

**Reference preparation**. See **Reference Material.**

**Response,** *n* - The extent to which an assay signal is modulated by the analyte.

**Secondary matrix reference material,** *n* - A reference material whose values have been assigned by a formal process of value transfer from a primary reference material, and which is to be used to assign values to tertiary reference materials.

**Sensitivity,** *n* - In quantitative testing, change in response of a measuring instrument divided by the corresponding change in the stimulus. In qualitative testing, the test method's ability to obtain positive results in concordance with positive results obtained by the reference method. In a clinical context, sensitivity is 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).

**Signal,** *n* - The measured response of the assay system to the analyte.

**Specific activity,** *n* - In immunoassay, detectable events per unit mass.

**Specificity,** *n* - An antiserum quality defining its reactivity with defined antigens. In a chemical context, the extent to which the assay responds only to (all subsets of) a specified analyte and not to other substances present in the sample. In a clinical context, the percent test negativity in the absence of disease (or specified clinical condition).

**Standard,** *n* - A material against which other materials can be measured (syn., reference material/preparation). Or, (preferred) an authoritative document setting forth criteria for performance and characteristics.

**Titer,** *n* - The reciprocal of the dilution factor required to produce a defined outcome in a defined system. In a defined system, it is usually proportional to the analyte concentration.

**Value transfer,** *n* - A formal protocol for the assignment of analyte values to one reference material from a reference material of higher order.

## 3 Qualification and Assessment of Immunogens

The preparation of immunogens represents the first step in antiserum preparation. The source of materials must be selected to ensure that idiotypic and allotypic specificity does not interfere with antiserum performance in clinical testing. Therefore, all known immunogenic subsets must be included in both the immunogen source material and in the library of human samples which makes up the representative population sample. In the event that synthetic immunogens are to be used, it is critical that the synthetic epitope represent a conserved epitope and that phenotypic variations do not affect the performance of the assay.

### 3.1 Isolated Naturally Occurring Immunogens

Several types of in vitro modifications have been observed with serum apolipoprotein A-I (and apoA-I containing particles) stored under alkaline conditions.[19,20,21] These modifications, which can include deamidation of asparagine and glutamine, can interfere with the binding of specific anti apoA-I monoclonal antibodies.[22] It has been reported that certain monoclonal antibodies bind preferentially to the modified epitopes that have resulted from deamidation of apoA-I.[19,20,23] There are also monoclonal antibodies that react specifically with oxidized LDL.[24,25] It is also known that apoB-100 degrades with storage, freezing, and lyophilization with potential loss of antigenic epitopes which may be relevant to the reaction of monoclonal antibodies. In addition, purified apoB-100 aggregates irreversibly with loss of exposed epitopes,[26] and apoA-I has proven to be subject to a variety of processing variables not fully understood at present.

#### 3.1.1 Phenotypic Variants

The existence of inherited variants of most, if not all, apolipoproteins has been documented. With some, such as apoE, DNA base substitutions (mutations) have been shown to result in specific amino acid substitutions, and secondarily, altered isoelectric focusing, DNA restriction fragment polymorphism (RFLP) analysis, or both. With other variants molecular size is altered, secondary to amino acid substitutions or to post-translational proteolysis.

In the case of Lp(a), the molecular size is primarily the result of variation in the number of DNA-encoded "kringles" [loops in the protein primary sequence (backbone) similar to those of plasminogen] for the (a) segment.[27,28]

In each case, ability to stimulate antibody production or reactivity with antisera may differ from one variant to another, particularly when monoclonal antibodies are used. Indeed, monoclonal antibodies which react with a polymorphic epitope on a protein may not recognize some variants at all. In addition, polymorphism-dependent antigen size effects may be seen in immunodiffusion assays (e.g., RID) or in immunonephelometry/turbidimetry.

In the case of Lp(a), antibodies must not react with plasminogen. Likewise, polyclonal and monoclonal antibodies to apoB-100 must react with epitopic determinants in the carboxyl-terminal portion of the molecule and not the N-terminal portion which is identical in both apoB-100 and apoB-48.[29]

Antisera ideally should be tested for reactivity with all known polymorphic variants — those which have gene frequencies $\geq$ 0.01 — and any discrepancies noted in package inserts. In addition, potential problems with molecular size differences should be discussed in detail.

### 3.2 Synthetic Immunogens

Recombinant DNA technology has made it possible to clone DNA: a specific DNA fragment is integrated into a virus or plasmid so that it can be amplified in bacteria or yeasts (eucaryotic cells). Recently, this technology has been used to synthesize recombinant apolipoproteins that are representative of the sequence of the native protein. These recombinant proteins can be used as immunogens for the stimulation of polyclonal or monoclonal antibodies.[29] Such products might also be used as reference materials if they prove to have identical reactivity to that of the native apolipoprotein.

### 3.3 Source of Immunogens

All polymorphic variants (i.e., phenotypes) should be included in the immunogen, when appropriate, to prevent bias in antibody specificity.[30,31] The source of the immunogen may favor one or another genetic subset if it does not represent the matrix toward which the routine use of the antibody is directed. The intended use of antibodies raised against such an immunogen must be clearly stated so as to avoid misuse in the clinical testing environment.

### 3.4 Native Versus Delipidated Immunogens

In most cases, except normal LDL, lipoprotein particles within a specific density range (e.g., 1.006-1.063),[32] bear more than one apoprotein. Producing an antibody to a single apolipoprotein requires further purification processes, often including delipidation. The epitopes exhibited by the apolipoprotein may vary with the amount of associated lipid.

## 4 Qualification and Assessment of the Immunochemical Specificity of Antibody— (Monoclonal and Polyclonal)

### 4.1 Proof of Antiserum Specificity — Qualitative Assessment

#### 4.1.1 Methods

Assessment of the specificity of polyclonal antisera should be performed by sensitive methods, such as crossed two-dimensional immunoelectrophoresis[33,34] or immunoblotting.[35,36] The use of less sensitive and less precise methods, such as immunoelectrophoresis and immunodiffusion (Ouchterlony), is to be discouraged.

Monoclonal antibodies should be tested for specificity by competitive inhibition techniques, using radioimmunoassay, enzyme immunoassay,[37] or immunoblotting. The binding properties of the selected antibodies should be well characterized to be sure that the epitope(s) reacting in a particular assay are expressed on all particles of a given type in all samples.

Antigen sources should include fresh serum, plasma, and freshly purified apolipoproteins when such can be shown to be stable and to approximate closely the native state. When cross-reactivities are suspected or are known to exist, the appropriate antigen-containing samples must be included; for example, polyclonal or monoclonal antisera must be tested for

reactivity to plasminogen (see Section 3.1.1). In addition, intact and denatured antigens should be tested in many cases. For example, deamidation of apolipoprotein A-I occurs spontaneously in direct relation to the temperature and duration of storage. Therefore, monoclonal, and perhaps polyclonal, antisera to apoA-I should be tested against fresh ("native") and stored samples (see Section 6.3).

#### 4.1.2 Contaminant Detection Levels

Several concentrations of each antigen source should be tested against serial dilutions of antiserum in order to avoid antigen or antibody excess. In crossed immunoelectrophoresis, gels should be allowed to incubate for at least 15 to 30 minutes following the second electrophoresis (into the antibody-containing gel) to permit further diffusion of antibodies, thus increasing the size and intensity of immunoprecipitates.[33,34] If for any reason traditional immunoelectrophoresis is used, diffusion should proceed for a minimum of 18 hours at room temperature. Although thorough washing to remove the nonreactive proteins is essential, excessive washing and/or the use of acidic washing solutions (such as unbuffered saline) may dissociate some immune complexes, with the loss of the more soluble precipitates.

Sensitive fixation and staining methods are essential; suggested methods include perchloric acid or trichloric acid-sulfosalicylic acid fixation followed by silver, colloidal gold, or Coomassie Brilliant Blue R250 or G250 stains. Ponceau S, amido black, fast green, and similar stains are not acceptable, nor is indirect illumination under dark-field conditions.

Contaminant antibodies or erroneous specificities are often totally undetectable in nephelometric and turbidimetric assays. Therefore, monospecificity should be documented by two-dimensional crossed antigen-antibody electrophoresis or similarly sensitive techniques. In practice, low concentrations of contaminating antibodies rarely cause difficulty in these procedures; however, they can contribute to bias, or imprecision, or both, in many quantitative methods, for example in immunonephelometry, immunoturbidimetry, immunofluorescence and ELISA, and can produce confusing patterns in RID and "rocket" electrophoresis.

#### 4.1.3 Materials

Antigen-containing solutions should also vary to cover the range of potential samples to be tested (e.g., serum, plasma, urine, and cerebrospinal fluid) and should be used at several dilutions (e.g., 1:2, 1:4, etc.).

#### 4.1.4 Nonspecific Precipitin Reactions

The Following is a list of major plasma proteins that can potentially contribute to nonspecific precipitin reactions with antisera because of their relatively high concentrations in analytical samples:

- Albumin
- $\alpha_1$-Antitrypsin
- $\alpha_2$-HS Glycoprotein
- $\alpha_2$-Macroglobulin
- Antithrombin III
- C-reactive protein
- Ceruloplasmin
- C1(q,r,s)
- C3
- C4
- C5
- Fibrinogen
- Fibronectin
- Haptoglobin
- Hemoglobin A
- Hemoglobin F
- Hemopexin
- IgA
- IgD
- IgG
- IgM
- Ig light chains
- Orosomucoid (∝, -Acid Glycoprotein)
- Plasminogen
- Prealbumin (Transthyretin)
- SP3
- Transferrin

#### 4.1.5 Apolipoprotein Cross-Reactivity

Apart from the desired specificity, the absence of cross-reactivity with the following proteins must be demonstrated in all cases:

- apoA-I
- apoA-II
- apoB-48
- apoB-100
- apoC-II
- apoC-III

- apoD
- apoE
- apo(a)
- Plasmin/Plasminogen

### 4.1.6 Permissible Phenomena

The following phenomena do not constitute nonspecificity in an antiserum to be used for immunochemical analysis, and are considered permissible.

#### 4.1.6.1 Cross-Species Reactivity

The fact that a reagent against a protein from one animal species cross-reacts with its analog in another species is anticipated and may not be considered a deterrent. Although speci-ficities for a single animal species can be produced in some cases, cross-reactivity is common.

#### 4.1.6.2 Precipitation of Plasma

The formation of a precipitate or the clotting of plasma samples by an antiserum does not indicate nonspecificity from an immuno-chemical standpoint. However, the manufacturer should so indicate if the possibility of such precipitation may interfere with use of the antiserum in assays of plasma samples, particularly with immunoturbidimetry or nephelometry.

#### 4.1.6.3 Removal of Contaminating Antibodies —Absorption/Adsorption

The great majority of polyclonal antisera are absorbed or adsorbed with serum or other body fluids to remove unwanted antibodies. Two methods are in common use: Absorption by the addition of soluble antigen insolution and Adsorption by the exposure of the antiserum preparation to antigen immobilized on a solid support.

As a result of Absorption, free antigen or soluble antigen-antibody complexes may remain in solution and may react with other antisera in many gel immunodiffusion techniques. This does not constitute nonspecificity of the antiserum; however, it is recommended that solid-phase Adsorption be used to minimize this problem. The manufacturer should so indicate if liquid absorption is used and warn against the problems related to the use of the antiserum in gel diffusion (see Section 11.3).

#### 4.1.6.4 Use of Agents that Enhance Im-munoprecipitation

Although nonimmunological precipitation of antisera may occur with the use of polyethylene glycol (PEG) or other similar enhancing agents, it is undesirable that such precipitates occur unpredictably. In as much as enhancing agents, principally PEG, are frequently used in immunoassays, this charac-teristic of an antiserum must be addressed and documented by the manufacturer.

#### 4.1.6.5 Unusual Assay Conditions

Certain forms of support media, particularly the highly purified agaroses, may not be conducive to some immunoprecipitin reactions in the presence of unusual conditions of pH or ionic strength. Consequently, the user or manufacturer should pretest such support media and recommended buffers with the antisera in question.

#### 4.1.6.6 Species of Antiserum Production

The species in which the antiserum was produced must be specified (see Section 4.3.4.).

## 4.2 Monoclonal Antibodies

Monoclonal antibodies (Mabs) can be powerful immunodiagnostic tools. However, these anti-bodies must be selected and characterized to satisfy specific and defined assay parameters. Some Mabs may be sensitive to detergents, to changes in pH and/or ionic strength of the buffers, to the temperature of the immunological reaction, and to the presence of other constituents in aqueous media.

To generate Mabs of high affinity, careful attention must be directed to the route and dose of immunogen. It is desirable to screen for hybridomas producing IgG antibodies. Hybridomas secreting IgM antibodies should be rejected.

The specificity of the Mabs must be determined. Lack of specificity may on occasion be attributed to the presence of contaminants in the immunogen and/or to inadequate screening. However, totally unexpected cross-reactivities with apparently unrelated molecules have been detected with Mabs to several proteins.

**NOTE:** Recently, a series of proteins that cross react with apolipoprotein S have been identified.[9] Moreover, several apoproteins (A-I, A-II, C-I, C-II, C-III and E) appear to be derived from a common ancestral gene[37] and have, as a result, considerable secondary structural homology. The formation of amphipathic helical structures is a common characteristic of apolipoprotein and may lead to similar epitopic expression. These considerations reinforce the need for stringent attention to antibody specificity.

The stringent criterion for Mab specificity to apolipoproteins is to verify that the antibodies react with the desired apolipoprotein or lipoproteins, and do not react with other purified apolipoproteins or other plasma proteins.

The most accurate test for specificity of Mab is the one- or two-dimensional immunoblotting technique.[38] To document the absence of cross-reactivity with other lipoproteins, apolipoproteins and plasma proteins, the Mab in question should be tested by immunoblotting against apolipoproteins, isolated lipoproteins, and whole plasma. In addition, Mabs directed specifically to apoB-100 should be tested for immunoreactivity with isolated apoB-48.

## 4.3 Antiserum Preparation

### 4.3.1 Fibrinogen Removal

Antiserum containing clottable fibrinogen will produce unanticipated precipitates upon mixing with serum or plasma samples. No antiserum should contain clottable fibrinogen.

### 4.3.2 Clarification of Antiserum

Whenever possible, antisera should be clarified by moderate speed centrifugation, ultra-filtration, and removal of large light-scattering lipoprotein particles and cryoproteins. Immuno-globulin fractionation is preferred, whenever feasible.

### 4.3.3 Removal of Antigen-Antibody Complexes

As noted under Section 4.1.6.3, antisera Absorbed or, to a lesser degree Adsorbed, with other proteins may contain soluble antigen-antibody complexes. These may persist even after vigorous centrifugation and filtration, only to precipitate upon their introduction into a medium containing agents which enhance immunoprecipitation, such as PEG. These complexes should be removed from all antisera and MUST be removed from products specifically developed for nephelometric and turbidimetric techniques. Alternatively, the possibility of nonspecific precipitates should be mentioned in package inserts, along with recommendations for further processing by the user.

Relevant methods for removal of antigen-antibody complexes include water dialysis, precipitation of antisera with 4 to 6 percent PEG 8000, immunoglobulin purification after Ab/Adsorption, or a combination of these. It is advisable that laboratorians test antisera for nonspecific precipitation in their own systems, whether immune complexes have been removed or not.

### 4.3.4 Reverse Reactions with Polyclonal Antisera

Reverse immunological reactions may occur when the antigen samples contain human antibodies to proteins present in the animal antiserum. For example, low affinity antibodies to ruminant IgM or milk proteins are frequently seen in sera from individuals with inherited deficiency of IgA.

Human antibodies to mouse Fab or Fc, bovine serum albumin, and other proteins have also been described. In addition, human rheumatoid factor may react with immunoglobulins from other species, and while this may be unavoidable, its occurrence must be assessed and documented. Fab fragments of antiserum have been used in some instances to circumvent the problem.

### 4.3.5 Pigment Interferences

Antiserum should not contain significant amounts of potentially interfering substances such as hemoglobin or bile pigments. In certain instances, pigments may be introduced to facilitate antiserum identification or to trigger certain mechanized analyses. In these cases, the package insert should specify the applications for which the antisera are not recommended and/or list the absorbing wavelengths of the substances added.

#### 4.3.6 Processing of Monoclonal Antisera

It is desirable to purify immunoglobulins from ascites fluids to remove all irrelevant proteins. Several methods for purification have been used, including ammonium or sodium sulphate precipitation, chromatography on DEAE cellulose, and high-performance chromatography. Of these, the latter is the preferred method.[39]

### 4.4 Quantitation of Nonspecificity, Undesirable Specificity, or Cross- Reactivity

All assays do not require the same degree of specificity. For example, the fact that an antiserum to apoA-I shows low titer and low affinity anti-apo(a) activity by radioimmunoassay makes it unsuitable for RIA, ELISA, and immunoblotting, but not for immunoprecipitin assays, including immunoturbidimetry or nephelometry.

In the case of antisera to the apolipoproteins, the manufacturer must insure that nonspecificity does not create a problem in all assays for which the antisera are intended and indicate that this is the case. The manufacturer should also indicate that the antisera are inappropriate for familiar methods in which nonspecific reactions may be seen.

## 5 Quantification of Antibody Titer

Methods of quantitative analysis: Quantitative specifications should be given for antisera to be used in immunochemical assays using an established technique. For example, antisera to be used in immunoprecipitin methods may be assigned Becker numbers[40] and antisera for RIA, ELISA, or FIA should have working dilutions.[41] The original (i.e., undiluted) antiserum should be high titer, since a satisfactory balance between clarity and titer may require substantial dilutions (e.g., greater than 1 to 30).[42] Performance in one class or type of assay cannot be compared to that in another system; titer specifications should be given for all recommended procedures (see Section 4.4).

## 6 Sample Handling and Preparation

### 6.1 Serum

The steps for handling and preparation of serum are as follows:

(1) Collect blood without prolonged application of a tourniquet, or after release of the tourniquet, into an evacuated red-top glass tube without anticoagulant (see NCCLS publication H3, *Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture).*

(2) Allow the sample to stand at room temperature for 30 to 45 minutes or until the clot has retracted.

Serum samples can be taken directly from above the retracted clot after a brief centrifugation. Prompt analysis is recommended (see Section 6.3).

### 6.2 Plasma

It should be noted that serum and plasma values are not equivalent. Blood anticoagulated by EDTA solutions (concentrations of 0.1 percent and 0.15 percent) result in values approximately 3.2 percent and 4.7 percent higher, respectively, than corresponding serum obtained during the same phlebotomy.[43]

### 6.3 Sample Storage

Analyses of apolipoproteins can be significantly affected by storage conditions, and it is recommended that tests be performed as soon as possible after separation. Light scattering techniques appear to be more sensitive than RID to length and temperature of storage and to freezing.[44] However, specimens usually may be held for up to four days at 0 to 4 °C for delayed analysis of apolipoproteins.

Samples for apoA-I testing may be stored frozen at -20 °C for no more than six months or at -70 °C for much longer, perhaps several years.[44] At -20 °C apoA-I will experience a 20 percent decrease in concentration.[45] ApoB levels appear to be stable at 4 °C for at least two weeks, but fall with freeze/thaw cycles, and significant changes can be detected even with a single cycle. Apo(a) appears to be markedly affected by storage conditions when quantified by RID but not by ELISA. While the recommendation that satisfactory analysis after one or two freeze/thaw cycles seems reasonable at this time, there is little published information regarding the stability of the other

apolipoproteins.[45] Therefore, freezing should be avoided if possible.

### 6.4 Interfering Substances

Patient samples received for analysis of lipids, lipoproteins, and apolipoproteins often present the analyst with a serum or plasma containing high concentrations of moieties that interfere with the analysis either directly, as the result of analyte levels being outside the dynamic range of an assay, or indirectly by introducing materials that confound the system of analysis. For example, turbid samples may render optical systems incapable of recovering a valid result; chylous samples may coat the inside surfaces of a pipetting system or cuvettes with material that prevents analysis of that sample or subsequent samples, and extremely chylous samples may introduce a volume occupying space that make interpretation of results difficult. Chylomicrons can usually be removed by storage overnight at 0 °C (on ice) and removal and discarding of the triglyceride (lipid) layer; if samples are still turbid, high speed centrifugation may be used. The loss of apolipoproteins by either of these procedures is trivial. In samples with extreme turbidity, alternative assay methods, such as RIA or ELISA, may be used. In rare instances, samples cannot be given realistic values by any method and values can only be recovered with the understanding that the analytic result cannot be placed in reference to a population.

### 6.5 Detergents

Accuracy in many assays for apolipoproteins requires the use of a detergent to reduce particle-size effects secondary to varying lipid content of the lipoprotein particles, to reduce background turbidity, to expose more antigenic determinants, or combinations of these. Some detergents, however, reduce the reactivity of either the antibodies or the antigen. Therefore, the effects of each specific detergent must be carefully evaluated in each test system and over the full reference range for the assay. If detergents are thought to be unnecessary, this should be confirmed by comparison of assays with and without detergent on a wide variety of fresh patient samples. This is true for both monoclonal and polyclonal antibodies.

## 7 Patient Preparation and Sampling

Success in obtaining accurate and interpretable results begins with patient sampling. Multiple specimens should be taken at least one week apart on an individual to reduce the short term biological variations normally experienced. Several controllable factors have been shown to affect lipid and lipoprotein levels. Some of these factors are shown below.

### 7.1 General Categories and Comments

#### 7.1.1 Position

Individuals that have been in the standing position may have serum analyte levels 4 to 10 percent higher than those of sitting or recumbent individuals.[46,47]

#### 7.1.2 Protracted Application of the Tourniquet

Prolonged venous stasis may increase apparent concentrations as much as 10 percent.

#### 7.1.3 Cigarette Smoking

Individuals who smoke have apoA-I levels lower than nonsmokers.[47]

#### 7.1.4 Alcohol Ingestion

Moderate alcohol ingestion may increase apoA-I levels.

#### 7.1.5 Duration of Fast Prior to Phlebotomy

While the biological variation in total cholesterol may be as much as 10 percent, much greater variation may occur in individual apolipoproteins between fasting and postprandial levels.[48]

#### 7.1.6 Concurrent Illness

Therapy and stress of active disease may affect apolipoprotein levels.

#### 7.1.7 Recent Weight Gain or Loss

Changes in weight due to dietary alteration or exercise may affect apolipoprotein levels.

### 7.1.8 Medications

Medications such as diuretics, beta-blockers, glucocorticoids, and gonadal steroids may affect results. The most commonly encountered medication in this category is the family of oral contraceptives which may elevate levels of apoA-I and apoB-100. The LDL cholesterol concentration tends to increase in oral contraceptive-treated women, but is slightly lower than the control post-menopausal women treated with estrogen alone.[49]

## 7.2 Specific Recommendations

The purpose of these analyses is to test the individual at a time that most accurately reflects a usual metabolic state and to minimize preanalytical effects (see Section 7.1). To introduce long-term prohibitions that are not characteristic of the individual's normal life style is considered artificial. There is one practical consideration which should be mentioned under this subject: The overnight fast reduces turbidity from chylomicrons, the presence of which adversely affects assay precision. It is advised, therefore, that the following conditions for obtaining patient samples be adopted:

(1) At least a 12- to 14-hour fast with nothing by mouth other than water, coffee, and essential medications. (Reasons relating to the technical feature of certain assays, e.g., sample clarity, are the overriding considerations in this recommendation). The meal preceding the fast should be of the customary diet for the subject.

(2) Minimal ingestion of alcohol during the 12 to 14 hours before phlebotomy.

(3) Sampling after subject is seated for at least 10 minutes.

(4) No cigarettes within one hour prior to phlebotomy.

(5) No interruption of medication programs. The type and duration of therapy must be indicated so that interpretation can be appropriately modified.

(6) Stable weight (+/- 4 lbs.) for preceding three weeks.

(7) No major illness or trauma within the preceding three months (e.g., myocardial infarction, stroke, major surgery, systemic infection, etc).

(8) No vigorous exercise for at least six hours before venipuncture.

## 7.3 Repeat Patient Sampling

Since both analytical and biological factors can contribute to result variability, repeated determinations of apolipoproteins should be made to reflect the true blood concentration under stable metabolic conditions of defined state of health. Repeat or confirmatory testing is essential in order to minimize misclassification of an individual and to document changes during intervention by diet or medication. At least two measurements at intervals of one to eight weeks are recommended, with averaging of the values obtained.

## 7.4 Population Screening

Apolipoprotein testing should not, for the present time, replace the measurement of total cholesterol, LDL-cholesterol and HDL-cholesterol, but rather should be used selectively in addition to these conventional laboratory tests. In the pediatric and adolescent age groups only children from high-risk families (i.e., family history of premature CAD before age 55 or a family history of hyperlipidemia) should be further evaluated by apolipoprotein measurement[50,51] when routine lipid measurements described above fail to yield a satisfactory diagnosis. If family history is positive, the traditional assays can make a diagnosis only if total cholesterol or LDL-C is massively elevated. There is a great perceived need for prospective studies to resolve the issue of incorporating apolipoprotein testing into coronary risk screening.

# 8 Immunochemical Methods

Only a few methods warrant specific attention because of their sufficiently widespread use: the major quantitative gel techniques, radial immunodiffusion (RID) and electroimmunoassay (EIA); two quantitative liquid-phase techniques— turbidimetry and nephelometry; and three solid-phase techniques— enzyme-linked immunosorbant assay (ELISA), radioimmuno-

assay (RIA), and fluorescence immunoassay (FIA).

Matrix effects are seen with all immunoassay techniques.[32] As a result, lyophilized standards and controls may behave differently from frozen or fresh patient samples. Such effects must be tested for each analyte in every immunoassay system. Manufacturers are urged to optimize materials so as to minimize matrix differences between calibrators, controls, survey materials, and patient samples.

### 8.1 Radial Immunodiffusion (RID)

The size of the immunoprecipitin ring is highly dependent on the effective particle/molecular size of the antigen (aggregate formation, as with the lipoproteins; molecular weight; conformation). Several manufacturers incorporate detergents in precast gels for apolipoproteins in order to disrupt lipoprotein particles and thereby reduce the size differences. However, molecular size differences of the apolipoprotein itself may also affect the rate of diffusion and apparent concentration. For example, high-molecular weight variants of apo(a) give relatively lower concentrations by RID, compared to ELISA or related techniques which are not size dependent.

The time, temperature, and humidity used for such assays must be appropriate to permit complete disaggregation and diffusion. The original Mancini technique which allows diffusion to proceed to completion over three to five days is recommended over the short, overnight incubation commonly employed. The latter is more affected by particle size variation.

### 8.2 Electroimmunoassay (EIA) (Laurell "Rocket Electrophoresis")[34]

EIA is affected by molecular size and configuration, but to a much lesser extent than is the case for RID. However, the use of high electromotive force to "drive" the antigens into the gels can result in clogging of the gel pores by samples with relatively large lipoprotein particles (VLDL and chylomicrons). Low voltages (and amperages) should therefore be used for the initial 15 to 20 minutes. The effects of detergents in the gels and/or in sample diluents must be evaluated for each assay performed by EIA.

### 8.3 Light-Scattering Immunoassays (Liquid Immunoprecipitan Assays)

Particle size also affects nephelometric and turbidimetric assays.[52] For example, VLDL -apoB may be overestimated in comparison to LDL-apoB in turbidimetric and fixed-point nephelometry assays, depending upon the angle of measurement. The reverse may be the case in rate-nephelometry[52] (see Section 6.5). In addition, relatively unexposed antigenic epitopes may react too slowly with specific antibody during the short reaction times used in these assays.[53] Several additives, including detergents, urea, guanidine, lipases, and the like, have been used to disrupt particles and to expose antigenic epitopes.[53,54] Lipemic sera present serious problems in light-scattering techniques (see Section 6.5).

### 8.4 Enzyme-Linked Immunosorbant Assay (ELISA)

The currently employed form of ELISA resembles conventional RIA.[12] This technique depends upon the reliable conjugation of an antibody with an enzyme. The stability of enzyme conjugates is now excellent, and their manufacture poses only minimal problems. In practice, most commercial manufacturers favor horseradish peroxidase (HRPO) due to the high turnover rate of this enzyme and the stability of the conjugates. Other enzymes can be used (e.g., alkaline phosphatase). The principle source of analytical variation in these assays is the high degree of dilution required.

### 8.5 Fluorescence Immunoassay (FIA)

In FIA, the radioactive tracers of RIA are replaced by one which emits light when stimulated by electromagnetic radiation of the appropriate wavelength. Advantages of FIA include high sensitivity and ease of operation without radiation hazard. The stability of fluorescent conjugates is now excellent, and their manufacture poses only minimal problems. Most quantitative fluorescence assays, however, have suffered from significant nonspecific background problems.

### 8.6 Radioimmunoassay (RIA)

The commonly employed competitive inhibition format of RIA combines high sensitivity,

economy of reagents, and reproducibility. In this method, a fixed amount of radiolabeled antigen competes for a limited amount of specific antibody with the "cold" unlabeled antigen in the sample or calibrator. A standard curve is constructed using known amounts of unlabeled antigen from which unknown antigen concentrations can be computed.[37] The assay can be carried out in a liquid medium, incorporating a separation step, or on a solid phase.

The overriding problem with RIA is the use of radioactive tracers, whose health effects have now become a serious issue for laboratory workers. Furthermore, disposal of radioactive waste has become sufficiently difficult that this alone is reason to seek alternate methods (see Section 8.4). The principle source of analytical variation in these assays is the high degree of dilution required.

### 8.7 Reference Method

As yet there is no widely accepted reference method for apolipoproteins; however, work is underway to develop such a method.[55]

### 8.8 Intermethod Differences[56]

The various immunochemical methods for assay of apolipoproteins may give differing concentrations in samples from genetically divergent individuals or populations, in different types of matrices, and with different methods of storage. These discrepancies may result from one or more of the following: particle size differences (see Section 8.3), genetically determined epitopic differences, degree of exposure of epitopes to degradation, interfering substances such as rheumatoid factor, matrix effects, error inherent in dilution schemes, etc. It is important to recognize in comparing values among samples and among assay methods that these problems may exist. Recent efforts to standardize analytical results have been summarized.[32]

## 9 Reference Materials

Reference materials for apolipoprotein measurements have been "fresh" frozen, lyophilized, or stabilized liquid preparations. Well documented stability studies of these various materials and their applicability to the major apolipoproteins have not been completed so that a recommendation cannot be given at this point. It can be stated that any matrix reference material must consist of a matrix that closely resembles human serum or plasma in the assay in question.

Some liquid materials with preservative or stabilizing agents have not proven stable at 4 °C for more than a few days for apolipoprotein testing. Frozen human serum preparations possess variable stability at -20 °C, but aliquots that are held at -50 °C to -70 °C under nitrogen gas to minimize oxidation demonstrate stability for about two years.[57]

Lyophilized serum specimens may show decreased immunoreactivity upon reconstitution with water, but the lyophilized serum may remain stable at this new apolipoprotein concentration when stored at -20 °C or below.[58,59]

Comparison of low triglyceride liquid material stored at -80 °C with a lyophilized sample of the same liquid serum pool frozen at -20 °C showed stability of value by an RIA for more than five years for apolipoproteins A-I and B.[60,61] Stability data at the recommended storage temperatures must be supplied to the users.

Reconstitution of lyophilized reference materials has proven to be a major problem in recovering precise results. The use of carefully and frequently calibrated automatic pipetting or diluting systems by the checking and correcting of volume by weight is recommended for the reconstitution of lyophilized specimens. Attention must be given by the manufacturers of lyophilized materials to the assignment of correct reconstituted values, since the total volume usually equals more than 1.00 mL after the user reconstitutes the vial with 1.00 mL of diluent. Lyophilized preparations are preferred for international reference materials because of the cost constraints and undesirability of distributing frozen materials.

Comparability of analytical results among laboratories was not improved when purified preparations of apoA-I and apoB were tested in comparison with secondary serum reference materials. Frozen "fresh" human serum has been found to serve as a suitable quality control reference material for intralaboratory studies,[26,32] and as national reference materials for

interlaboratory studies when shipment of frozen pools is not prohibitive. Whenever possible, these pools should be prepared from a sufficient number of individuals to ensure that different phenotypes are represented.

### 9.1 International Reference Material

A collaborative effort between the World Health Organization (WHO) and the International Federation of Clinical Chemistry (IFCC) with manufacturers of apolipoprotein diagnostic materials produced the WHO-IFCC First International Reference Reagents for apolipoproteins A-1 and B. The apo A-1 reference reagent is a lyophilized human serum and the apo B reagent is a stabilized liquid human serum. The WHO and IFCC offer standardization services to the manufacturers of apolipoprotein diagnostic products by an approved protocol. The Center for Disease Control and Prevention, Atlanta, GA, maintains a repository of these reference materials at -80 $^{0}$C at the request of WHO.

### 9.2 National Reference Material

National Reference Material may be frozen serum pools instead of lyophilized materials.

Any National Reference Material should transfer the International Units or tentative concentration values from the accepted International Standard.

### 9.3 Manufacturers' Reference Materials

All manufacturers should insure that values assigned to their reference materials, controls, quality control samples and survey materials intended for use as serum calibrators or for quality control systems are traceable to the National Reference Material and therefore indirectly to the internationally accepted standard.
In circumstances where the analytical method is considered to be sensitive to the physical or chemical state of external reference materials, international or national, the manufacturer should standardize the immunoassay to produce accurate values on fresh human specimens over the same dynamic range, demonstrate parallelism over the dynamic range, and determine traceability to the reference methods and materials that define the accuracy base as noted in Section 9.1.

## 10 Data Management

### 10.1 Units

While some attempt has been made to express apolipoprotein values in mmol/L, it is recommended that g/L or arbitrary Units/L be used at the present time. This should be reevaluated in fewer than five years to determine whether it will be possible or appropriate to assign molar SI units.

### 10.2 Precision

Precision for apolipoprotein quantification will vary with the technology used and the method selected for apolipoprotein determinations; the manual methods are generally less precise than the fully automated systems. While most apolipoprotein analytical systems (except RID) have reported intralaboratory precision of 5 to 10% CV, precision of 3 to 5% CV or less is achievable with some semiautomated and automated technologies. Surveys conducted by CDC indicate that the principle source of error in apolipoprotein testing programs is among laboratory variation (12% for apoA-I and 17% for apoB).[32] Whether precision can be significantly improved in all clinically applicable apolipoprotein assays remains to be seen. It is anticipated that the IFCC collaborative effort will result in significant improvement in both intra- and interlaboratory performance.[32,43,59]

### 10.3 Linearity

Empirical, apparently linear, quantitative relationships can be confirmed by several approaches. A plot or fit of the data may appear to be linear in that no systematic differences between the data and its functional (or graphical) fit are observed. A plot of the residuals (delta = observed — computed) with respect to the variable should not show systematic behavior. If a reasonable number of data points— at least six— more or less equally distributed over the range of interest, show no systematic deviation from the expected values, linearity can usually be assumed. A smaller number of data points (three or four) may also be used, as long as they are widely spaced.

Systematic trends may be less obvious, however, and apparently good fits can occur when the linear relationship is not valid. In some

cases, a relatively narrow portion of the assay range may be practically linear, whereas the extremes are not. In this case, value transfer may be performed within this range as long as the points measured are bracketed by closely-spaced calibration points, reducing the effect of nonlinearity. In any case, multiple replicates of each calibration point are essential.

A graphical presentation of the above approach may be used, such as the drawing of error bars (e.g., 95% confidence bars) for each plotted point. If these bars intersect the fitted line in a random manner, a linear fit probably is justified.

Correlation coefficients are sometimes used for judgment of fit of an equation to a data set. While these are good tests of correlation, they are not good tests for linearity. The 95% confidence limits for points (e.g., using error bars) and for the regression line in comparison with these parameters for other models (e.g., parabolic or logarithmic curves) are better estimates of validity of fit.

### 10.4 Calibration and Accuracy

Calibration is the establishment of a dose response curve, which is then used to calculate (e.g., by interpolation) concentrations of the analyte in clinical samples. Accuracy, which depends upon the calibration curve and the appropriateness of assigned values in calibrants, refers to the nearness of patient (or control) assays to the true values. As the limits of the range of assay are approached, accuracy usually decreases; in addition, the spacing of calibrator concentrations too far apart decreases accuracy in the midpoints between calibrator levels. The accuracy of patient results can be increased in several ways, such as increasing the number of calibration points, increasing the number of replicates of each point, diluting all clinical samples to fall in the midpoint of the curve, etc.

**Note:** It should be emphasized that "spiking" (i.e., addition of a purified antigen to a calibrator) may result in uncertainty because of differences in recovery and reactivity of purified antigen, compared to native material. Accordingly, spiking should be used only when other approaches (e.g., concentration of serum or use of "pathological" samples) are not feasible and when documentation of comparability with the native analyte is adequate.

Uncertainty may also be related to appropriateness of the reference material itself. If accurate reference materials physically and immunochemically identical to the unknowns (or with insignificant dissimilarity) can be prepared, measurement would be simplified in that analytical uncertainty would depend entirely on the precision of comparison. Reliable and accurate calibration can only be achieved with the availability of an authoritative reference method to assign an accurate value to the reference material. At the present time, no statement can be made as to the characteristics and protocol for an authoritative International/National Reference Material. It is anticipated that at the completion of the IFCC cooperative effort a consensus will have been achieved on:

(1) Interim values for apoA-I and apoB
(2) A protocol for value transfer
(3) Characteristics of the accepted candidate(s) when applied to various analytical methods.

### 10.5 Reference Cutoff

Adequate data on apolipoprotein levels for both sexes and for various age ranges are lacking. Therefore, the appropriate cut-off points to use for clinical significance are poorly defined. Reference intervals must take into account several factors including age, sex, race and the new reference material (see Section 7, Patient Preparation and Sampling[32] and Appendix A).

### 10.6 Data Management and Reporting

Evidence is accumulating that apolipoprotein testing provides more clinically useful information for assessing CAD risk than conventional lipid or lipoprotein-cholesterol measurements. For the present, apoA-I and apoB measurements, especially when used as apoA-I:apoB ratios, are better discriminators of CHD risk assessment. More studies are necessary to recommend the cut-off points which will insure high positive and negative predictive values that will be accurate for males and females of all age groups. The prospective studies in progress will help provide these answers.

Clinical data, while somewhat limited, suggest that apoA-I/apoB or apoB/apoA-I ratios are more

sensitive and specific for assignment of CAD risk, with higher predictive values than the use of either apo level alone.

## 11 Package Inserts and Product Evaluation

The FDA labeling regulation requires that both intended use and limitations be stated by the manufacturer.

The following are issues that should be considered from the user's perspective in evaluating the product.

### 11.1 Immunogen

The source of immunogen should be described in general terms.

#### 11.1.1 Specificity

From a practical point of view, polyclonal antisera against naturally occurring antigens are never truly monospecific even following Ab/Adsorption.

Statements of specificity are therefore operational and method specific and very much dependant upon the relative concentration of the antigen in question. The level of specificity required is also method dependent; nonspecificity in sensitive systems such as RIA does not always disqualify a reagent from use in a less sensitive form of analysis, such as immunoturbidimetry.

However, the absence of interference in each proposed technique must be documented by the manufacturer using clinical samples, purified antigens, enriched preparations or samples naturally or artificially depleted in a given analyte. Recommendations regarding both applicable and nonapplicable uses should be stated in the package insert.

#### 11.1.2 Methods of Ab/Adsorption

Methods of Ab/Adsorption should be indicated on the label or in the package insert. If human proteins remain in the antiserum, the possibility of unexpected reactions in gel methods should be addressed as well.

#### 11.1.3 Cross-Species Reactivity

Cross-species reactivity should be stated when known. If there is a high likelihood of problems related thereto, as with immunoblotting procedures, users should be advised to test for cross-species reactivity in their own systems.

### 11.2 Further Processing

If further processing is required for antiserum to be used in special techniques, the recommended methods should be given. For example, soluble antigen/antibody complexes should be removed before their use in nephelometric or turbidimetric systems using PEG (see Section 4.3.3).

### 11.3 Freedom from Infectious Agents

The methods used for documentation of determination of freedom from infectious agents in human materials, as indicated by FDA guidelines, should be specified.

### 11.4 Traceability to Authoritative Reference Materials

Statements defining the trail of value assignment for units or mass, and the methods by which value transfer has been accomplished, should be detailed. It is expected that reference to several materials may produce varying results with assay methods which are sensitive to matrix effects; therefore, all values should be listed.

### 11.5 Calibration

The recommended calibration procedure should specify the type of calibrator to be used and whether it will perform in a parallel manner to "fresh" clinical materials. The description should indicate the number of points on the dose-response curve and whether a linear and parallel relationship were attained.

### 11.6 Equivalence Studies

Comparability results of the manufacturers' method with an agreed upon interim reference method on "fresh," frozen, lyophilized or stabilized serum pool should be described. These studies should document equivalence or traceability to an accepted reference material. Any matrix effects should be mentioned.

### 11.7 Limitations

The manufacturers should clearly indicate that a product is designed for use with certain matrices and not with others and the circumstances that are known to produce aberrant results.

## 12 Interpretation of Values

Serum levels of lipids, lipoproteins, and apolipoproteins vary widely among individuals within a given population and among different populations. With the exception of an association of pancreatitis and certain visual abnormalities with extraordinarily high triglyceride levels, there are no acute adverse consequences of high levels. The optimal apolipoprotein levels for good health are not known. Similarly, the consequences of altering apolipoprotein levels are likewise uncertain. Thus, results of apolipoprotein analyses must be interpreted with respect to other members of the same population, until results of intervention trials, in which apolipoproteins are measured prospectively, become available. By comparing results with a normal reference population, surveyed by the same or comparable methodology, relative CAD risk can be determined. All apolipoprotein levels vary according to age, race, and sex. It is recommended that the cut-off points for CAD significance be at the 75th percentile for apoB and at the 25th percentile for apoA-I. Moreover, concurrent illness and medications can also alter apolipoprotein levels and must be taken into account in interpreting the results of these studies.

At present, the finding of a particular apolipoprotein level does not dictate a particular therapeutic regimen. Apolipoprotein levels are primarily of value in establishing risk, in the diagnosis of inherited abnormalities of lipid metabolism, and in assessing results of management of individuals.

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## Appendix

**Reference Intervals**

The most common means of establishing references ranges is to use "healthy" subjects representing a proper sampling of age and sex. Assuming that the distribution of values is Gaussian, the upper limits of normal can be selected mathematically. The difficulty with this approach in the case of CAD related apolipoprotein disorders is that silent disease is ubiquitous and the individuals contributing to the population base are not fully evaluated as to their coronary status by angiography. Many individuals with severe CAD are completely asymptomatic until the sudden event of vascular collapse. It is inevitable then that all populations will include a significant number of individuals with silent CAD.

The past experience with serum cholesterol reference ranges reflects just such a sampling error explaining the previously reported reference ranges of 160-330 mg/dL (1.6-3.3 g/L; 4.1-8.5 mmol/L). This upper value is far outside what is now considered to be the upper limit. The NIH National Cholesterol Education Program, Expert Panel on Detection and Treatment of Cholesterol has redefined the upper limit as less than 200 mg/dL (2.0 g/L; 5.2 mmol/L) for all adults over 20 years of age. This new more aggressive cutoff is at about the 40th percentile of the general asymptomatic population. The net result is that 60% of all adults are considered to have undesirably high levels. Thus a major change in the perception of what levels of apolipoproteins minimize CAD risk is required before apolipoprotein values can be used to assign CAD risk in a scientific manner.

## Summary of Comments and Subcommittee Responses

I/LA15-P: *Apolipoprotein Immunoassays: Development and Recommended Performance Characteristics; Proposed Guideline*

General

1. I have looked through the NCCLS document and it seems to me there are three important considerations:

    1. Lack of an agreed upon material for standardization from which calibrators can be derived.

    2. Uncertainty as to the significance of allotypic variation in the amino acid composition of apolipoproteins and how much of a problem this will be with respect to immunoassays.

    3. Lack of information with respect to clinical risk categories for apolipoproteins. This must await the results of prospective studies along the lines of Framingham.

    I do feel that apolipoproteins are a "wave of the future" with respect to assessment of cardiac risk but I am not sure the future has yet arrived. If there is some interest on the part of the clinicians and we do get a nephelometer I have no objection to doing it in-house.

- **The subcommittee agrees with the three considerations as summarized by the commentor.**

Foreword

2. In the 3rd paragraph, delete "serum" or substitute "serum or plasma."

- **The statement has been revised to include "plasma."**

3. Should apolipoprotein(a) read lipoprotein(a) or should Lp(a) read apo(a)?

- **The subcommittee believes the statement is correct as written because only the apo forms have been well studied.**

4. It seems odd that only cholesterol has a reference for its predictive value.

- **The subcommittee would welcome suggestions for additional references.**

5. A question on terminology: Does "hyperbetalipoproteinemia' imply elevated apo B? If not, either "hyperapobetalipoproteinemia" or perhaps "hyperapolipoproteinemia B" should be included for its predictive value.

- **The subcommittee believes the hyperbetalipoproteinemia indirectly implies an elevated apoB.**

6. In the last sentence of the second paragraph on page xiii, are "hyper apoB" and "apo(a)" protein variants? "ApoE variants" is redundant.

- **"Hyper apoB" and "apo(a)" are protein variants. The subcommittee does not agree that "apoE variants" is redundant in this context; it is used only as an example.**

Section 2.0

7. Definition (10) of Antigenic Determinant. "A determinant can be either a linear sequence of amino acids or conformational." Since antigens can be non-amino acids (e.g., carbohydrates), and nonlinear, a more accurate statement might be "A determinant can be determined either by primary composition or conformational relationships."

- **The statement was revised to state, "A determinant can be determined either by primary composition or conformational relationships."**

8. Definition (26) of ELISA. Could the presence of the analyte also be detected by an antigen-enzyme conjugate?

- **The subcommittee agrees that an antigen-enzyme conjugate can also detect the presence of an analyte. The definition has been revised.**

Section 3.1

9 In the ninth line, replace "undergone" with "resulted from."

- **The suggestion has been incorporated.**

Section 4.1.1

10. In this section, as well as Section 11.1.1, specificity of an antibody, no matter if it is monoclonal or polyclonal is always operationally defined. There is no reason to believe *a priori* that definition in another assay system even if it is "more sensitive" is adequate. Matrix factors such as pH, ionic strength and dielectric constant may alter both the conformation of the antigen and the conformation of the antibody.

- **The subcommittee believes that less sensitive and less precise methods should be discouraged.**

Section 4.1.5

11. Many of the proteins requested for cross-reactivity studies are cost prohibitive. Could nonreaction with affinity-purified serum be also used to show specificity?

- **The subcommittee does not recommend the use of affinity-purified serum.**

Section 4.1.6.6

12. This should include a reference to Section 4.3.4, Reverse Reactions with Polyclonal Antisera.

- **A reference to Section 4.3.4 has been added.**

Section 6.2

13. I think some anticoagulants are not satisfactory for apolipoprotein quantitation, e.g., heparin.

- **The guideline has been revised with the recommendation to avoid heparin as an anticoagulant.**

Section 6.3

14. I believe apo A-I needs to be frozen sooner than four days if it is not tested.

- **The subcommittee is not aware of data to support this statement. If the appropriate data become available, the area committee will consider the recommended change during the next revision of the document.**

Section 6.4

15. Regarding the removal of chylomicrons, and "The loss of apolipoproteins by either of these procedures is trivial." This statement needs a literature reference.

- **The statement, which is not intended to be definitive, was developed from the collective experience of the subcommittee.**

Section 7.3

16. The need for repeat or confirmatory testing is clearly indicated. I then looked for the interval, and was surprised that the recommendation is to 'average' the values obtained. First of all, isn't it possible to specify a more definite interval for repeat or confirmatory testing? At least give one interval to be used in eliminating misclassification, and one (or a series) appropriate for documenting changes with intervention therapy. And the idea of averaging the values just doesn't make any sense to me. With one high reading and one low reading say a week apart, the question would seem to be whether one is 'wrong' or not.

- **Migration to the central tendency, which is equal to averaging, is commonly used. If the physician believes the difference between the two test results is significant and causes question as to whether one test result is "wrong," then a third test is usually indicated.**

Section 11.6

17. Equivalence studies are to be compared to an "interim reference method." This requirement appears to be impossible to implement.

- **This recommendation has been useful with the IFCC reference method.**

## Index

## Related NCCLS Publications*

**DI1-A2** **Glossary and Guidelines for Immunodiagnostic Procedures, Reagents and Reference Materials; Approved Guideline (1992)**. Establishes "generic guidelines" that may be applied to different types of immunodiagnostic agents and methodology.

**DI2-A2** **Immunoprecipitin Analyses: Procedures for Evaluating the Performance of Materials; Approved Guideline (1993)**. Common terminology and basic methodology for immuno-diagnostic procedures.

**DI4-T** **Enzyme and Fluorescence Immunoassays: Tentative Guideline (1986)**. Guidelines for enzyme and fluorescence immunoassay of macromolecular analytes.

**LA1-A2** **Assessing the Quality of Radioimmunoassay Systems; Approved Guideline (1994)**. Definitions and procedures for properly assessing radioimmunoassay systems.

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

**NOTES**