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Protocols for Determination of Limits of Detection and Limits of Quantitation; Approved Guideline



This document provides guidance for determining the lower limit of detection of clinical laboratory methods, for verifying claimed limits, and for the proper use and interpretation of the limits.

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

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Abstract

NCCLS document EP17-A—*Protocols for Determination of Limits of Detection and Limits of Quantitation; Approved Guideline* provides protocols for determining the lower limit of detection of clinical laboratory methods, for verifying claimed limits, and for the proper use and interpretation of these limits. This document also provides guidance for determining lower limits of quantitation based on a laboratory's goals for performance at low-levels. This applies to all quantitative procedures, even if the reported result is qualitative. EP17-A is intended for use by clinical laboratories and by manufacturers of *in vitro* diagnostic tests.

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Foreword

Laboratory methods have many performance characteristics that must be understood and assessed for their appropriate use. The performance characteristics for any method describe the method's capability to reliably measure the amount of an analyte in a subject's sample (although for some analytes, reliable detection is sufficient). Two such critical performance characteristics are defined at the lower end of the measurement scale. The first is the smallest amount that the method can reliably detect to determine presence or absence of an analyte. This is the limit of detection (LoD). The second characteristic is the smallest amount the method can reliably measure quantitatively. This is the limit of quantitation (LoQ).

The limits of detection and quantitation are critical because detecting extremely small amounts of an analyte can be necessary to define disease states, screen for disease, identify significant exposure, or to reveal the presence or absence of toxins, pollutants, carcinogens, contaminants, infectious agents, and illicit drugs. Some of these applications may effectively use the examination method as a qualitative or semiquantitative (ordinal scale) procedure. Although the discrimination point, or cutoff value, for a qualitative method is rarely designated by the developer to be equivalent to the lowest detectable amounts of the analyte, knowledge of the limit of detection informs the choice of a cutoff, so the procedures in this document should be applicable.

The LoD and LoQ are also important in laboratory examinations for tumor markers, hormones, agents of infectious diseases, therapeutic drugs, and other tests where low values separate subjects into different disease or exposure categories. Because of the statistical sophistication of these protocols, it is expected that this document will be used primarily by manufacturers and clinical laboratory supervisors.

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. In light of this, NCCLS recognizes that harmonization of terms facilitates the global application of standards and deserves immediate attention. Implementation of this policy must be an evolutionary and educational process that begins with new projects and revisions of existing documents.

The Subcommittee on Limits of Detection has made every effort to use globally accepted terms wherever possible. These are described in Sections 2.3.1 and 2.3.2 which discuss some commonly used terms that are not employed in this document, and in Section 3. Users are encouraged to comment on the choices made by the subcommittee, in the hope of eventually reaching full international harmonization of these terms and concepts.

Key Words

Limit of blank, limit of detection, limit of quantitation, nonparametric statistics

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This guideline was prepared by NCCLS, as part of a cooperative effort with IFCC to work toward the advancement and dissemination of laboratory standards on a worldwide basis. NCCLS gratefully acknowledges the participation of IFCC in this project.

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Protocols for Determination of Limits of Detection and Limits of Quantitation; Approved Guideline

1 Scope

NCCLS document EP17 specifies recommendations for determining the lower limit of detection of clinical laboratory methods, for verifying claimed limits, and for the proper use and interpretation of the limits. It also provides guidance for determining lower limits of quantitation based on a laboratory's goals for performance at low-levels. EP17 may be applied to all measurement procedures (even if the reported result is qualitative), most notably those for which the medical decision level is low (i.e., approaching zero).

The intended users of this guideline are clinical laboratory supervisors and manufacturers of *in vitro* diagnostic tests.

2 Introduction

There have been a great number of procedures proposed for establishing the lower limits of detection and quantitation (LoD and LoQ) in laboratory measurement procedures. This document does not intend to review them all, nor does it attempt to find agreement among them. The needs of the clinical laboratory are unique in that the recommended procedure must apply to analytical devices as they are used in the laboratory setting. Usually, this means using the device as it is, often as set up and maintained by a manufacturer or its representatives.

The most commonly recommended statistical protocols for LoD and LoQ, including the ISO protocol 11843, Parts 1 to 4,[1-4] assume that the raw instrument output at low levels can result in negative concentrations. These protocols also assume that the signals for blank samples are normally distributed around some low average value (usually zero). However, many common clinical laboratory instruments report analyte levels in positive numbers only, and basic instrument analytical signals are not retrievable, so the preferred parametric models are not appropriate.

This protocol is intended for use with methods that report in mass or concentration units, and, in particular, in which there is clinical need or interest in very low concentrations (approaching zero). EP17 is intended for all methods, but it was designed specifically for use with methods that report "zero" or positive values only. Manufacturers or developers of methods who have access to the basic instrument response should consider use of ISO 11843-1[1] and either 11843-2[2] or 11843-3,[3] depending on whether or not linear calibration data are available; these documents describe the internationally recognized procedures for determining the LoD. Usually, the expected limits of detection for the EP17 protocol are the same as for the ISO model, but the ISO procedure should return values with lower uncertainty. However, it is important to remember that the use of the parametric ISO procedures requires the validity of the assumptions of normality (Gaussian) at all tested levels, including the blank material.

Proper use of the ISO protocol will also require statistical sophistication that is often beyond the capabilities of most laboratories. The EP17 protocol is intended to be applied and understood without straining the resources available to most clinical laboratories. The procedures are based on the concepts used in conventional LoD procedures, but are practical for use with common clinical laboratory devices.

This protocol requires knowledge of the true (or accepted) levels of the measurand in samples being tested. There is little utility in estimates of precision around an unknown value. It is also important to recognize that modern electronic instruments do not possess continuous functions relating sensor and voltage or current output. The random error measured in the region of a blank may be truly random, but it

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could also be due to explainable factors. Before spending much effort pursuing statistical descriptions of results on a blank material, it is important to first define the issues related to the analyzers themselves and the environment in which they are used.[5] The following NCCLS documents EP5—*Evaluation of Precision Performance of Clinical Chemistry Devices*, EP6—*Evaluation of the Linearity of Quantitative Measurement Procedures: A Statistical Approach*, EP7—*Interference Testing in Clinical Chemistry*, and EP9—*Method Comparison and Bias Estimation Using Patient Samples*, or equivalent procedures, should be used to fully describe the characteristics of the procedure prior to the implementation of this protocol.

2.1 Path of Development and Use

The instrument manufacturers (or method developers) are responsible for accurately establishing the lower limit of detection of the clinical tests performed by their systems. They are also responsible for determining the upper and lower limits of the measuring range for the methods and the precision of results at these limits. Manufacturers are also responsible for ensuring that the clinical laboratories are able to validate the established LoDs for their methods, for all of the analytes in the systems.

The laboratory director is responsible for ensuring that the laboratory properly verifies the LoD of the methods used by the laboratory, and properly determines the LoQ to meet the laboratory's needs. This should be carried out as a part of the general evaluation of performance comprising various aspects of precision, trueness, linearity, etc. The limits can change as the laboratory experiences changes in personnel, reagents, and equipment. Therefore, validations should be repeated periodically to ensure they are current. The director is then responsible for relaying to the physician the analyte LoDs, LoQs, and any other limitations of the analytical results reported, which should include the uncertainty of the reported results that are near the LoD.

For proper use of laboratory test results in diagnosis and monitoring of disease or therapeutic measures, it is one of the physician's responsibilities to understand the limitations of the clinical laboratory methods, including the analytical LoDs, LoQs, and the uncertainty of results near those limits. If an analytical result approaches the LoD, the physician must understand that the relative uncertainty of that result can be quite large and use it with due caution. Analytical results much larger than the laboratory's LoD usually have lower uncertainty and can be used with more confidence.

2.2 Common Practices

Currently, many devices report LoD and LoQ that come from a simple model, using only measurements of blank samples. In this simple model, it is assumed that the highest possible value from a blank is the threshold for determining the presence of the analyte, and therefore is the lower limit of detection. Most commonly, a method developer will make repeated measurements of a blank sample, calculate the mean and standard deviation of these measurements, multiply this SD by some factor, add that to the mean of the replicates, and call this value the LoD and possibly the LoQ. (One traditional definition in analytical chemistry is to use a factor of 10 times the SD to determine the LoQ and a factor of 2 or 3 to determine the LoD). This model has two deficiencies. First, it depends on the assumption that the distributions of results for low-levels and blanks are symmetric and normally distributed. Some parametric models make the additional assumption that replicate measurements on low-level samples have the same standard deviation as on the blank samples. A major defect of this approach is that the model does not distinguish between apparent and true (or actual) concentrations of the analyte. That is, it ignores the fact that a sample with a true concentration exactly at the LoD (as determined by this model) will have only a 50% chance of returning a value that is interpreted as showing the presence of the analyte. This is discussed in more detail in Section 4.1. The effect of this is that many commonly reported LoD/LoQ values are lower than they would be if more statistically rigorous procedures were used.

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2.3 Terminology

Because of the widespread use of the LoD and LoQ concepts, a variety of terms are in common use. This document does not attempt to explain or reconcile all of these terms. Terms used in this document are defined in Section 3. However, there are two common terms that have nonstandard use in the clinical laboratory that should be discussed here, to avoid confusion.

2.3.1 Nonstandard Use of "Critical Value"

The term "critical value" is used in ISO 11843 as the highest result that can reasonably be expected from a blank sample (a sample with concentration at or near zero). It is the instrument (method) response, above which the sample can be considered to have a positive value of the measurand. However, this term is commonly used in medical laboratories for test results that indicate an important medical condition. Therefore, in this document, this response threshold is called the "limit of blank" (LoB; see below).

2.3.2 Nonstandard Use of "Sensitivity"

The term "sensitivity" will not be used in this document, due to the existence of several alternative common uses of that term. "Limit of detection" is preferred due to its more precise definition and common use. In many clinical laboratories and diagnostic applications, "sensitivity" or "analytical sensitivity" are used interchangeably with "limit of detection," "lower LoD," or "detection limit." However, "sensitivity" is also used in several other ways, some of which are preferred uses in their areas. In different applications, "sensitivity" might be used alone or with modifiers.

The most common use is consistent with the IUPAC definition of "analytical sensitivity," which is "the slope of the calibration curve." The calibration function relates the mean of the measured concentrations to the actual (known) concentrations; the steeper the slope of the calibration curve, the more sensitive the assay is to slight changes in amount of analyte. This is not the same as the limit of detection. Indeed, even if the slope of the calibration curve is steep, the lowest concentration that can be reliably detected can be large if there is a high variation of the measured signal. On the other hand, a method with a moderate analytical sensitivity (moderate slope of the calibration curve) and very low random variation of the signal can have a low limit of detection.[6]

Other uses include "functional sensitivity," which is used to describe the interassay precision at very low analyte concentrations, for certain diagnostic assays with high precision requirements at low concentrations. The terms "diagnostic" or "clinical" sensitivity are used to characterize or compare the performance of diagnostic tests in relation to prespecified clinical information (e.g., presence or absence of a target analyte related to a disease).

2.3.3 Other Limits at Lower Levels

There are several different types of limits in common use. The limits that are described in this document are defined in Section 3 ("limit of blank" [LoB], "limit of detection" [LoD], and "limit of quantitation" [LoQ]). However, there are two other types of limits: the lower end of the linear range and the lower end of the measuring range. It is possible that four of these five limits could all be the same (with the model considered in this document, the LoB is lower than the LoD), or they could all be different. Most often, some of them will be the same, but not all.

In describing these concepts, it is important to distinguish between the "true" amount of a substance that is actually in the sample, and the individual measurement result. In this document, we use the terms "actual concentration" to describe what is truly in the sample, and "measured concentration" or "result" to describe values that the laboratory will observe when using a particular analytical method.

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The relationship among these limits is discussed below.

- The lowest is the "limit of blank" (LoB), which is the highest value we expect to see in a series of results on a sample that contains no analyte. (This limit is described in more detail in Section 4.) It is important to note that the LoB refers to an observed test result, while all of the other limits refer to actual concentrations of the analyte.

- The next lowest is the "limit of detection" (LoD), which is the actual concentration at which an observed test result is very likely to exceed the LoB and may therefore be declared as "detected."

- The "limit of quantitation" (LoQ) is the lowest actual concentration at which the analyte is reliably detected *and* at which the uncertainty of the observed test result is less than or equal to the goal set by the laboratory, or by the manufacturer of the method. The goal for uncertainty (or for bias and imprecision) must accompany LoQ claims, or be available for the laboratory's records.

- The lower end of the measuring range (LMR) is the lowest level at which defined conditions are met. These defined conditions include all stated characteristics of the method, including bias and imprecision, uncertainty, and often other characteristics.

- The lower limit of linear range (LLR), is the lowest concentration at which the method response has a linear relationship with the true concentration (see NCCLS document EP6—*Evaluation of the Linearity of Quantitative Measurement Procedures: A Statistical Approach*). This also requires a goal for nonlinear error, which must accompany any claims about linearity.

It will always be the case that LoB < LoD ≤ LoQ. The other limits may not have a consistent relationship to each other. The relative placement of these limits will be determined by the goals for quantitation, for nonlinear error, and for the defined characteristics.

2.4 Discussion of Limits of Detection and the Tie to Qualitative Tests

The limit of detection is also an important performance criterion for qualitative tests where there is an underlying continuum of instrument signal, yet the result is reported as "positive" or "negative" (or "present" or "absent"). For example, ELISA methods use a function of optical density and other instrument responses to distinguish between positive and negative responses. For these tests, the limit of detection can be estimated using the concepts described here. The relationship among the limit of detection, the discrimination point between positive and negative results (also called the cutoff), and the instrument response at concentrations near zero, should be fully documented by the manufacturer or developer and communicated to users. This does not apply to tests where the discrimination point is well above the limit of detection, such as for pregnancy tests.

These qualitative tests may often have a narrow dynamic range and reach saturation of the signal at rather low concentrations. However, all qualitative tests with an underlying continuum have a transition area from no response or background signal to full response, and the discrimination point or cutoff is generally designated by the manufacturer to be in this area. For some tests, manufacturers define a different cutoff for positive and negative results, creating a zone where the response is "indeterminate."

This indeterminate area is called the "95% interval" in NCCLS document EP12—*User Protocol for Evaluation of Qualitative Test Performance.* In EP12, however, it is not a statistical confidence interval around the cutoff point; instead, the endpoints of this interval are the concentrations at which repeated results are 95% positive or 95% negative, respectively. Note that repeated tests of the concentration exactly at the cutoff point yield positive results 50% of the time and negative results for the other 50% of the time. In this protocol, the LoB is the same as the cutoff point, the upper endpoint of the 95% interval

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is the same as the LoD (where truly positive samples produce a positive signal 95% of the time), and the lower end of the interval is zero (no analyte present in the sample).

Knowledge of the limit of detection for a qualitative test, combined with frequent analysis of a low positive control, allows the laboratory to monitor the consistency of performance of the method near the cutoff concentration. It is possible to experience an unobserved increase in the limit of detection of a qualitative test, for example, due to changes in reagents. This can increase the rate of false-negative results, because samples with detectable levels of an analyte may have results that fall below the cutoff, while samples at higher concentrations may still give correctly positive responses. Consequently, the experimental determination of the limit of detection is important for controlling the quality of many qualitative tests.

3 Definitions

Accepted reference value – A value that serves as an agreed upon reference for comparison, and which is derived as a) a theoretical or established value, based on scientific principles; b) an assigned or certified value, based on experimental work of some national or international organization; c) a consensus or certified value; based on collaborative experimental work under the auspices of a scientific or engineering group; and d) when a), b), and c) are not available, the expectation of the (measurable) quantity, i.e., the mean of a specified population of measurements (ISO 3534-1).[7]

Accuracy – Closeness of agreement between a test result and the accepted reference value; **NOTE:** The term accuracy, when applied to a set of test results, involves a combination of random components and a common systematic error or bias component (ISO 3534-1)[7]; See **Trueness.**

Analyte – Component represented in the name of a measurable quantity (ISO 17511).[8]

Alpha (α) error//Type I error//False positive – Probability of falsely rejecting the null hypothesis that a substance is not present when it is true.

Beta (β) error//Type II error//False negative – Probability of falsely accepting the null hypothesis that a substance is absent, when in fact the substance is present at the designated level.

Bias – Difference between the expectation of the test results and an accepted reference value (ISO 3534-1)[7]; **NOTE:** Bias is a measure of **Trueness** (VIM93).[9]

Blank – Sample that does not contain the analyte of interest, or has a concentration at least an order of magnitude less than the lowest level of interest.

Limit of blank (LoB) – Highest measurement result that is likely to be observed (with a stated probability) for a blank sample; **NOTE:** a) LoB is not an actual concentration from which measurements are obtained; indeed, the actual concentration at which a positive signal is assured is called the limit of detection. Similarly, LoB is the lowest value expected from a sample that contains a level of the analyte equal to the LoD with stated probability; b) This is the same as the "lower limit of determination": lowest result obtained by a stated measurement procedure that can be given with a statement of **uncertainty of measurement.**[10] Also called "critical value" (ISO 11843).[1-4] (See Section 2.3.1.)

Limit of detection (LoD) – Lowest amount of analyte in a sample that can be detected with (stated) probability, although perhaps not quantified as an exact value (revised from WHO-BS/95.1793)[11]; **NOTE:** Also called "lower limit of detection", "minimum detectable concentration" (or dose or value), and sometimes used to indicate "sensitivity" (see Section 2.3.2).

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Limit of quantitation (LoQ)//Lower limit of quantitation – Lowest amount of analyte in a sample that can be quantitatively determined with stated acceptable precision and trueness, under stated experimental conditions (modified from WHO-BS/95.1793)[11]; **NOTE:** Also called "lower limit of determination" and "lower end of the measuring range."

Linearity – The ability (within a given range) to provide results that are directly proportional to the concentration (amount) of the analyte in the test sample (modified from WHO-BS/95.1793)[11]; **NOTES:** a) Linearity typically refers to overall system response (i.e., the final analytical answer rather than the raw instrument output); b) The linearity of a system is measured by testing levels of an analyte which are known by formulation or known *relative to each other* (not necessarily known absolutely); when the system results are plotted against these values, the degree to which the plotted curve conforms to a straight line is a measure of system linearity.

Measurand – Particular quantity subject to measurement (VIM93)[9]; **NOTE:** 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.

Measurement error//Error of measurement – The result of a measurement minus a true value of the measurand; limits (VIM93)[9]; **NOTE:** a) Formerly, the term **total error** was used in NCCLS documents.

Measuring range//Working range – Set of values of measurands for which the error of a measuring instrument is intended to lie within specified limits (VIM93)[9]; **NOTE:** Formerly, the term **reportable range** was used in NCCLS documents.

Precision – Closeness of agreement between independent test results obtained under stipulated conditions (modified from ISO 3534-1).[7]

Quantity – Attribute of a phenomenon, body, or substance that may be distinguished qualitatively and determined quantitatively (VIM93).[9] **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.

Reference material (RM)//Reference preparation – Material or substance, one or more of whose property values are sufficiently homogeneous and well established to be used for the calibration of an apparatus, the assessment of a measurement method, or for assigning values to materials (VIM93).[9]

Repeatability – Precision under conditions where independent test results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment within short intervals of time (ISO 5725-1).[12] **NOTE:** Formerly, the term **within-run precision** was used in NCCLS documents.

Traceability – Property of the result of a measurement or the value of a standard whereby it can be related to stated references, usually national or international standards, through an unbroken chain of comparisons all having stated uncertainties (VIM93).[9]

Trueness – Closeness of agreement between the average value obtained from a large series of test results and an accepted reference value (ISO 3534-1)[7]; **NOTE:** The measure of trueness is usually expressed in terms of bias. (ISO 3534-1).[7]

Uncertainty of measurement – Parameter, associated with the result of a measurement, that characterizes the dispersion of the values that could reasonably be attributed to the measurand; **NOTES:**

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a) The parameter may be, for example, a standard deviation (or a given multiple of it), or the half-width of an interval having a stated level of confidence; b) Uncertainty of measurement comprises, in general, many components. Some of these components may be evaluated from the statistical distribution of the results of series of measurements and can be characterized by experimental standard deviations. The other components, which can also be characterized by standard deviations, are evaluated from assumed probability distributions based on experience or other information; c) It is understood that the result of the measurement is the best estimate of the value of the measurand, and that all components of uncertainty, including those arising from systematic effects, such as components associated with corrections and reference standards, contribute to the dispersion (VIM93).[9]

Verification – Confirmation, through the provision of objective evidence, that specified requirements have been fulfilled (ISO 9000).[13]

4 Procedures for Determining and Verifying the Limit of Detection (LoD) for a Measurement Procedure

4.1 Overview

The classic approach for determining LoD, as reviewed by Currie,[14] is discussed briefly in this section.

For a given assay, a method developer (or manufacturer/modifier of a method) prepares a series of results on blank samples and a series of results on very low-level samples. The results for the blank samples are used to determine a response that is rare, and therefore provide a threshold where higher values can be interpreted to indicate a positive sample. This point and the standard deviation of the low sample measurements are used to find the concentration where measurements are very likely to exceed the highest expected value of the blanks. Probability distribution density curves of repeated measurements of a blank and of a sample with a low concentration of analyte are schematically shown in Figure 1.

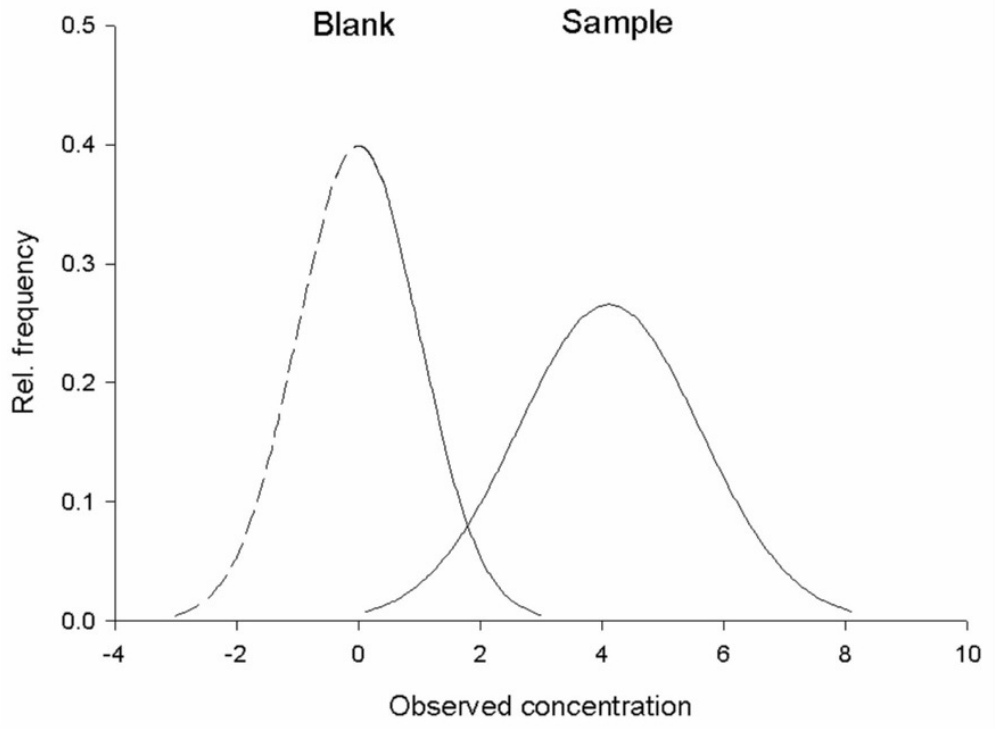

Figure 1. Distributions of Observed Concentrations for the Blank and a Sample With a Low Amount of Analyte. The dashed line corresponding to concentration less than zero indicates that some instruments do not report values less than zero.

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4.1.1 General Approach for Determining the Limit of Blank

The dispersions of the results from blanks and low-level samples are due to random measurement error, which often is lowest for the blank sample. In Figure 1, the mean of the blank measurements is close to zero with a symmetrical distribution. While this type of signal can occur internally in the instrument, many instruments automatically convert negative value signals to zero or a small positive value or suppress those so that only nonnegative concentration values are provided as output. It is assumed that values exceeding the 95th percentile of the distribution of values on truly blank samples deviate significantly from blank measurements. When a sample produces an observed value that exceeds this limit, it may be declared to contain an amount of analyte that exceeds zero.

Using this limit, we have a 5% probability of having a truly blank sample give a value that indicates the presence of the analyte. This is commonly called Type I error (α error). At the same time, we observe that measurements of a sample with a low concentration of analyte can fall below this limit; and so would be declared to be indistinguishable from the blank measurements. If we conclude that no measurable analyte is present in these cases, we commit an error commonly known as Type II error, the associated error risk being denoted by β, hence the phrase "β error." Method developers may set α and β appropriately, depending on the relative costs of the errors.

Recently, the International Organization for Standardization (ISO) recommended a definition of the minimum limit of detection in relation to stated levels of Type I and II errors.[1] The default levels for these errors were set to 5%, i.e., $\alpha = \beta = 5\%$. An α value of 5% corresponds to using the 95th percentile of the distribution of blank values as the limit for declaring a measured value significantly higher than the blank. Given a Gaussian distribution of blank values (see Figure 1), this limit corresponds to:

$$LoB = \mu_B + 1.645\ \sigma_B, \quad (1)$$

where μ_B and σ_B are the mean and standard deviation of the blank measurements, respectively.

For the situation where values less than zero are not reported or the blank values are non-Gaussian (with an asymmetric distribution of blank values), the 95th percentile has to be estimated by another approach. The most straightforward procedure is to apply a nonparametric principle based on the ordered values.[15] Having ranked N_B values according to magnitude, the 95th percentile may be estimated as the value of the position ($N_B(95/100) + 0.5$) ordered observation.[16,17] In case of a noninteger value, interpolation is carried out between adjacent ranked values (see example). The limiting percentile (Pct_B) of the blank distribution, which cuts off the percentage α in the upper tail of the distribution, is called the limit of blank (LoB), i.e.,

$$LoB = Pct_{B\ 100-\alpha}. \quad (2)$$

4.1.2 General Approach for Determining the Limit of Detection

In order to address the Type II error, one has to consider the minimum sample concentration, equal to LoD, that provides results that have a specified probability of exceeding the LoB. If the Type II error level β is set to 5%, then 95% of the measurements exceed the LoB, when an actual sample concentration is at the LoD. Figures 2a and 2b illustrate two cases, one with an actual sample concentration equal to LoB and another with an actual sample concentration at a level such that the 5th percentile of the distribution of sample concentration measurements equals the LoB. In the first case, 50% of the sample measurements are below the LoB, and the other 50% exceed the LoB. Only the latter 50% would be declared as significantly exceeding the blank value, i.e., containing a detectable amount of the analyte, and therefore $\beta = 50\%$. In Figure 2b, on the other hand, 95% of the measurements with sample having analyte concentration equal to LoD exceed LoB and are declared as measurements obtained with a sample containing a detectable amount of the analyte. Thus, only 5% of the measurements are erroneously declared not significantly different from the blank, and $\beta = 5\%$, which is the Type II error risk (the β risk). The LoD is the actual concentration of this sample; it is the lowest actual concentration that can be

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detected reliably. Usually, the low-level sample distribution is Gaussian and the 5th percentile of the distribution corresponds to the LoB:

$$LoB = \mu_S - 1.645\ \sigma_S, \quad (3)$$

where μ_S and σ_S are the mean and standard deviation of the population of the low sample measurements.

Overall, we thus have:

$$\mu_S = LoD = LoB + 1.645\ \sigma_S.$$

If the distribution of blank values is Gaussian, then $LoB = \mu_B + 1.645\ \sigma_B$ and, consequently,

$$LoD = \mu_B + 1.645\ \sigma_B + 1.645\ \sigma_S. \quad (4)$$

In case the sample distribution is not Gaussian (and cannot be transformed to Gaussian), it may be possible to estimate the LoD nonparametrically, as discussed in Section 4.3.2.2; otherwise, it is necessary to produce samples near the tentative LoD, and check for the lowest level where 5% or fewer of the observed measurements are below the LoB.

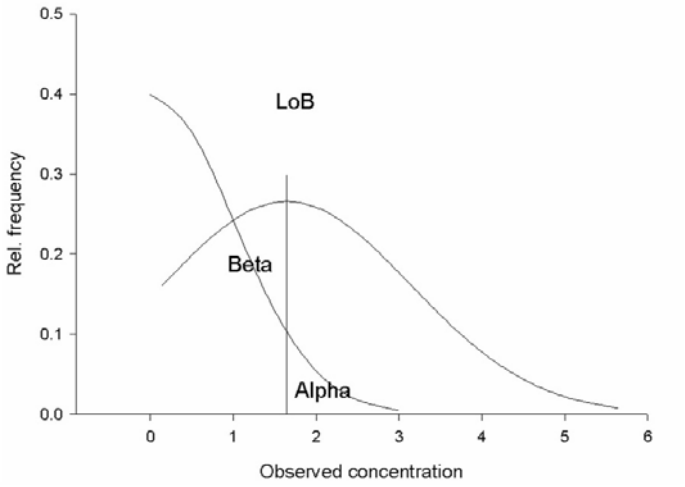

Figure 2a.

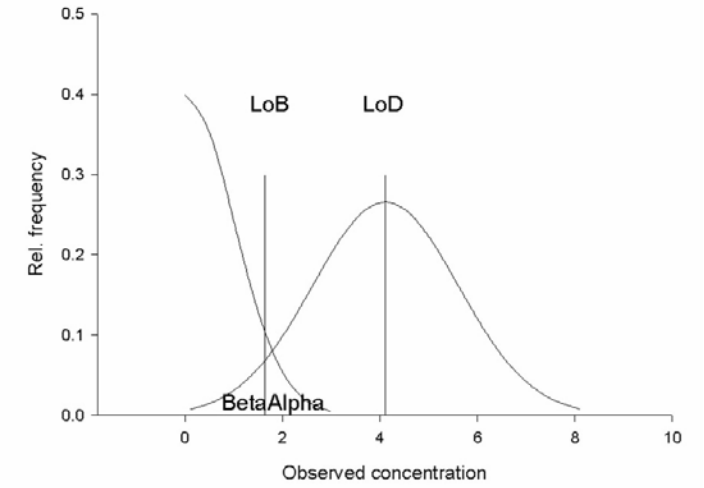

Figure 2b.

Figure 2. Distributions of Replicates for Blank Samples (left curve in both figures) and Two Hypothetical Low-Level Positive Samples (2a and 2b). When the actual concentration of analyte in the sample equals LoB, 50% of the measurements exceed LoB (a). With an actual sample concentration equal to LoD, (100% - β) (95%) of the sample measurements exceed LoB (b).

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4.2 Study Design

The designs discussed below assume a single system or set of laboratory equipment, operating in stable conditions (no reagent lot changes or major changes in calibration). However, the recommendations include considerations for variability due to time (or run) and differences between subjects.

Manufacturers should consider performing the LoD study on two or more instruments with two or more lots of reagents to attempt to capture the variability expected with different instrument/reagent systems. If the results from different systems are substantially different, the reasons should be investigated. If the results from different systems are essentially the same, the largest LoD should be used as the estimate. This suggestion must be balanced with cost and practical considerations. In any design, the results should be accompanied by a complete description of the study and the parameters that were investigated.

4.2.1 Number of Samples

In determining the optimal number of samples for establishing or verifying an LoD, the goal is to require only the necessary number of samples and measurements, and thereby allocate resources most efficiently between blank and low-level samples. It is understood that more samples and more measurements provide better estimates with less uncertainty, so the total number of measurements used will be limited by budget concerns and sample availability. In a statistical design, the optimal ratio between the numbers of blank and sample measurements is related to the uncertainty of the estimated LoB and the variability between sample measurements (in this protocol, called SD_S).

Nonparametric estimation of the LoB is about half as efficient as a parametric estimation procedure.[18] That is, the parametric and nonparametric procedures have the same expected result, but when using the same number of measurements, the parametric estimates have smaller uncertainty (if the data are Gaussian). The uncertainties of percentile or standard deviation estimates will also be proportional to the dispersion of the distributions. Thus, with nonparametric estimation of the LoB and parametric estimation of the LoD, the number of blank measurements should exceed that of the low sample measurements. However, most commonly, the dispersion of low-level sample measurements will exceed that of the blank measurements. Overall, an equal number of blank and low-level sample measurements would be best for most cases.

When the number of blank sample measurements is the same as the number of low-level measurements, then the uncertainty of the LoD declines with the inverse of the square root of the number of measurements (perhaps adjusted for the number of different samples). The relationship between sample size and uncertainty of LoD is described by Linnet and Kondratovich.[18] Appendix A shows the formula to estimate the standard error of the LoD, as a function of the number of results for the blanks and the low-level samples, and the standard deviation of those replicates. Appendix A also shows a simplified formula to determine N, when the same number of replicates is to be used for blanks and low-level samples, and the standard deviation of the low-level sample measurements is related to the standard deviation for the blanks.

As a reasonable compromise between precision and costs, a minimum number of *60 measurements* (on both blank and low-level samples) is suggested for establishing the LoD. This would typically be performed by the developer of the method. To verify a claimed LoD, use *a minimum of 20 results* at the claimed level and, if necessary, at the LoB. This is usually performed by the laboratory or user.

4.2.2 Characteristics of Blanks and Low-Level Samples

When possible, blanks and low positive samples must be commutable with natural samples, e.g., for a drug assay it might be a serum or plasma sample free of drug and not just a buffer solution. Because artificial and spiked samples may behave differently than subject samples, the reader is advised to review

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NCCLS documents EP7—*Interference Testing in Clinical Chemistry* and EP14—*Evaluation of Matrix Effects* before using samples that are not natural subject samples.

In order to ensure that the measurements are representative, it is preferred to compile measurements from a number of samples, rather than a single sample. Matrix differences exist from sample to sample, and instead of repeatedly measuring one particular sample, a set of five or more samples is preferable. These measurements should be spread over several days so that the assessment reflects the performance of the analytical method over a range of typical laboratory conditions, including (where appropriate) different analysts and equipment. For verifying claims, the time period need not include changes in reagent lots or major equipment maintenance. For establishing claims, the time period *should* include changes in reagent lots.

4.2.2.1 Considerations for Blank Samples

For endogenous compounds, blanks might be samples that are stripped of the component, e.g., by precipitation by an antibody, by enzymatic degradation, or by adsorption to charcoal, etc. For hormones, blank samples may be provided from diseased subjects or subjects with suppressed levels due to pharmacological treatment. For tumor markers, samples from nondiseased subjects may be appropriate. If a residual level of analyte is unavoidable, it should be an order of magnitude lower than the limit of the analytical range for the assay.

4.2.2.2 Considerations for Low Positive Samples

For low-levels of endogenous compounds, it is preferred to use a set of subject samples with concentrations in the low range. If it is necessary to spike a sample with the analyte (for example, for a drug), it is best to spike a set of samples from different subjects, rather than just one sample or a pool. A pooled SD_S estimate can then be derived from repeated measurements with the set of samples, e.g., 12 measurements of each of five samples (see example below). Measurements should be carried out on different days, so that SD_S reflects the total analytical variation.

In order for estimates of the LoB and LoD to be meaningful, the measurement values should be traceable to SI if possible,[3] or to some known level. If a reference method for the analyte exists, it should be used to compare measurements in the low range. Traceability can also be demonstrated with measurements of spiked samples, considering the traceability of the spiking substance.

4.2.3 Results

The measurements can be of any characteristic or property, such as concentration or activity, as long as the quantity being measured (the measurand) is clearly defined. When *the measurand* differs from the *analyte* of interest (for example, an *enzyme's activity* is not the same as a mass concentration of *the enzyme*), the quantity must be clearly defined. This is essential for understanding differences in LoD for different methods, or under different measurement conditions.

4.3 Procedure to Determine and Verify the LoB and LoD

The procedures to establish the performance characteristics and to verify performance claims differ in complexity and in the number of measurements that are needed. The procedures are based on the same model and the same tolerances for Type I and Type II error.

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4.3.1 Procedure to Determine or Establish the LoB

It is recommended that a minimum of 60 blank measurements be carried out. The LoB is estimated from repeated (N_B times) measurements of one or several blank samples. Using several samples may help to ensure that a sample that has some noticeable amount of analyte is not used as a blank. As stated above, if the data appear to be Gaussian, then use parametric procedures to estimate the LoB as in equation (1). If the data are non-Gaussian (as when truncated at zero), use equation (2), and the procedure described below.

The measurements are ordered according to their value, and the appropriate percentile (p) is estimated as the value of the observation with rank value as determined below[15-17]; in this case, p = (100 - α) = 95:

$$\text{LoB} = \text{Result at position } [N_B(p/100) + 0.5] = \text{Result at position } [0.95^* N_B + 0.5] = P_{(1-\alpha).} \quad (5)$$

If this is a noninteger value, linear interpolation is carried out. For example, if p = 95 and N_B = 60, then N_B (p/100) + 0.5 = 57 + 0.5 = 57.5, and the 95th percentile would be the average of the 57th and 58th ranked observations. If N_B = 65, then $N_B(p/100)$ + 0.5 = 62.25, and the 95th percentile would be a combination of the 62nd and 63rd ranked observations, calculated as $X_{62} + 0.25(X_{63} - X_{62})$.

These steps are shown in the first flowchart, from A to B.

4.3.2 Procedure to Determine the LoD

To determine the LoD, the standard deviation of sample measurements (SD_S) is obtained from repeated measurements (N_S) of sample(s) with a relevant low concentration, i.e., a concentration in the range from LoB to approximately 4 x LoB. It is recommended that a minimum of 60 results be obtained from low concentration samples. The preferred procedure is to take several low-level samples (four to six) and calculate a pooled estimate of precision at these levels. Prior to pooling the separate estimates of precision, they should be tested for consistency with a conventional F test (two samples) or Cochran's test, [19] if there are more than two samples. If the test fails, the root cause needs to be investigated by the manufacturer/developer. It can indicate instability of the reaction or sample affecting the variability. A *tentative* estimate, LoD_t, is then obtained as:

$$LoD_{tent} = LoB + c_\beta SD_S \quad (6)$$

where SD_S is the estimated standard deviation of the sample distribution at a low-level and c_β is derived from the 95th percentile of the standard Gaussian distribution (and the correction factor), which is applied because the SD_S is a biased estimate for the population standard deviation σ_S. If the number of measurements N_S is not too small, c_β =1.645/(1-1/(4 x f)), where f is the degrees of freedom of the estimated standard deviation SD_S.

For example, if 60 results (N_S = 60) obtained from five (K = 5) low concentration samples, then degrees of freedom, f, for the pooled estimated standard deviation SD_S is 55 (f = N_S - K) and c_β = 1.645/(1-1/220)=1.653.

NOTE: It is not necessary or desirable to obtain all measurements on exactly the same low concentration sample. This is because it is only necessary to estimate SD_S (the SD for measurements at low concentrations). Using multiple samples will help account for typical subject variability. This procedure is demonstrated below in Section 4.3.3.

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4.3.2.1 Considerations for the Standard Deviation

It is important then to consider whether the SD_S at the measured level(s) is likely to be the same as at the LoD_t level. In estimating the LoD, a common problem is that the sample standard deviation is not constant, because it frequently increases with the sample concentration. However, over a limited range of low concentrations, it should be approximately constant and the outlined procedure is appropriate (see example below). If you can assume a constant SD_S, then the $LoD = LoD_{tent}$.

Similarly, if the relative standard deviation (or CV) is constant, it may be used to estimate SD_S at a trial LoD, and thereby determine the LoD using the above formula. Otherwise, a more complicated approach may be undertaken, in which it is assumed that the sample standard deviation is a function of the concentration level.[1,2]

4.3.2.2 Considerations for the Distribution Shape

If the low sample data are not Gaussian, then it may be possible to transform the data to make it more Gaussian, for example, by logarithmic transformation. If this is done, the LoD is calculated in transformed units, and then converted into original units by the reverse transformation process (e.g., exponential).

If it is not possible to produce Gaussian data, but the SD_S is reasonably constant, then a nonparametric dispersion measure might be used. This nonparametric estimate can be calculated as the distance from the β percentile of the distribution of measurements to the assigned value (or accepted reference value) of the low sample. Call this distance $D_{S,\beta}$; $D_{S,\beta}$ is analogous to $c_\beta * SD_S$, therefore:

$$LoD = LoB + D_{S,\beta}. \quad (7)$$

If the SD_S is not constant, and if it is not possible to produce Gaussian data, then a nonparametric "trial-and-error" procedure must be used. This is a process whereby samples are prepared at a tentative assumed LoD level(s), and a series of measurements are obtained (over time and operators). The β-percentiles are calculated, as the percentage of observations below the LoB. The LoD is the lowest level material where the β-percentile is 5% or less.

These steps are shown in the second flowchart, for procedures B to D.

4.3.3 An Example of Determining the LoD

We consider here a hypothetical hormone assay, for which the manufacturer or a research laboratory wants to estimate the LoD. The default values $\alpha = \beta = 5\%$ are used. Suppose that the manufacturer has ten samples available from subjects lacking the hormone due to disease or pharmacological suppression. Ten measurements are performed on each blank sample on ten different days to ensure that the total assay variation is reflected. Only nonnegative values are provided by the assay, and the distribution of the 100 blank measurements is skewed because of truncation (see Figure 3). Thus, the LoB is estimated nonparametrically as the 95th percentile of the measurements. The 15 highest blank values are displayed in Table 1. The 95th percentile corresponds to the 95.5 ordered observation (=100 x (95/100) + 0.5). Linear interpolation between the 95th and 96th observation yields an LoB estimate of 0.0544 U/L (= 0.0539 + 0.5 x (0.0548-0.0539)).

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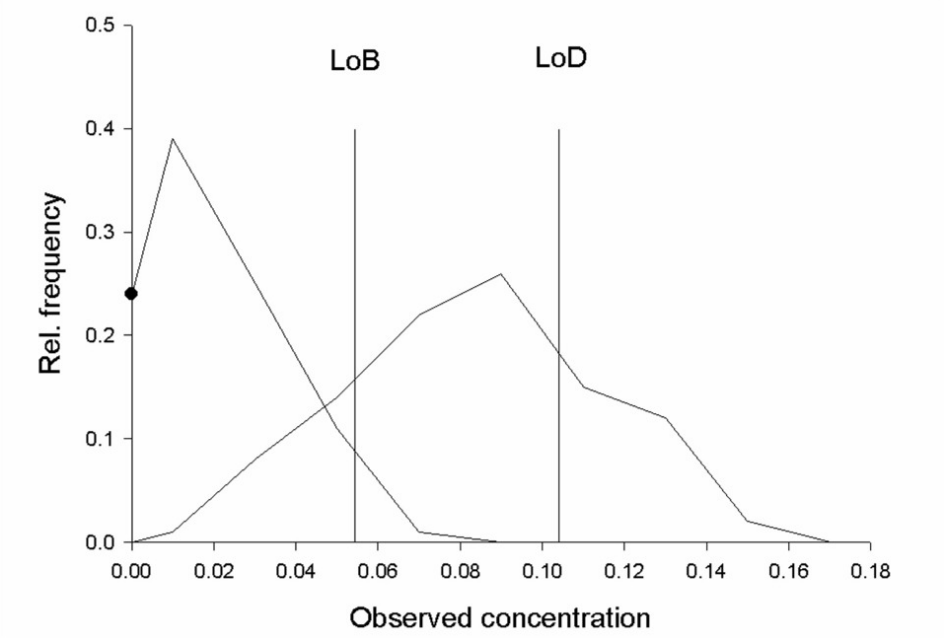

Figure 3. Recorded Distributions of 100 Blank and 100 Sample Values for the Hypothetical Hormone Example. The estimated LoB (= 95th percentile of the distribution of blank values) and the estimated LoD are indicated. SD_S was derived from the distribution of sample values (actually as a pooled estimate of sets of ten measurements that are here merged together).

Table 1. Upper 15 Blank Values (U/L) for the LoD Establishment Example

Ranks	Blank values
100	0.0653
99	0.0603
98	0.0560
97	0.0554
96	**0.0548**
95	**0.0539**
94	0.0538
93	0.0488
92	0.0488
91	0.0470
90	0.0468
89	0.0451
88	0.0443
87	0.0440
86	0.0426
85	0.0419

Samples with low concentrations (in the range of 0.05 to 0.20 U/L) are obtained from subjects. We suppose here that one sample is obtained from each of ten subjects, and that each sample is assayed ten times. A visual inspection suggests that the distributions are symmetric and close to a bell shape so that parametric statistics can be used. A visual review determined that the SD_S estimates were not related to the level in these samples. Thus, a pooled estimate of the SD_S (= 0.0299) was computed as described in Appendix B (in this case, the square root of the average of the variances). The degrees of freedom are 10 x (10 - 1) = 90 and $\alpha = 0.05$, so $c_\beta = 1.6449/(1-1/360)) = 1.6494$. An estimate of the LoD is then obtained:

$LoD = LoB + c_\beta SD_S = 0.0544 + 1.6494 \times 0.0299 = 0.104$ U/L.

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4.3.4 Procedure to Verify a Claimed LoD

A laboratory may want to ensure that an assay fulfills the claim of a given LoD stated by a manufacturer, rather than establishing its own LoD. If available, the laboratory should use the LoB provided by the manufacturer, but should verify this LoB with at least 20 replicates of a blank material. If no more than three replicates exceed the claimed LoB, then this LoB should be used. If the LoB is not available from the manufacturer, then estimate the LoB as described in Section 4.3.1. Then perform repeated measurements of sample(s) with concentration equal to the claimed LoD, and estimate the proportion of results exceeding the LoB. It is recommended that a minimum of 20 sample measurements be used and that these measurements, where possible, come from a variety of samples and over several days. If the recorded proportion is in agreement with the expected value (1-β; default 95%), that is, if "95%" is contained within the 95% confidence limits for the recorded proportion, then the data support the claim of the LoD. It is possible to have more than one measurement result in 20 below the LoB and still meet this criterion. Table 2 displays for the sample sizes 20 to 1,000 the lower bounds for the recorded proportion that are in agreement with the expected 95% proportion.[18] An example of verification is shown in Section 4.3.5.

If the recorded proportion is not in agreement with the expected 95%, then the claimed LoD is not verified. The user could consider contacting the manufacturer or establishing an LoD independently, as described in Section 4.3.2. This might be accomplished by using existing data and rerunning a low positive sample at a slightly higher concentration.

Table 2. Lower 95% Confidence Bounds for Observed Proportions of Results Exceeding the LoB With the Expected Proportion 1-β = 95% (modified from Linnet & Kondratovich[18])

N	Lower bound of observed proportion (%)
20	85
30	87
40	88
50	88
60	88
70	89
80	89
90	90
100	90
150	91
200	92
250	92
300	92
400	93
500	93
1,000	94

These steps are shown in the third flowchart, for procedures C to D.

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4.3.5 An Example of Verifying a Claimed LoD

A given analytical procedure is claimed by the manufacturer to have an LoD of 45 U/L with $\alpha = \beta = 5\%$. The user carries out 25 blank measurements (five measurements of five blank samples over five days) and 25 measurements of samples spiked with 45 U/L of the analyte (five measurements of five samples over five days). The measurements are recorded in Table 3 and displayed in Figure 4. Visual inspection reveals that the distribution of blank values is asymmetric, and accordingly, the LoB is estimated nonparametrically. The blank values are ranked according to size as displayed in Table 3. The 95th percentile corresponds to the 24.25 ordered observation (= 25 x 0.95 + 0.5). Linear interpolation between the 24th and 25th observations yields LoB = 19.17 U/L (= 18.01 + 0.25 x (22.65 - 18.01)).

Table 3. Values for the LoD Verification Example

Ranks	Blank values	Sample values
1	0	18.80
2	0	19.02
3	0	26.63
4	0	26.91
5	0	31.08
6	0	33.99
7	0	35.11
8	0	35.90
9	0	36.12
10	1.08	41.67
11	1.92	43.90
12	2.38	46.32
13	2.98	47.77
14	3.80	47.99
15	4.78	48.83
16	7.30	54.67
17	8.81	57.30
18	10.31	59.10
19	11.29	61.17
20	13.48	61.96
21	14.39	62.97
22	16.97	66.44
23	17.40	73.44
24	**18.01**	73.80
25	**22.65**	75.71

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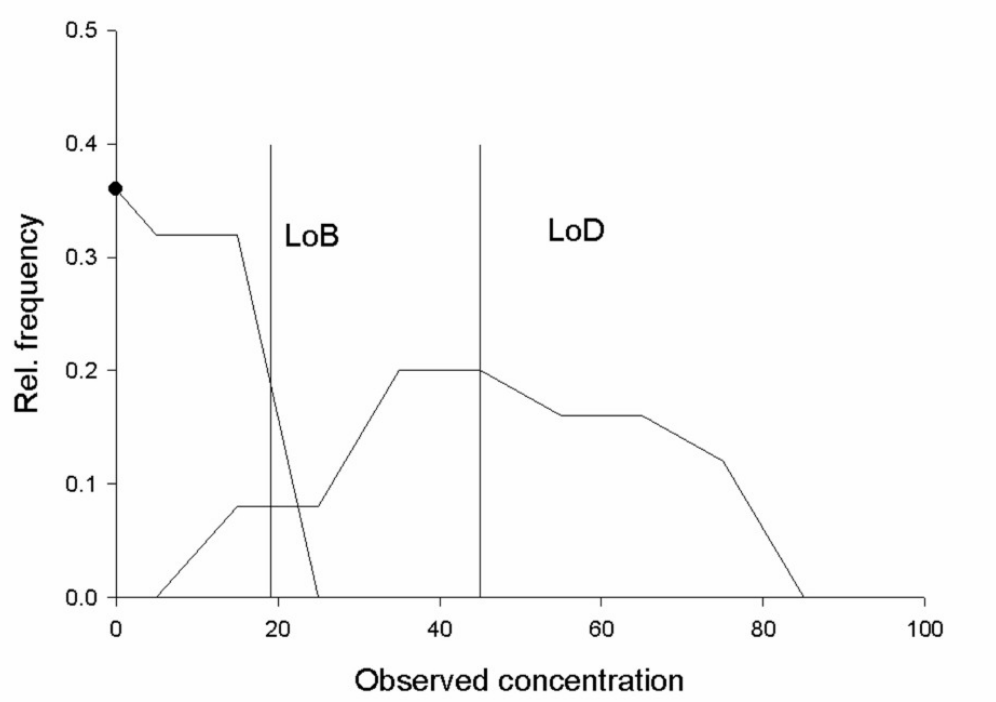

Figure 4. Example Illustrating the LoD Verification Procedure. Distributions of 25 blank measurements and 25 measurements of samples were spiked with the analyte so that the actual concentration equals the claimed LoD of 45 U/L. The LoB was estimated to 19.17 U/L (95th percentile of the blank distribution). Ninety-two percent (23/25) of the sample measurements exceeded the LoB.

A set of measurements of five spiked samples with actual concentration of 45 U/L is shown in Table 3. The proportion of sample measurements that exceed the LoB is 92% (= 23/25). From Table 2, it is observed that 92% is higher than the lower bound (85%) for agreement with 95% for the sample size of 25. Thus, the observed proportion is in accordance with the expected one of 95% according to the claim, and the present evaluation does not contradict the claim.

5 Limit of Quantitation (LoQ)

The LoQ is the lowest actual amount of an analyte that can be reliably detected (the LoD), *and* at which the total error meets the laboratory's requirements for accuracy (acceptable for clinical use). Depending on the defined goal for error, the LoQ could be equal to the LoD or it could be much higher. It could not be lower than the LoD. The LoQ should be determined as part of the development of a method and it may be claimed by manufacturers (perhaps labeled differently). Laboratories may wish to establish their own LoQ, or verify a manufacturer's claim. It may not be necessary to determine the LoQ for every method, if the uncertainty (or total error) of measurement can be determined for low-levels. In these instances, it may be acceptable to report the estimated uncertainty of every low-level result, and allow the user to interpret whether it is suitable for use.

Establishing goals for accuracy (total error) is beyond the scope of this document. These might originate from, for example, perceived or expressed customer requirements, external regulations, convention, or manufacturer claim. Usually, these are expressed as the maximum amount of measurement error that can be tolerated to be fit for use.

The procedure for estimating the total error for a method follows the most current edition of NCCLS document EP21—*Estimation of Total Analytical Error for Clinical Laboratory Methods.* Alternatively, laboratories may choose to estimate the uncertainty of their measurements following other international consensus procedures.[5,12,20] Regardless of the procedure used, it must be recognized that error and relative

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error can be different than at higher levels, and error can change significantly with small changes in levels.

In the absence of commercial or certified reference materials at sufficiently low-levels, reference materials may be prepared by spiking or by appropriate dilution of samples of known concentration or activity, where linearity into the region of lower concentrations has been adequately determined. Please see the most current edition of NCCLS document EP6—*Evaluation of the Linearity of Quantitative Measurement Procedures: A Statistical Approach.*

5.1 Establishing the Limit of Quantitation

The test results from the LoD study should be used to estimate bias and imprecision for that level of analyte. For this protocol, a minimum of 40 replicates is recommended, from three to five different samples and determined from at least five runs. The difference between the mean of the replicates (if one sample is used) and the accepted reference value is an estimate of bias. If more than one sample is used at that level, then the average of the differences is the estimate of trueness. The total standard deviation of the 40 results (one sample) or the pooled precision estimate SD_S (see Appendix B) is the estimate of precision. Combine these to get an estimate of total error at that level, using Bias + 2 x SD_S = Total Error (if Bias is negative, use Total Error = - (Bias - $2SD_S$)). If this estimate is less than the defined goal for total error, then: LoQ = LoD.

This procedure ensures that, for samples with a true value equal to the LoQ, there is an approximate 95% probability that the test result will be sufficiently accurate. If a higher probability is needed, then SD_S should be multiplied by a larger factor; for example, if Bias + $4SD_S$ and Bias - $4SD_S$ are within the goal (and error is Gaussian), then more than 99.5% of results will be fit for use.

If the goal is not met at this level, then slightly higher levels must be tested. Suitable reference materials must be obtained, similar to the materials used to determine the LoD. As before, the actual concentration must be known by independent means, either by testing with a reference method, by spiking, or, where appropriate, by dilution. If the materials are manufactured, then the uncertainty of the dilution factor and/or spiking process should be included with the estimates of uncertainty or for total error derived from the tests on the material.

If a laboratory determines an LoQ that is higher than the lowest level of the assumed measuring range (or reportable range), then the measuring range may not be suitable for the intended use in the laboratory. If the lower 95% confidence limits for the estimates of bias and imprecision obtained from this exercise are greater than what has been claimed for the method, then the manufacturer should be contacted.

5.2 Verifying a Claimed LoQ

If a laboratory wishes to confirm an LoQ or if the error cannot be assumed to be Gaussian, then an alternative procedure can be used to check a claim for LoQ. This requires all the considerations in Sections 5 and 5.1 to be followed, with the exception of calculating SD_S and estimating total error, and it is acceptable to use a minimum of 25 replicates. In this case, the replicates on each sample are compared to the reference value for that sample and the goal for error. The number of such results that exceed the error goal is a measure of the suitability of the method at that level. Table 2 can be used to determine the number of "unacceptable" results that could be observed, if a criterion of 95% is used. For example, if there are a total of 30 replicates (over some number of samples), then Table 2 tells us that at least 87% of results (26.1, rounded up to 27) must be within the error goal. Therefore, if zero to three results have excessive error, the LoQ can be assumed to be verified. If there is concern about this result (in this example, allowing 10% of samples to have excessive error), then more replicates should be obtained.

If this criterion is not met, then the claimed LoQ should be questioned. These steps are shown in the fourth flowchart, for procedure D to the end.

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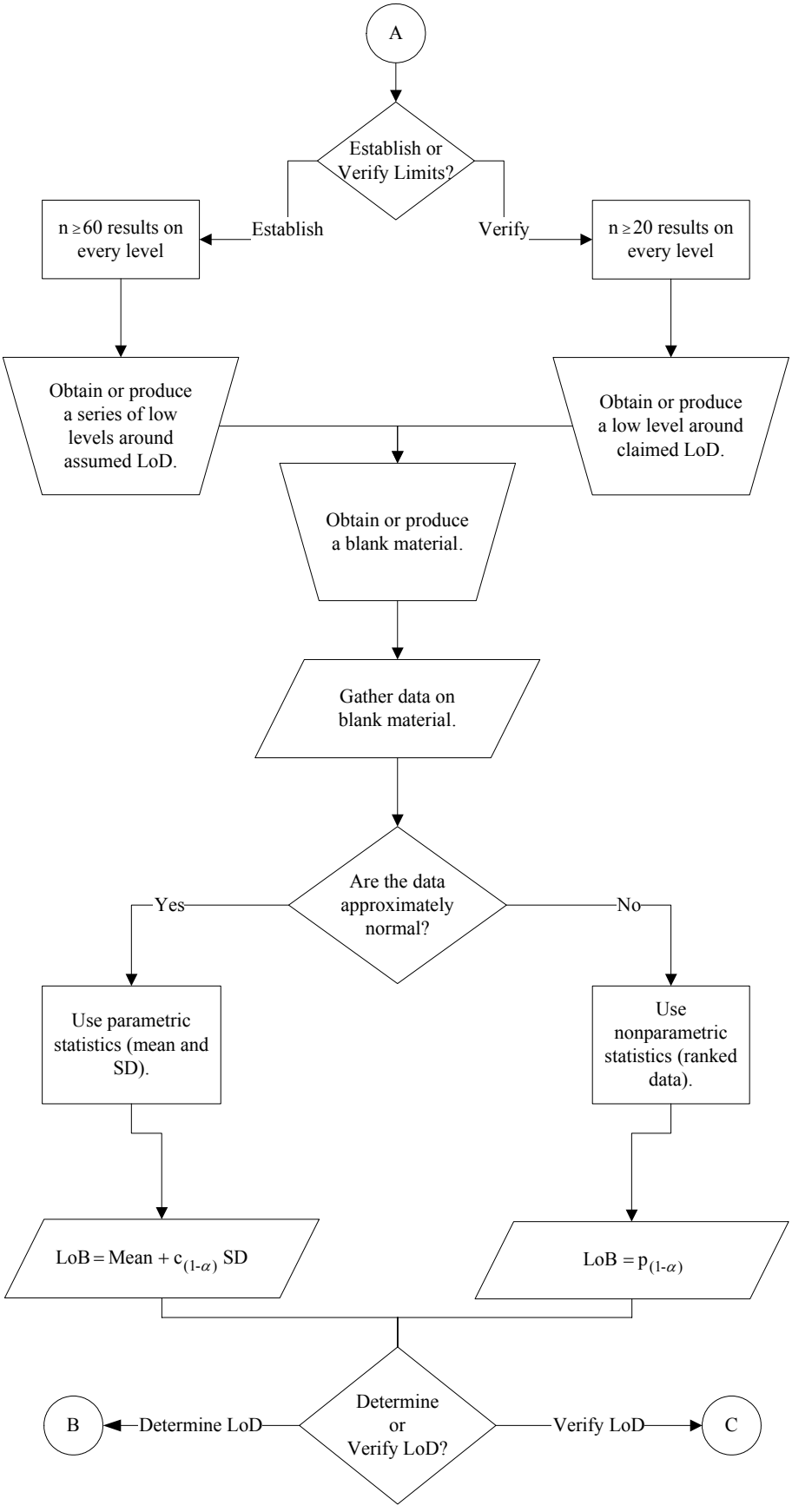
Procedure to Determine LoB



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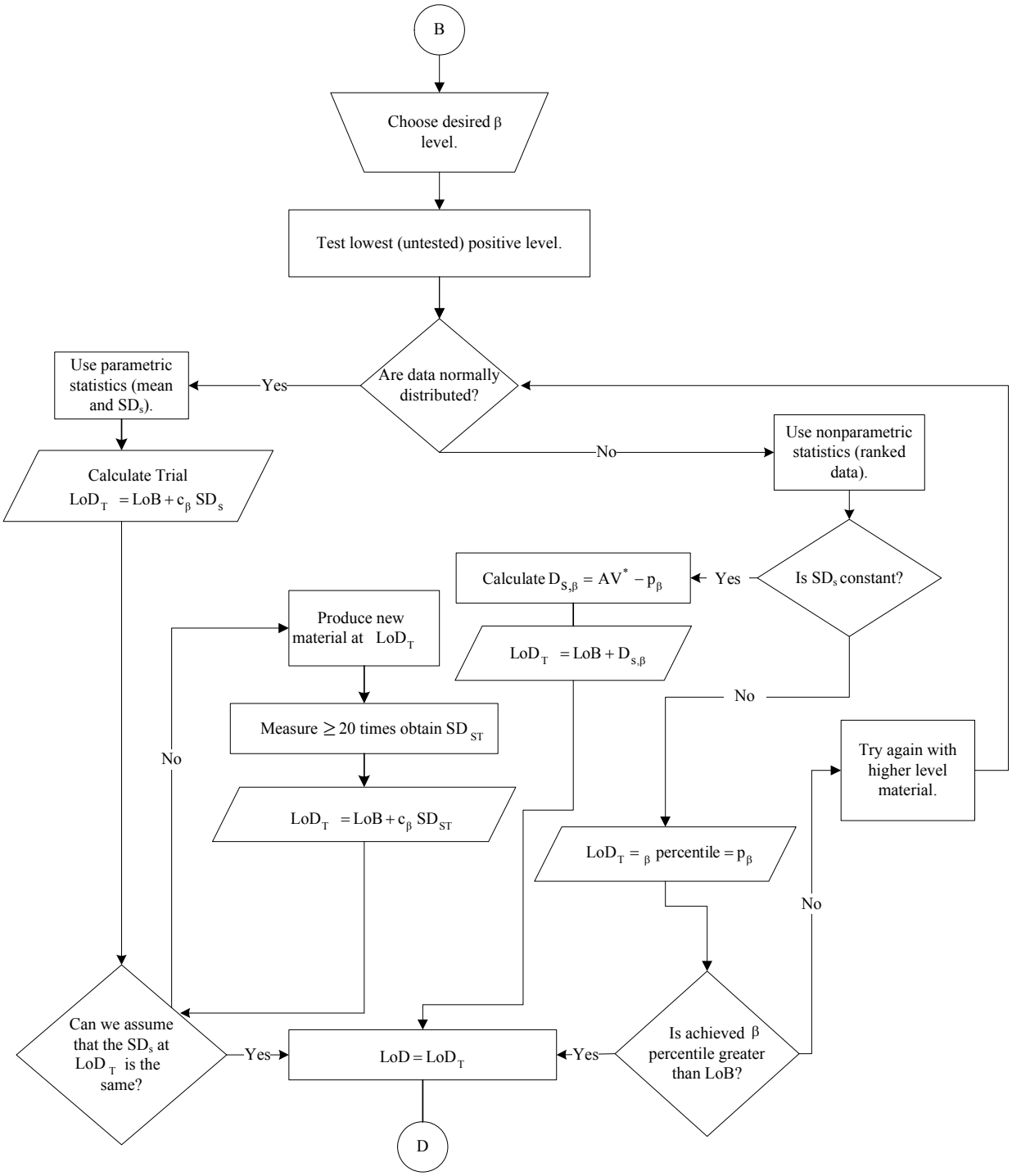
Procedure to Determine LoD



*AV = Assigned value of low concentration material (see Section 4.3.2.2)

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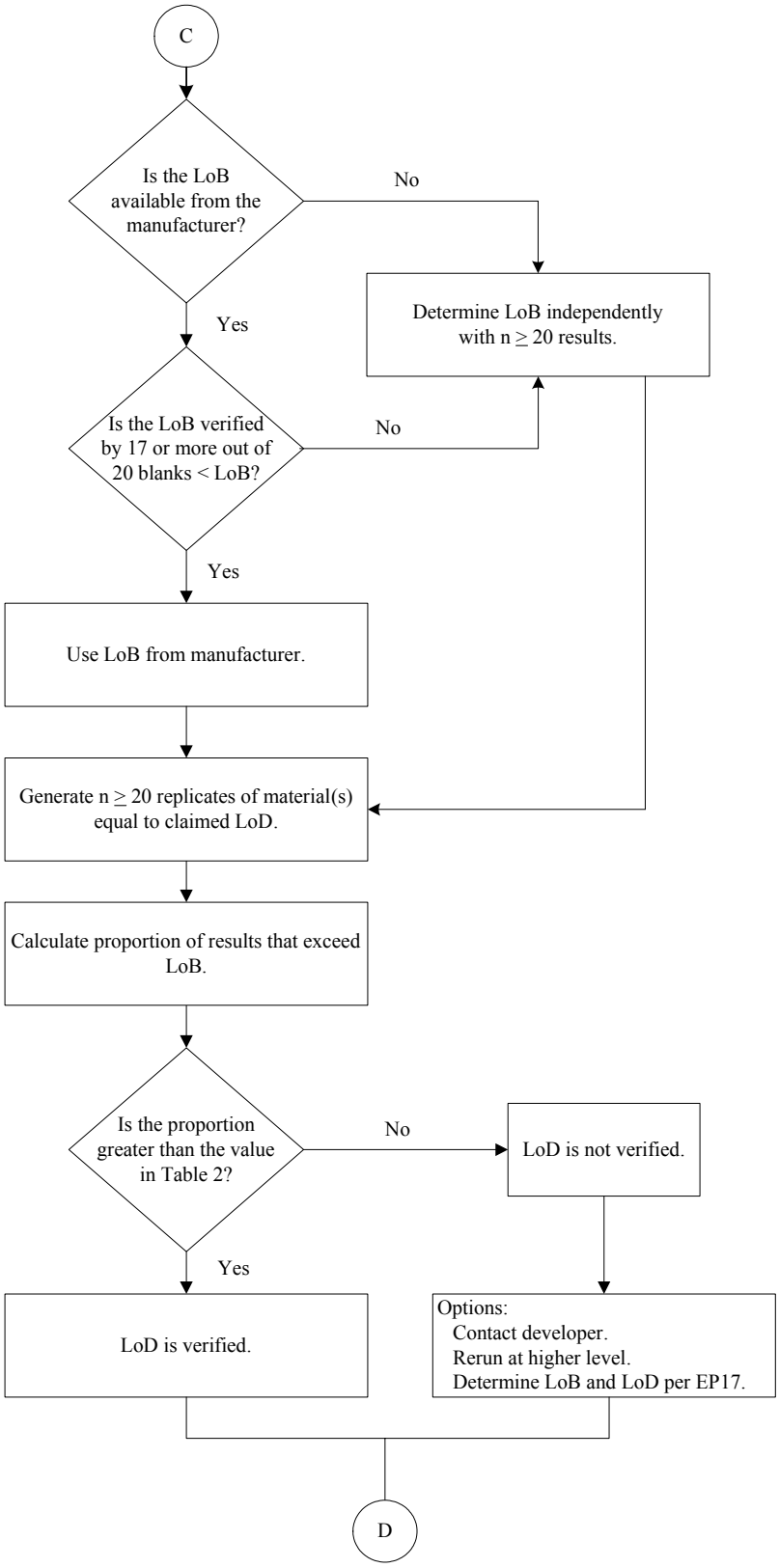
Procedure to Verify a Claimed LoD
(Assume β = 5%)



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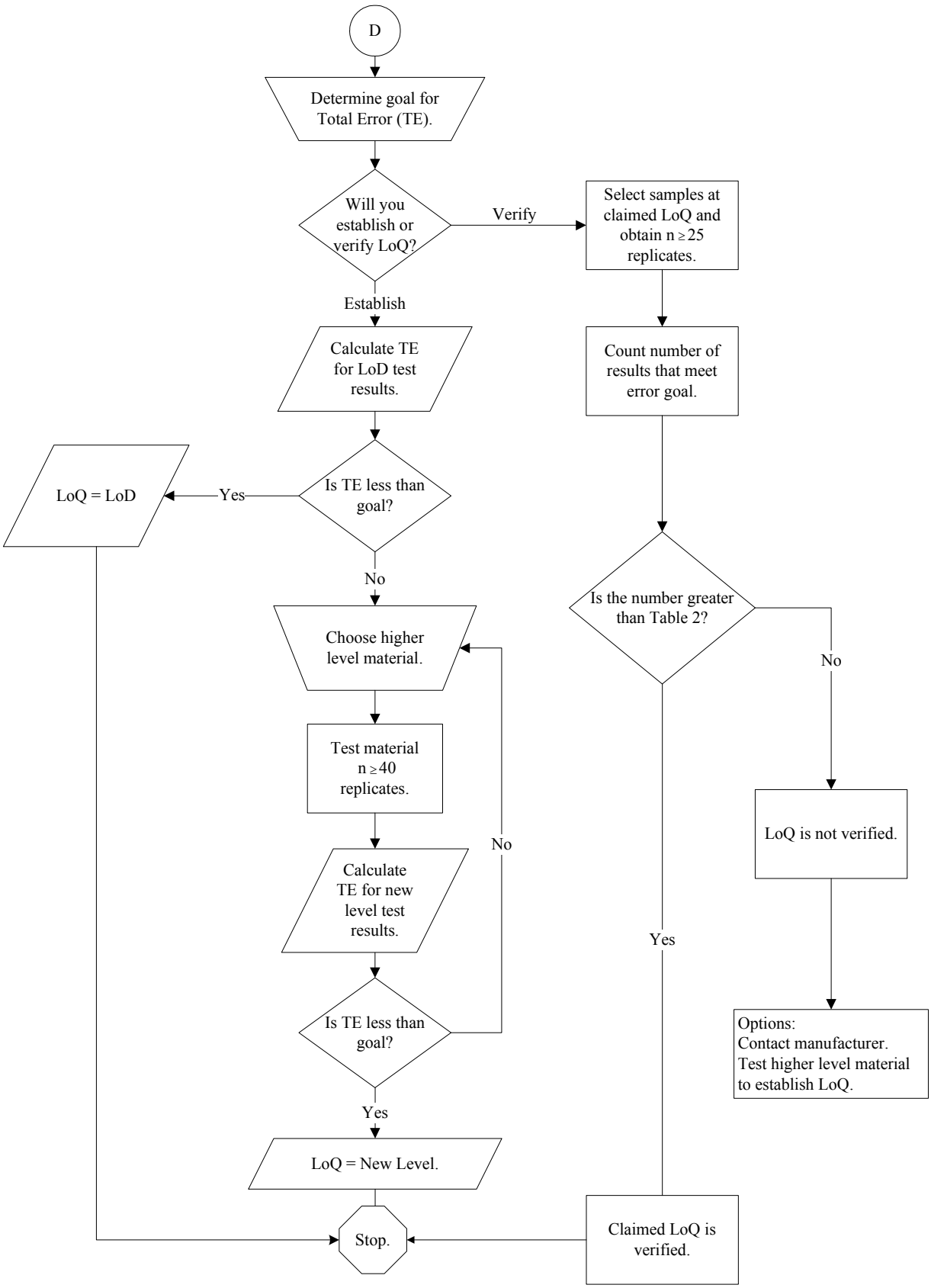
Procedure for LoQ



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6 Reporting Results

6.1 Reporting Intervals for Quantitative Results

Having established the various limits according to this protocol, a manufacturer or method developer may wish to report LoB, LoD, and LoQ in the package insert or method description. If the manufacturer or method developer chooses to report these limits, they must be accompanied by all alpha and beta probabilities, goals for stated levels of precision and trueness, and other design characteristics.

Reports to clients should depend on where the observed result lies relative to the limits and on the particular laboratory procedures. If a laboratory wishes to report the most complete information, including a "gray zone" of uncertain quantitation, then the following is appropriate:

Result ≤ LoB	report "not detected; concentration < LoD"
LoB < Result < LoD	report "analyte detected; concentration < LoQ"
LoD ≤ Result < LoQ	(a) report "analyte detected; concentration < LoQ"[a]; or (b) report the result with a caution about possibly higher uncertainty
Result ≥ LoQ	report the result

If a laboratory chooses to report only quantitative results or "less than" determinations, or report in the simplest terms, then the following is appropriate:

Result ≤ LoB	report "concentration < LoD" or "not detected"
LoB < Result < LoQ	report "concentration < LoQ"[a] or "detected"
Result ≥ LoQ	report result as measured

[a] The "gray zone" of uncertain quantitation exists on both sides of LoQ, so this statement is not exactly correct. However, this recommendation is preferred for practicability.

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These reporting schemes and the various limits are shown in Figure 5.

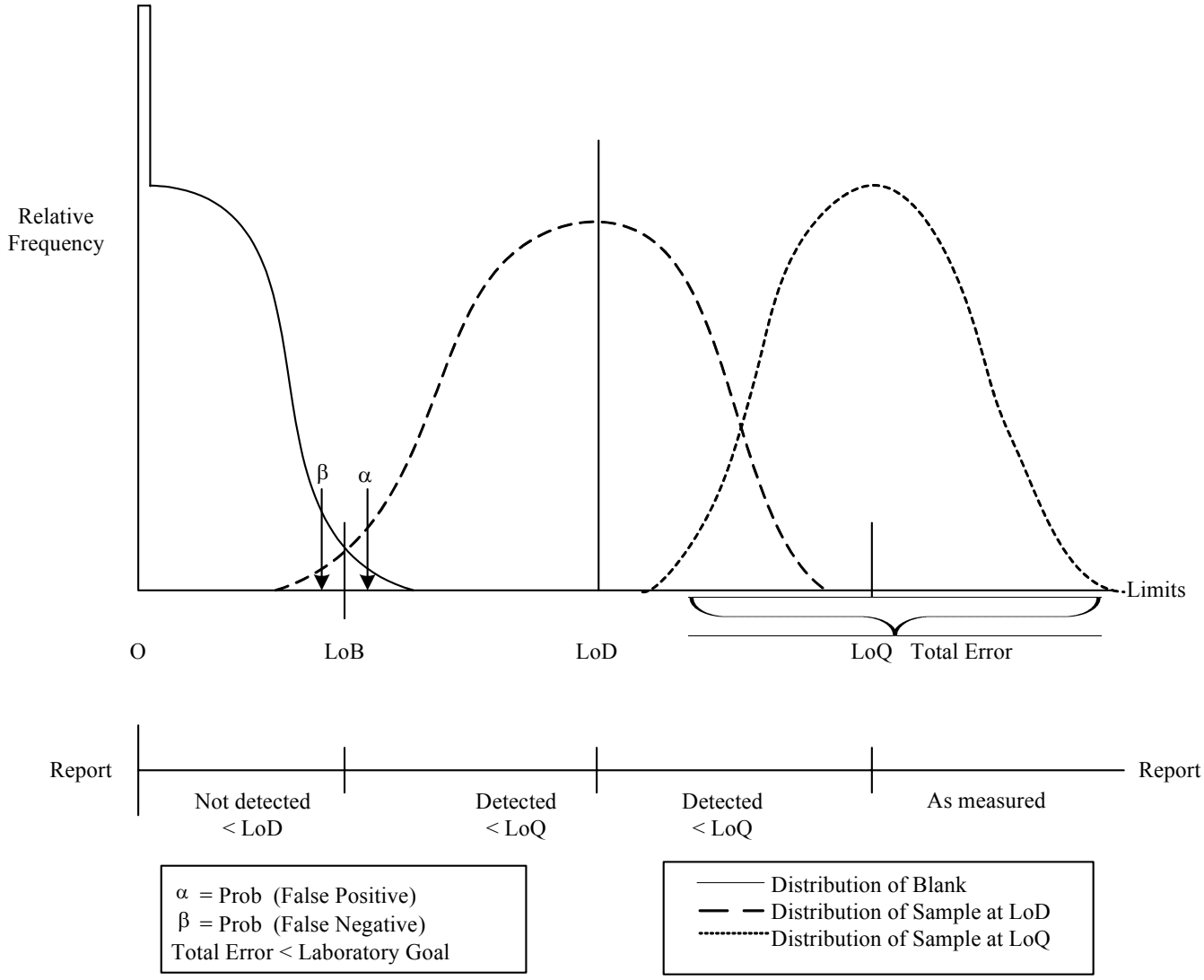

Figure 5. Distribution of Results for Blank, Low Positive at LoD, and Low Positive at LoQ (Report recommendations are shown for results at various points relative to limits.)

6.1.1 Example

For a method where the LoB is 6 mmol/L, the LoD is 8 mmol/L, and the LoQ is 10 mmol/L. The following are options for reports that could accompany various test results:

For a laboratory reporting the most complete information:

Result	Report
5 mmol/L	"substance not detected; concentration < 8 mmol/L"
7 mmol/L	"substance present, cannot be quantified; concentration < 10 mmol/L"
9 mmol/L	(a) "substance present, cannot be quantified; concentration < 10 mmol/L"; or (b) "result = 9 mmol/L, but should be interpreted with caution due to a higher level of uncertainty" (report uncertainty, if requested)
11 mmol/L	"result = 11 mmol/L" (report uncertainty or goal, if requested)

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For a laboratory reporting only quantitative results:

Result	Report (1)	Report (2)
5 mmol/L	"concentration < 10 mmol/L"	"not detected"
7 mmol/L	"concentration < 10 mmol/L"	"detected; < 10 mmol/L"
9 mmol/L	"concentration < 10 mmol/L"	"detected; < 10 mmol/L"
11 mmol/L	"concentration = 11 mmol/L"	"11 mmol/L"

6.1.2 Cautions on Interpretation of Quantitative Results

Measured values less than the LoQ but greater than the LoB may be used to show that the analyte is present, but the actual measured levels should not otherwise be used for clinical interpretation. Results between the LoD and LoQ could be reported if there is a caution regarding higher uncertainty in the result.

LoD and LoQ may be the same, or there may be some difference, depending on the uncertainty at the LoD and the laboratory's goals. If there is a gap, one cannot assume that the response between the LoD and the LoQ is linear, or even that it is a monotonic increasing function. Even though the assumption of monotonicity is true in most cases, instances may occur where that relationship does not exist, such as discontinuities, zero values, etc. Without clear documentation of a monotonic relationship, interpretations about concentrations in this range are not recommended.

In routine testing, the LoD and LoQ should be used as above to decide on how to report results. There are other situations, such as when the average of replicates is used as a subject's result, or for scientific studies, when laboratories should record the measured concentrations (if available), irrespective of whether the values are below or exceed the LoD. If truncated results are used in these situations, any averages will be biased.

6.2 Example of a Claim in Labeling

Reports of LoD should be as follows, unless the method developer provides alternative wording that contains this information. If any claim is made for conformance with this protocol it must include the actual measurand, the level of confidence (α and β), and the LoB.

Based on n determinations—n_1 blank determinations and n_2 positive determinations—the limit of detection for measurand is x^*, with a stated probability (e.g., 95%) of obtaining a positive response (a result that exceeds the LoB) at that level ($1-\beta$) and a stated probability of obtaining a negative response on blank samples ($1-\alpha$). An LoB of y^* was used.

*[mmol/L, mg/L, activity units, or other appropriate units].

For example:

"The limit of detection (LoD) for C-reactive protein is 3 mg/L, determined consistent with the guidelines in this NCCLS protocol and with proportions of false positives (α) less than 5% and false negatives (β) less than 5%; based on 130 determinations, with 70 blank and 60 low-level samples; LoB = 1 mg/L."

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[18] Linnet K, Kondratovich M. A partly nonparametric procedure for determination of the limit of detection. *Clin Chem.* 2004;50:732-740.

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Appendix A. Standard Error of LoD and Estimation of Sample Size

Linnet and Kondratovich* have described the formula for the standard error of the LoD when α and β are set to 0.05. The formula is as follows:

$$SE(LoD) = \sqrt{\left[\left(2.114^2 \times \frac{\sigma_B^2}{N_B}\right) + \left(1.645^2 \times \frac{\sigma_S^2}{2(N_S - K)}\right)\right]}$$

With σ_B = (pooled) SD of results on blank sample(s)
σ_S = (pooled) SD of results on low-level positive sample(s)
N_B = number of blank measurements
N_S = number of low positive sample measurements
K = number of low-level positive samples

For example, if $\sigma_B = 1.0$, $\sigma_S = 1.5$, $N_B = N_S = 100$, and K=10

SE(LoD) = √[(4.469(1/100)) + (2.706(2.25/180))] = 0.2802

If we assume that $N_B = N_S - K = N$ and we assume that $\sigma_S/\sigma_B = m$, then the equation simplifies to:

$$SE(LoD) = \sqrt{\frac{\left(4.469\sigma_B^2 + 1.3530\sigma_B^2 m^2\right)}{N}}$$

$$SE(LoD) = \sigma_B\sqrt{\frac{\left(4.469 + 1.3530m^2\right)}{N}}$$

If a goal is specified for the SE(LoD) (call it G), and α and β are set to 0.05, then the required sample size is calculated as follows:

$$N = \frac{\sigma_B^2\left(4.469 + 1.3530m^2\right)}{G^2}$$

For example, in the above situation where $\sigma_B = 1.0$ and $\sigma_S = 1.5$, then $m = 1.5$.

If we have a goal of SE(LoD) ≤ 0.30, then

$N = 1(4.469 + 1.3530(1.5)^2) / (0.3)^2 = 83.48$.

So, it would require at least 84 replicates of the blank sample and about the same number (84 + K) of replicates of low positive sample in order to meet the design requirements.

* Linnet K, Kondratovich M. A partly nonparametric procedure for determination of the limit of detection. *Clin Chem.* 2004;50:732-740.

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Appendix B. Estimation of SD_S

Procedure to Calculate the Pooled SD_S From Replicates of More Than One Sample

When SD_S is estimated from repeated measurements of several samples, a pooled estimate is formed from the weighted average of the variances (squared SD_S) as:

$$SD_S^2 = (n_1 SD_{S1}^2 + n_2 SD_{S2}^2 + n_3 SD_{S3}^2 + \ldots\ldots + n_n SD_{Sn}^2)/(n_1 + n_2 + n_3 \ldots\ldots\ldots + n_n)$$

where n_1, n_2, …. refer to the degrees of freedom for the subsamples ($n_1 = N_1 - 1$, etc.).

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Appendix C. Example

This data set is for a manual method for total mercury in blood, measured as μg/L. There are 117 measurements on a variety of blank samples, but with no indication of samples or replicates, so all measurements are considered independent. This set includes values less than zero. A subset of these results (n = 30) had originally been used to establish an LoD based on the conventional Mean + 3SD model; this limit was set at 0.14 μg/L. The raw data have a symmetric, Gaussian appearance, as shown in Figure C1.

NOTE: The original 30 results must have had a much lower SD and possibly a smaller mean, since the claimed LoD is quite small for the distribution of all 117 results; in fact, 11 of the 117 results (9.4%) were larger than the claimed LoD.

If the distribution indeed was normal, then only 0.27% of the results, which would be less than 1 out of 117, would exceed the above LoD. This shows that estimates obtained with relatively small sample sizes are not reliable.

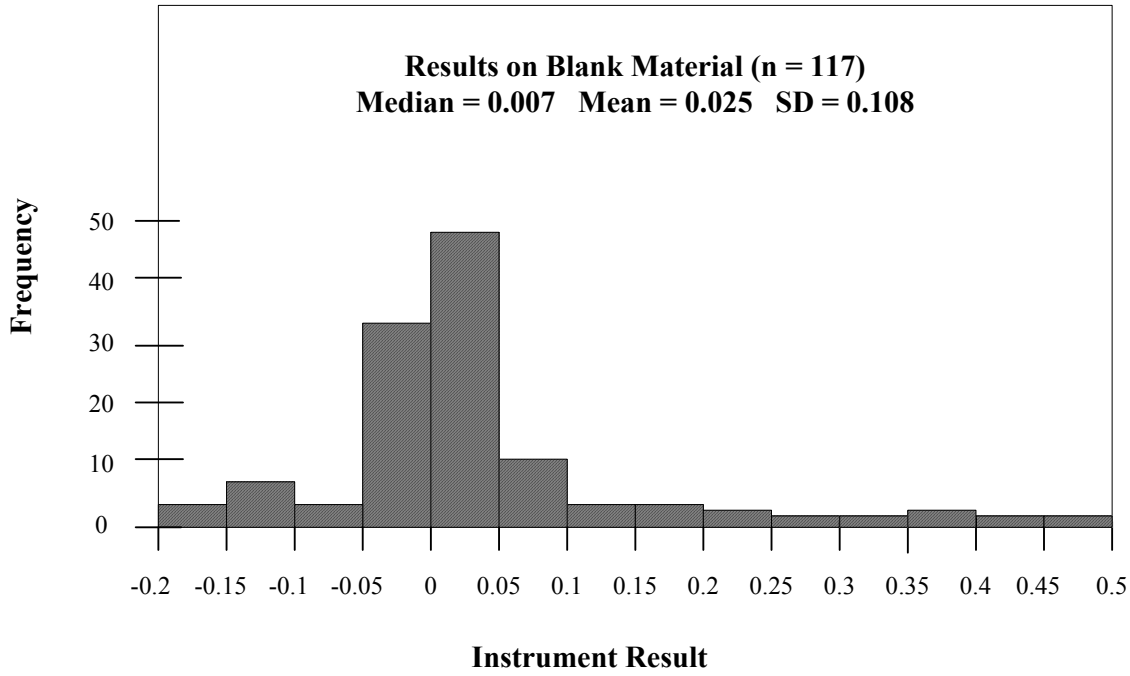

Figure C1. Mercury in Blood: Distribution of 117 Results on Blank Material

Part 1. Parametric Calculations on Raw Data

Figure C1 shows the distribution of all 117 blank results. From these data, we calculate:

Raw median = 0.065
Raw mean = 0.025
Raw SD_B = 0.108

These data can be used to determine an LoB following the parametric procedure described in this document, as in Equation (1):

$$\mathbf{LoB = \mu_B + 1.645\ \sigma_B} \quad (1)$$

Since $\boldsymbol{\sigma_B}$ is unknown, we must use the estimated standard deviation, SD_B, with the degrees of freedom of 116 and $c_{.95,116} = 1.6449/ (1 - 1/464) = 1.648$

LoB = 0.025 + 1.648(0.108) = **0.203 μg/L**

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Appendix C. (Continued)

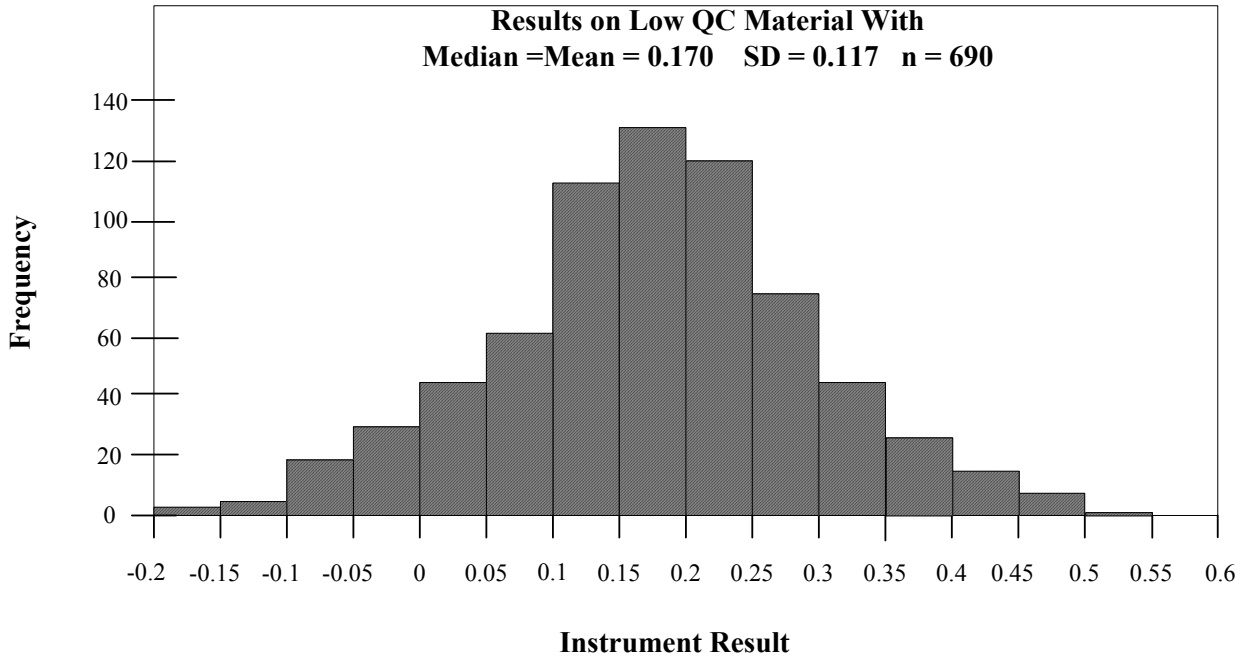

Figure C2. Mercury in Blood: Distribution of 690 Results on Low-Level QC Material

There were also 690 results taken over a long time period, for a stable low-level control material at a level near the claimed LoD. In total, these raw results also are adequately Gaussian, and contain results less than zero. These results are shown in Figure C2, and the calculated statistics are as follows:

Raw median = 0.170
Raw mean = 0.170
Raw SD_S = 0.117

These statistics can be used to calculate the parametric LoD, as described in Equation (6):

$$\mathbf{LoD_t = LoB + 1.645\ \sigma_S} \quad (6)$$

LoD = 0.203 + 1.6449/(1-1/2756)(0.117) = **0.396 µg/L**

Note that the mean for the low QC data is much lower than the calculated LoD. In fact, 425 of the results (β = 62%) are lower than the LoB and therefore would not provide an answer from the laboratory that analyte is detected. A sample that is truly at 0.17 µg/L has a 38% chance of producing a result that exceeds the LoB (β = 62%). Therefore, following the procedures in Section 4.3.3, we see that the claimed LoD of 0.140 µg/L is not supported by these data, and the low positive sample tested is too low for reliable detection.

Given the distance from the tested level and the estimated LoD, we must also consider whether the SD_S at levels near 0.40 µg/L is the same as for the level calculated at 0.17 µg/L. This will be checked later in this example.

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Appendix C. (Continued)

Part 2. Nonparametric Calculations on Truncated Data

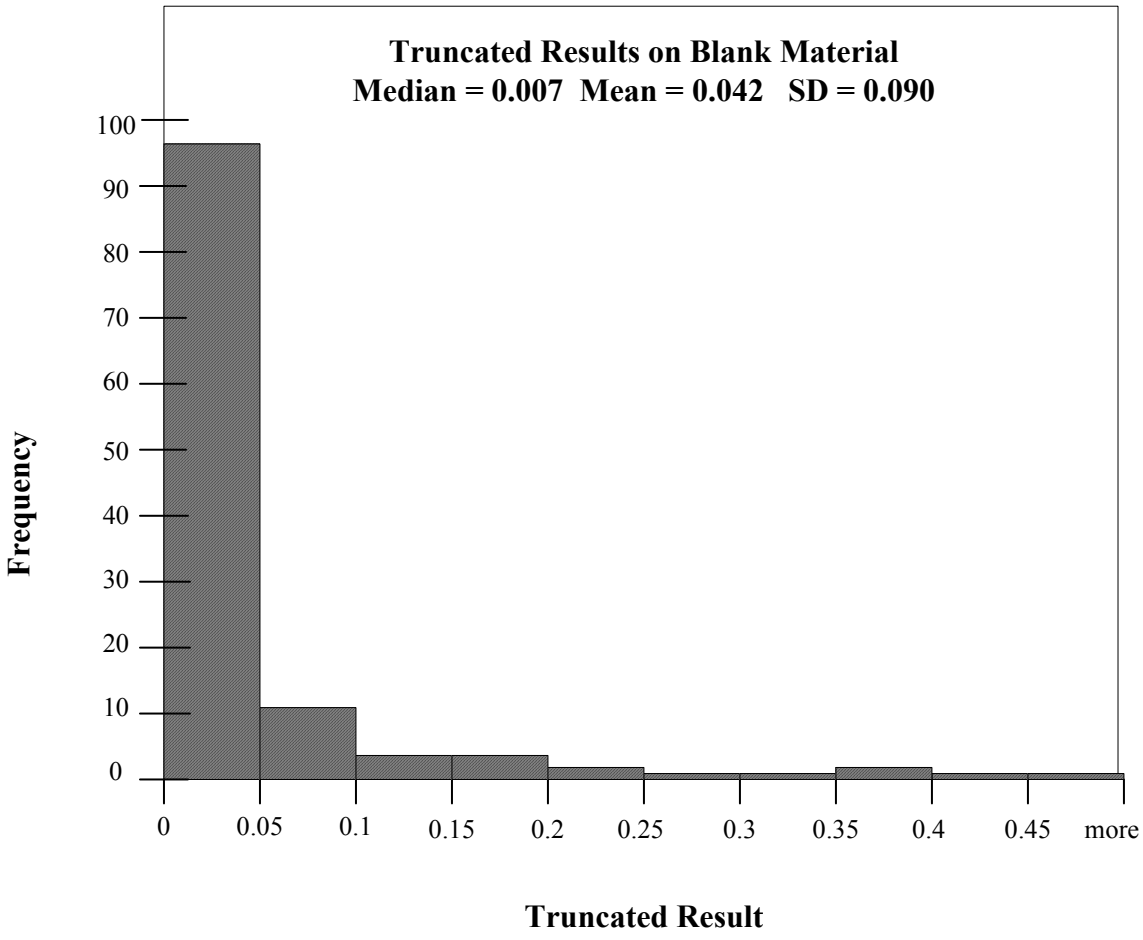

Figure C3. Truncated Results on Blank Material, with Results Less Than Zero Made Equal to Zero

If this method reported only positive results, the distributions would be somewhat different. Figure C3 shows the distribution of the same blank measurements as in Figure C1, but with negative values counted as zero. Note the increase in the mean and decrease in SD, as is typical for truncated data; the median does not change (since more than half of the raw results were positive):

Truncated median = 0.065
Truncated mean = 0.042
Truncated SD_B = 0.090

The nonparametric LoB can be determined by calculating the 95th percentile of the blank distribution, as described in Equation (2), with data taken from Table C1 (highest 15 results on blank):

$$\mathbf{LoB = Pct_{B\ 100-\alpha}} \quad (2)$$

LoB: 95th pctl = 117 x 0.95 + 0.5 = result at position #111.65 = 0.220 + 0.65(0.250 - 0.220) = **0.239 µg**/L

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Appendix C. (Continued)

Table C1. 15 Highest Results on Blank Material

Rank	Result
103	0.081
104	0.110
105	0.110
106	0.120
107	0.160
108	0.170
109	0.180
110	0.210
111	**0.220**
112	**0.250**
113	0.300
114	0.351
115	0.358
116	0.400
117	0.540

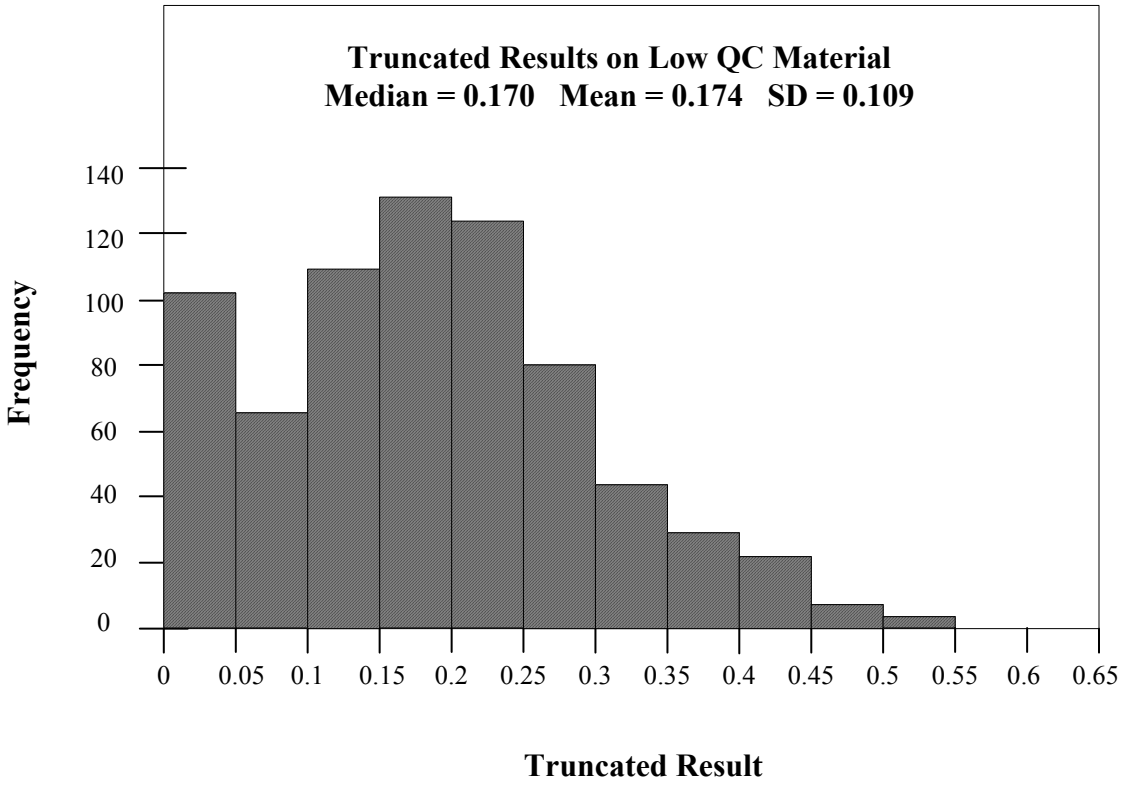

Figure C4. Truncated Results on Low-Level QC Material

Figure C4 shows the truncated data for the low-level QC material. There is a smaller impact from the truncation at zero, but the effect of raising the mean and lowering the SD can still be seen:

Truncated median = 0.170
Truncated mean = 0.174
Truncated SD_S = 0.109

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Appendix C. (Continued)

Since the data on low-level material are not Gaussian (after the truncation), there are two choices for determining the LoD:

1) Use the nonparametric estimate $D_{S,\beta}$, the distance between the assigned value (or median) of the low-level material and the 5th percentile of the distribution of low-level responses, as described in equation (7):

$$\mathbf{LoD = LoB + D_{S,\beta}} \quad (7)$$

with $D_{S,\beta}$ calculated as the median minus the result in position (690 x 0.05 + 0.5) = position 35.

Unfortunately, with these data, rank positions 1 to 52 are zero, so the distance estimate cannot be determined. It is clear, however, that the distance will be greater than the median, so:

LoD > LoB + $D_{S,\beta}$ = 0.239 + 0.170
LoD > 0.409 µg/L

2) The last option is to estimate SD_S using samples at a higher level, and then if data are reasonably Gaussian, the SD can be used to estimate the LoD, using the nonparametric LoB.

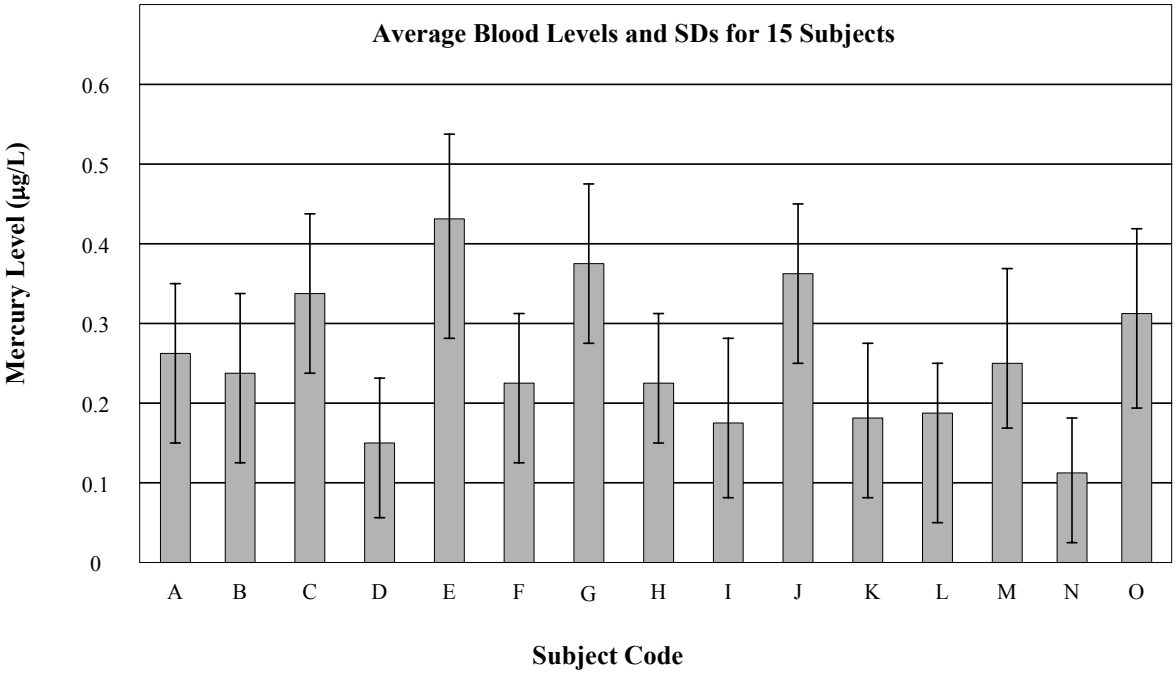

Figure C5. Means and Standard Deviations for 15 Subjects, Each With 20 Results

This was accomplished, as depicted in Figure C5, which shows the means and SD's for 15 subjects with low-levels of mercury in blood. For each sample, the laboratory obtained a series of 20 results over a period of at least four days. The figure shows the means and +/- 1 SD limits around the means. The actual means and SD's are given in Table C2. We can see that there is little, if any consistent difference in SD at different concentrations of mercury in blood. We therefore choose the four subjects with sample averages closest to 0.400 µg/L (subjects C, E, G, and K) and pool those values to obtain an estimate of SD's at that level. The data for these four subjects are shown in Table C3.

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Appendix C. (Continued)

In this example, it is apparent that there is no relationship between concentration and standard deviation at low-levels. The Cochran test shows no significant differences between SD's for any subject. Therefore, at the option of the analyst, the entire set of 15 subjects could be used to generate a more reliable estimate of SD_S. In this example, the overall pooled SD_S is 0.0978 with 15(20 - 1) = 285 degrees of freedom. The LoD would be calculated as:

LoD = 0.239 + 1.65(0.098) = 0.401 μg/L

Table C2. Means and Standard Deviations for Total Mercury in the Blood of 15 Subjects

Subject	Average	Standard deviation
A	0.248	0.0997
B	0.227	0.1079
C	**0.341**	**0.0927**
D	0.141	0.0926
E	**0.411**	**0.1296**
F	0.219	0.0892
G	**0.381**	**0.1004**
H	0.225	0.0826
I	0.183	0.1018
J	**0.364**	**0.1059**
K	0.185	0.0927
L	0.190	0.0538
M	0.258	0.1107
N	0.107	0.0720
O	0.309	0.1127

Table C3. Results for Four Subjects With Levels Closest to 0.400

Replicate	Subject C	Subject E	Subject G	Subject J
1	0.3825	0.26954	0.4112	0.160
2	0.280	0.460	0.4403	0.390
3	0.279	0.2611	0.4025	0.430
4	0.4978	0.280	0.4317	0.370
5	0.3086	0.2501	0.4719	0.190
6	0.390	0.271	0.3313	0.310
7	0.5457	0.251	0.3081	0.530
8	0.350	0.210	0.2131	0.360
9	0.390	0.460	0.4859	0.350
10	0.221	0.620	0.4480	0.420
11	0.2892	0.501	0.2352	0.40
12	0.2419	0.461	0.4726	0.340
13	0.2686	0.402	0.4320	0.210
14	0.3156	0.480	0.2252	0.500
15	0.25314	0.5559	0.5300	0.440
16	0.2985	0.5830	0.3793	0.340
17	0.2982	0.5193	0.5126	0.390
18	0.3002	0.5211	0.3103	0.420
19	0.4739	0.370	0.350	0.210
20	0.4424	0.501	0.2320	0.520

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Appendix C. (Continued)

$SD_S^2 = (n_1 SD_{S1}^2 + n_2 SD_{S2}^2 + n_3 SD_{S3}^2 + \ldots\ldots + n_n SD_{Sn}^2)/(n_1 + n_2 + n_3 \ldots\ldots + n_n)$

$= 19(0.0927^2 + 0.1296^2 + 0.1004^2 + 0.1059^2) / (4 \times 19) = 0.0117$

$SD_S = \sqrt{0.0117} = 0.108\,\mu g / L$

Therefore, $LoD = LoB + c_\beta(SD_S)$ with f = 4(20 - 1) = 76 degrees of freedom

$LoD = 0.239 + 1.6449/(1 - 1/304)(0.108) = 0.417\ \mu g/L$

Part 3. Limit of Quantitation

Assume there is no bias, or that the result is traceable to the method (this is called an "empirical method").

Assume further that the laboratory has determined a goal for total error of 0.250 μg/L at levels below 0.5 μg/L.

Check to see if the goal is met:

For the test subject sample data,

$2SD_S = 2 \times 0.108 = 0.216$ μg/L, and the total error is the interval: -0.216 μg/L to 0.216 μg/L.

The total error is within the total error goal (indeed, the interval: -0.216 to 0.216 is within the interval: -0.250 to 0.250).

So, the goal is achieved, and LoD = LoQ.

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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 Subcommittee Responses

EP17-P: *Protocols for Determination of Limits of Detection and Limits of Quantitation; Proposed Guideline*

General

1. This document is very complicated to read. It could be simplified.

- **Evaluating quantitative measurement procedures is sometimes complex. A sentence has been added to the Foreword and the Scope statement to indicate that this document is intended for use by manufacturers and clinical laboratory supervisors.**

2. We could not agree internally that this document would be of benefit to all affected stakeholders.

- **The NCCLS process is designed to benefit the majority of its membership across all constituencies.**

3. It is intensely detailed and not readily usable by the average laboratory technologist.

- **See the response to Comment 1.**

4. The document indicates it is acceptable to report "not detected" without giving a "<" concentration. It appears that it is inappropriate; a "<"along with the concentration is an important piece of information for interpreting the current results; and this information could prove useful if it were necessary to review the results at a later date.

- **In Section 6.1, the document gives two options for reporting "not detected." In both of these options, the "<LoD" is added as recommended information. The laboratory needs to decide how to report results less than LoB, but the actual result and information about the LoD should always be available from the laboratory upon request.**

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NOTES

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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. The approach is based on the model presented in the most current edition of NCCLS document 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 (i.e., operational aspects that define how a particular product or service is provided). 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

EP17-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 on the following page.

Documents & Records	Organization	Personnel	Equipment	Purchasing & Inventory	Process Control	Information Management	Occurrence Management	Assessment	Process Improvement	Service & Satisfaction	Facilities & Safety
					X EP5 EP6 EP9 EP12				EP7		

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

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Related NCCLS Publications*

EP5-A **Evaluation of Precision Performance of Clinical Chemistry Devices; Approved Guideline (1999).** This document provides guidance for designing an experiment to evaluate the precision performance of clinical chemistry devices; recommendations on comparing the resulting precision estimates with manufacturer's precision performance claims and determining when such comparisons are valid; and manufacturer's guidelines for establishing claims.

EP6-A **Evaluation of the Linearity of Quantitative Measurement Procedures: A Statistical Approach; Approved Guideline (2003).** This document provides guidance 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.

EP7-A **Interference Testing in Clinical Chemistry; Approved Guideline (2002).** This document provides background information, guidance and experimental procedures for investigating, identifying, and characterizing the effects of interfering substances on clinical chemistry test results.

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

EP12-A **User Protocol for Evaluation of Qualitative Test Performance; Approved Guideline (2002).** This document contains a protocol that optimizes the experimental design for the evaluation of qualitative tests, to better measure performance and provide a structured data analysis.

* 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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