

Limit of Blank, Limit of Detection and Limit of Quantitation

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Summary

- Limit of Blank (LoB), Limit of Detection (LoD), and Limit of Quantitation (LoQ) are terms used to describe the smallest concentration of a measurand that can be reliably measured by an analytical procedure.
- LoB is the highest *apparent* analyte concentration expected to be found when replicates of a blank sample containing no analyte are tested.
$$\text{LoB} = \text{mean}_{\text{blank}} + 1.645(\text{SD}_{\text{blank}})$$
- LoD is the lowest analyte concentration likely to be reliably distinguished from the LoB and at which detection is feasible. LoD is determined by utilising both the measured LoB and test replicates of a sample known to contain a low concentration of analyte.
$$\text{LoD} = \text{LoB} + 1.645(\text{SD}_{\text{low concentration sample}})$$
- LoQ is the lowest concentration at which the analyte can not only be reliably detected but at which some predefined goals for bias and imprecision are met. The LoQ may be equivalent to the LoD or it could be at a much higher concentration.

Introduction

Sensitivity, Analytical Sensitivity, Functional Sensitivity, Lower Limit of Detection, LoB, LoD, and LoQ are terms used to describe the smallest concentration of a measurand that can be reliably measured by an analytical procedure. There has often been a lack of agreement within the clinical laboratory field as to the terminology best suited to describe this parameter. Likewise, there have been various methods for estimating it. Clinical laboratorians have perhaps been lax in dealing with this analytical issue because, in many cases, the ability of a laboratory test to detect a very small amount of measurand is not clinically significant. For example, the medical decision levels for glucose and cholesterol are so far above the lower analytical limits of these tests that it is highly unlikely that clinical action will depend on measurements of these analytes at such low concentration. Nevertheless, it is important to fully characterise the analytical performance of every clinical laboratory test in order to understand its capability and limitations, and to ensure that it is “fit for purpose.” Moreover, defining the limits of an assay at low concentration is directly related to its dynamic range, or analytical measurement range.

To provide a standard method for determining LoB, LoD and LoQ, Clinical and Laboratory Standards Institute (CLSI) has

published the guideline EP17, Protocols for Determination of Limits of Detection and Limits of Quantitation.¹ The Figure taken from their document illustrates the distinction of LoB, LoD and LoQ values. Typically, LoQ will be found at a higher concentration than LoD, but how much higher depends on the specifications for bias and imprecision used to define it. ‘Analytical sensitivity’ defined as the slope of the calibration curve is sometimes used as a synonym for LoD. However, because LoD may well reside at some concentration below the linear range of an assay, where the calibration curve is no longer valid, this usage should be avoided.

All of these parameters are related but have distinct definitions and should not be confused. The intent is to define the smallest concentration of analyte that can be *detected* with no guarantee about the bias or imprecision of the result by an assay, the concentration at which *quantitation* as defined by bias and precision goals is feasible, and finally the concentration at which the analyte can be quantitated with a *linear* response.

Limit of Blank

EP17 defines LoB as the highest *apparent* analyte concentration expected to be found when replicates of a sample containing no analyte are tested.¹ Note that although the samples tested to define LoB are devoid of analyte, a blank

(zero) sample can produce an analytical signal that might otherwise be consistent with a low concentration of analyte.

LoB is estimated by measuring replicates of a blank sample and calculating the mean result and the standard deviation (SD).

$$\text{LoB} = \text{mean}_{\text{blank}} + 1.645(\text{SD}_{\text{blank}})$$

Assuming a Gaussian distribution of the raw analytical signals from blank samples, the LoB represents 95% of the observed values. (Note: Typical modern clinical analysers don't routinely display the actual analytical signal but automatically convert it to a concentration value. The raw analytical signal is preferable for establishing LoB as analysers may report all signal values below a certain fixed limit as "zero concentration").

The remaining 5% of blank values represent a response that could actually be produced by a sample containing a very low concentration of analyte. Statistically, this false positivity is known as a Type I (or α) error (Figure). Conversely, while a sample that actually contains analyte is expected to exceed the LoB, it must also be recognised that a proportion of very low concentration samples will produce responses less than the LoB, representing Type II (or β) error (Figure). Thus, EP17 acknowledges that the overlap of the analytical responses of blank and low concentration is a statistical reality, and uses LoB as a reasonable starting point for estimating the LoD.

Limit of Detection

Although reagent package inserts may state that an assay has a dynamic range that extends from zero concentration to some upper limit, typically an assay is simply not capable of accurately measuring analyte concentrations down to zero. Sufficient analyte concentration must be present to produce an analytical signal that can reliably be distinguished from "analytical noise," the signal produced in the absence of analyte.

LoD is the lowest analyte concentration likely to be reliably distinguished from the LoB and at which detection is feasible. It is therefore greater than LoB (Figure). A traditional and typical approach to estimate LoD consists of measuring replicates, usually $n=20$, of a zero calibrator or blank sample, determining the mean value and SD, and calculating LoD as the mean +2 SD. Variations of this approach use the mean plus 3, 4, or even 10 SDs to provide a more conservative LoD. The assumption is that if analyte is present, it will produce a signal greater than the analytical noise in the absence of analyte. This is a simple and quick method. The weakness is that there is no objective evidence to prove that

a low concentration of analyte will indeed produce a signal distinguishable from a blank (zero concentration) sample. As Needleman and Romberg noted, "It defines only the ability to measure *nothing*".²

An alternative approach utilises analysis of samples containing small but known concentrations of the substance of interest (be it a drug, hormone or other analyte).^{3,4} The advantage of this empirical approach is that objective data is used to compare the analytical response of blank and low concentration samples to determine conclusively what concentration of analyte is necessary to distinguish its presence from its absence. Various analytical specifications (e.g. a minimum signal-to-noise ratio for a chromatographic method or a minimum absorbance requirement for a spectrophotometric procedure) can be applied to ensure that the LoD is meaningful and clearly distinguishable from a negative or blank sample.

As defined in EP17, LoD is determined by utilising both the measured LoB and test replicates of a sample known to contain a low concentration of analyte.¹ The mean and SD of the low concentration sample is then calculated according to:

$$\text{LoD} = \text{LoB} + 1.645(\text{SD}_{\text{low concentration sample}})$$

Again assuming a Gaussian distribution of the low concentration samples, 95% of values will exceed the previously defined LoB, and only 5% of low concentration samples will produce values below the LoB and erroneously appear to contain no analyte. Manufacturers are expected to establish the LoB and LoD using two or more instruments and reagent lots to capture the expected performance of the typical population of analysers and reagents. A recommended practical number of LoB and LoD samples to be used by a manufacturer to *establish* these parameters is 60, while a laboratory *verifying* a manufacturer's LoD (and possibly the LoB) is 20.

Once a provisional LoD is established, it can be confirmed by examining the observed values for samples containing the LoD concentration. Some LoD sample values are expected to be less than the estimated LoD (Figure), but when using 1.645 SD, no more than 5% of the values should be less than the LoB. If the observed LoD sample values meet this criterion, the LoD is considered established or verified. If more than 5% (roughly 1 out of 20 observations) of the LoD sample values fall below LoB, the LoD is too low and must be re-estimated (i.e. by testing a sample of higher concentration that will generate a higher mean and SD and thus a higher LoD).

This is an abbreviated and simplified description of the EP17 protocol. The guideline contains considerably more statistical

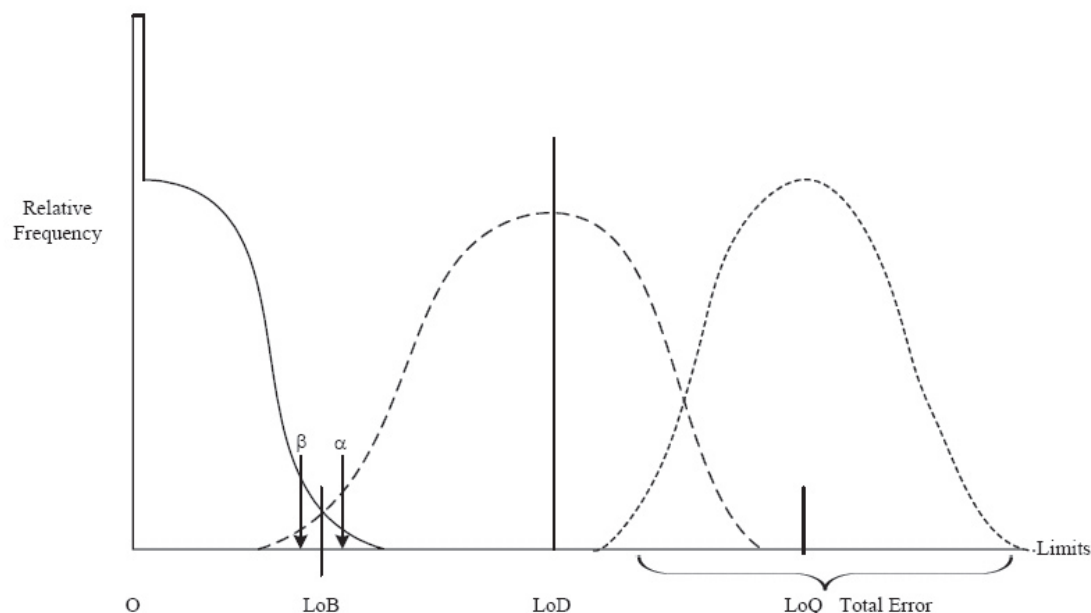


Figure. Relationship between LoB, LoD and LoQ. The solid line defines the LoB and represents the distribution of results for a blank specimen. As modern analysers seldom report results of less than zero, the frequency of “zero” results is artificially increased as illustrated. The LoB shown excludes a small proportion of blank results (“ α ”). The dashed line defines the LoD and represents the scatter (imprecision) of results for a specimen of low concentration. The LoD is then set so that only a small proportion (“ β ”) of these results will fall below LoB. The dotted line defines the LoQ and represents the distribution of results for a specimen of low concentration meeting the target for total error (imprecision *and* bias). It may be that this LoQ total target error is met by a specimen at the LoD concentration in which case $LoQ = LoD$. Otherwise, LoQ will have to be set after testing a specimen of higher concentration. Reproduced with permission, from NCCLS publication EP17-A, Protocols for Determination of Limits of Detection and Limits of Quantitation; Approved Guideline.

detail and guidance, including the use of non-parametric (non-Gaussian) techniques if necessary. Readers are encouraged to consult EP17 for a complete explanation of this method for establishing and verifying LoD.¹

Limit of Quantitation

LoQ is the lowest concentration at which the analyte can not only be reliably detected but at which some predefined goals for bias and imprecision are met. “Functional sensitivity” is defined as the concentration that results in a $CV=20\%$ (or some other predetermined % CV), and is thus a measure of an assay’s precision at low analyte levels (without addressing bias).⁵ It was originally developed as a clinical diagnostic tool to characterise thyroid stimulating hormone (TSH) assay performance in distinguishing euthyroid from hyperthyroid patients at low TSH concentrations. It can be expected that the LoD lies somewhere below an assay’s functional sensitivity.

The LoQ may be equivalent to the LoD or it could be at a much higher concentration (Figure); it cannot be lower than the LoD. A LoD provides an estimate of bias and imprecision at very low analyte concentration. If the observed bias and

imprecision at the LoD meet the requirements for total error for the analyte (i.e. the assay is “fit for purpose”) then: $LoQ=LoD$. If the analytical goals are not met at the LoD, a slightly higher analyte concentration must be tested to determine the LoQ.

The Table provides a brief summary of the features of the LoB, LoD, and LoQ.

Conclusions

It is important to fully characterise the analytical performance of clinical laboratory tests in order to understand their capability and limitations, and to ensure that they are “fit for purpose.” The terms LoB, LoD, and LoQ describe the smallest concentration of a measurand that can be reliably measured by an analytical procedure.

To *establish* these parameters a manufacturer would test a large number of sample replicates to increase the robustness and the statistical confidence of the estimate (Table 1). In addition, a manufacturer establishing the LoB, LoD, or LoQ should perform studies using more than one analyser and one lot of reagents to encompass the variability that users can expect to

Table.

Parameter	Sample Type	Replicates	Sample Characteristics	Equation
LoB	Sample containing no analyte, e.g. zero level calibrator	Establish: 60 Verify: 20	Negative or very low concentration sample that is commutable with patient specimens	$LoB = \text{mean}_{\text{blank}} + 1.645 (SD_{\text{blank}})$
LoD	Sample containing low concentration of analyte, e.g. dilution of lowest concentration calibrator	Establish: 60 Verify: 20	Low concentration samples, such as a dilution of the lowest non-negative assay kit calibrator or patient specimen matrix containing a weighed out amount of analyte, commutable with patient specimens	$LoD = LoB + 1.645 (SD_{\text{low concentration sample}})$
LoQ	Sample containing low concentration of analyte at the expected LOQ, e.g. sample with concentration at or above the LoD	Establish: 60 Verify: 20	Low concentration samples at or above the concentration of the LoD; analyte concentration must be sufficient to produce analytical signals that meet predetermined targets for bias, imprecision, and total error	$LoQ \geq LoD$

encounter in the field. Clinical laboratories can *validate* these parameters using a smaller number of samples and likely will use only one analyser and one lot of reagents.

LoB and LoD are important for tests used to discriminate between the presence or absence of an analyte (e.g. drugs, troponin, human chorionic gonadotrophin) and LoQ, to reliably measure low levels of hormones (e.g. TSH) for clinical diagnosis and management and should be incorporated as part of any method evaluation.

Competing Interests: None declared.

References

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