

Method Validation Essentials, Limit of Blank, Limit of Detection and Limit of Quantitation

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Development of robust, accurate, precise, and linear assays is at the heart of development activities for drug product and drug substances. Determining the limits of what an assay can reliably quantitate is a requirement of regulatory authorities globally. Generally, the guidance for limit of detection (LOD) and limit of quantitation (LOQ) can be found in the International Conference on Harmonization's (ICH) Q2 *Validation Of Analytical Procedures: Text And Methodology* (1); however other useful resources can be found (2, 3).

Limit of blank (LOB), LOD, and LOQ are commonly used terms used to describe the smallest concentration of an analyte that can be reliably measured by an analytical method. The purpose of this paper is to present the best practices relative to the study design and statistical/analytical approach to limit determination. One needs to know the concentration a method will reliably detect the signal from measuring an analyte versus the noise in the measurement method. An analogy of two people talking near a jet engine helps to illustrate the idea. LOB is analogous to no one talking, just the noise of the engine; LOD is when one person detects the other is speaking (lips are moving) but cannot understand a word they are saying as the engine noise is too high. With LOQ, the noise is sufficiently low that every word is heard and understood.

The following are several different types of assays, and the application of LOB, LOD, and LOQ to each type of method to determine the limits can be confusing to those that work on analytical method development and validation:

- Quantitative assays without background noise (calibration curve method)
- Quantitative assays with background noise (signal-to-noise method)
- Identification assays (signal-to-noise method)
- Potency assays (LOD/LOQ not required, ICH Q2 and *United States Pharmacopeia (USP) 1033*)(6)
- Visual assays (logistics method).

LOB, LOD, and LOQ approaches for each type of assay will be defined by a study design, typical number of determinations, and method of analysis.

Cubic nature of analytical methods

Analytical methods are generally considered to be cubic or sigmoidal in nature. At the low end of quantitation no signal can be detected, then as the signal increases, the detection and quantitation can be reliably determined, a linear transition follows and finally saturation of the method. From these curves, LOB, LOD, and LOQ of the method can be determined. Normally the curves are defined based on the signal from the method and the log concentration of a serial dilution scheme; however, other approaches may be appropriate (see Figure 1).

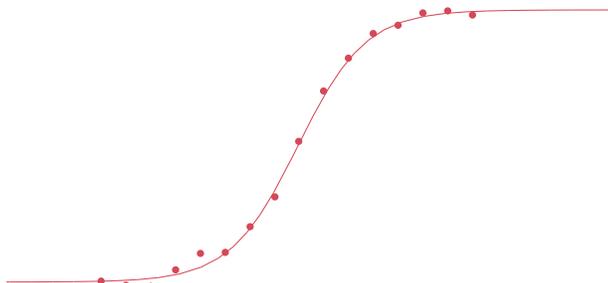


Figure 1: Nonlinear nature of analytical methods.

Multiple approaches for limit determination

There are multiple approaches for determining the detection limits. Blank evaluation is nice to have but often not required. ICH Q2 suggests the following methods for defining limits (1):

- Based on standard deviation of the blank
- Based on standard deviation of the response and the slope
- Based on visual evaluation
- Based on the signal-to-noise ratio.

Based on the standard deviation of the blank

Blank samples are good at evaluating the background noise of the method. Limit of blank helps to clarify the analytical error when the analyte is not in solution. Limit of blank is defined in EP17-A as (4, 5), "Highest measurement result that is likely to be observed (with a stated probability) for a blank sample; a) LOB is not an actual concentration from which measurements are obtained. ... Similarly, LOD is the lowest value expected from a sample that contains a level of the LOD with a stated probability."

This method of limit determination uses the mean and standard deviation of blank samples to set the limits. If normal distributions are not likely, other distributions may be used using the same K sigma multiplier. Sample sizes should be selected based on delta, power, and alpha. Normally 10 or more determinations are made at no concentration in the appropriate matrix (see Equation 1 and Figure 2):

$$\begin{aligned} \text{LOB} &= \text{Mean blank} + 1.645 * \text{Stdev blank (one sided 95\%)} \\ \text{LOD} &= \text{Mean blank} + 3.3 * \text{Stdev blank (one sided 95\% * 2)} \\ \text{LOQ} &= \text{Mean blank} + 10 * \text{Stdev blank (one sided 95\% * 6)} \\ &[\text{Eq. 1}] \end{aligned}$$

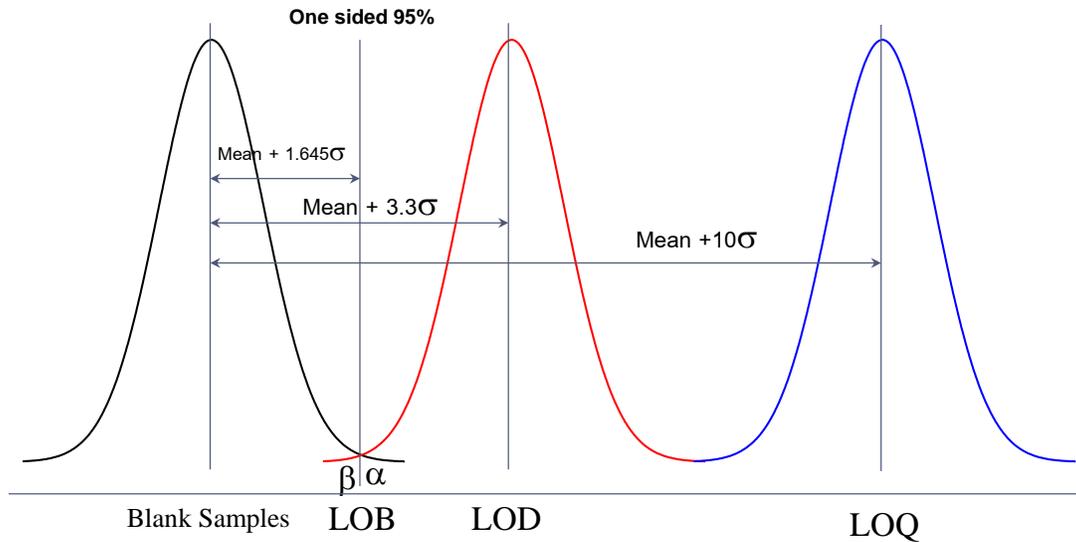


Figure 2: Limit of blank (LOB), limit of detection (LOD), and limit of quantitation (LOQ) based on the blank.

Limit of detection is generally evaluated for quantitative assays and impurities. ICH Q2 defines the LOD as, “The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value” (1).

The weakness of this approach is that it is not looking at a measured signal when setting the limits as the analyte is not in solution.

Based on standard deviation of the response and the slope

This approach to limit determination is good when the method does not exhibit background noise of any magnitude. A standard curve may be used to determine LOD and LOQ. Samples need to be taken in the range of the LOD and LOQ and not extrapolated into the range. Normally six or more determinations are made at five concentrations.

The detection limits may be expressed as below in Equation 2:

$$\begin{aligned} \text{LOD} &= 3.3 \sigma/\text{Slope} \\ \text{LOQ} &= 10 \sigma/\text{Slope} \end{aligned}$$

Where: σ = the standard deviation of the response at low concentrations
Slope = the slope of the calibration curve.

[Eq. 2]

The slope may be estimated from the calibration curve of the analyte. The estimate of σ is typically the root mean squared error (RMSE) or standard deviation of the residuals taken from the regression line. The slope is used to convert the variation in the response back to the scale of the theoretical concentration (see Figure 3).

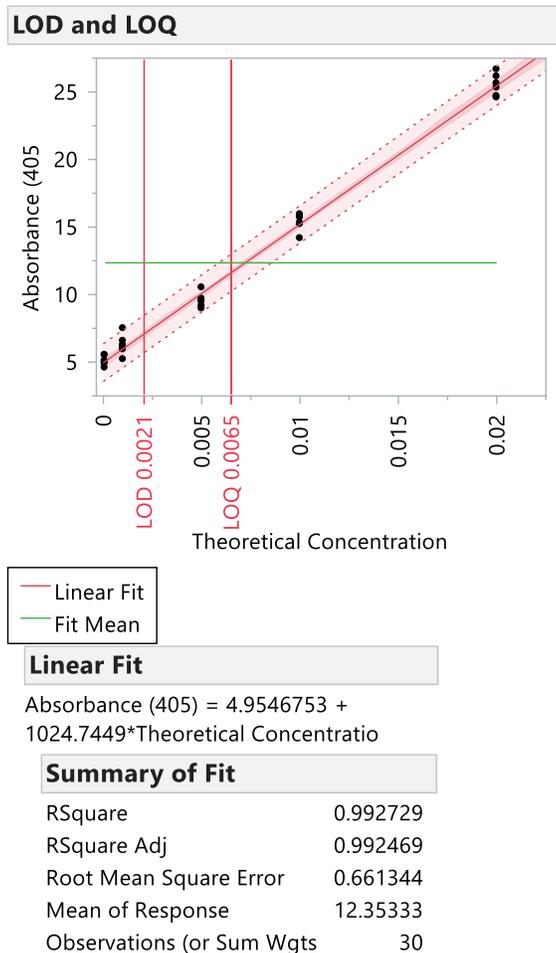


Figure 3: Limit of detection and limit of quantitation (LOQ) from a standard curve.

Parameter	Estimate	LOD/LOQ	K Sigma	Detection Limits
Root Mean Square Error	0.661	LOD	3.3	0.0021
Slope	1024.745	LOQ	10	0.0065

Based on visual evaluation

Visual evaluation may be done by an analyst or by an instrument. Concentration is varied, and the question is at what concentration can a shift in color, presence of aggregation or particles, or presence on a gel be detected. ICH Q2 states, “*The detection limit is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.*”

Typically five to seven concentrations are made from a known reference, and then the analyst or instrument is used to determine if it is detected. Six to 10 determinations are typical for each concentration. For each sample, the analyst or instrument either detects or does not detect the analyte. Nominal logistics are used in the analysis for a probability of detection. Normally on this type of analysis, LOD is set at 99% detection, and LOQ is set at 99.95% detection (see Figure 4).

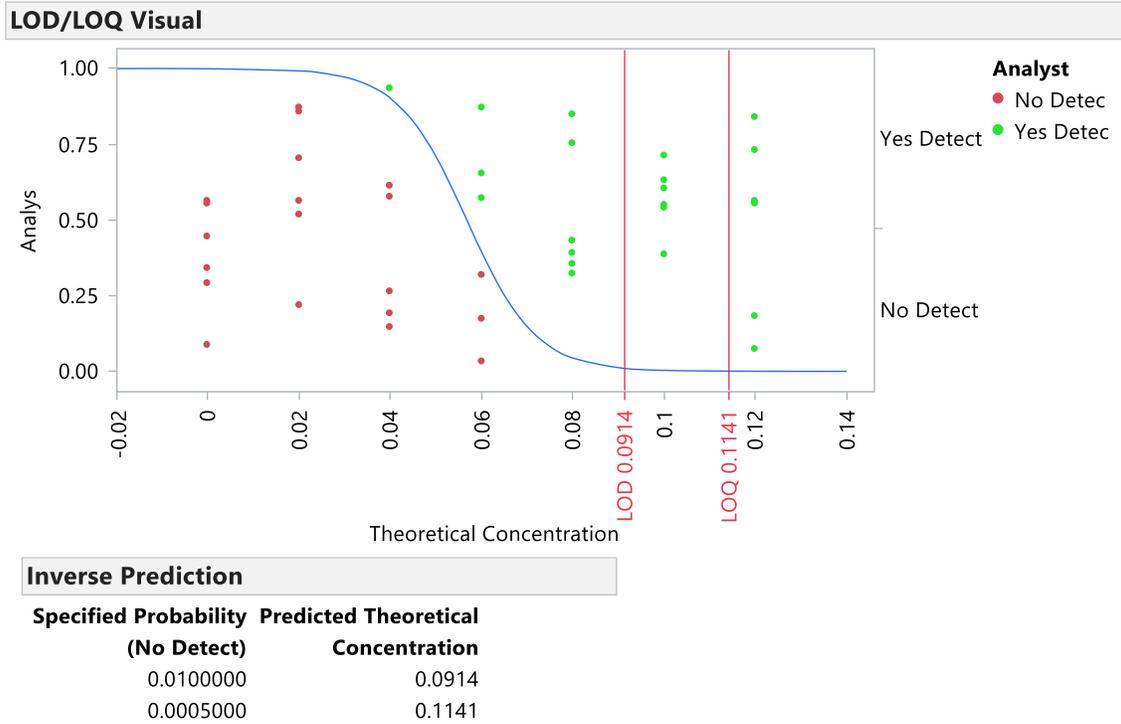


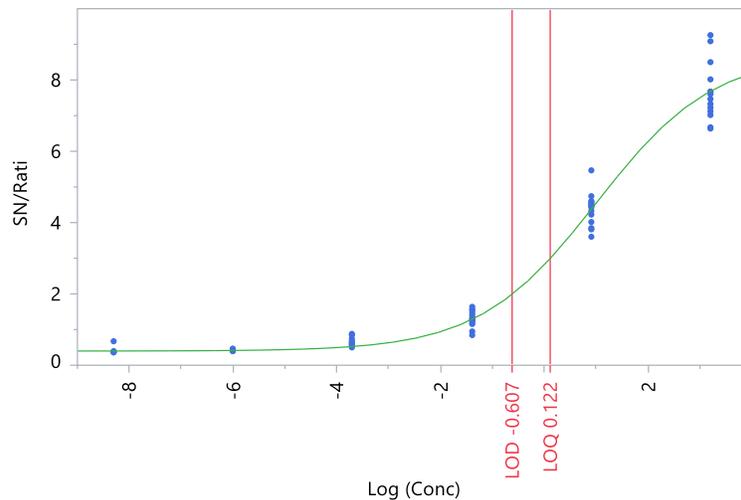
Figure 4: Logistics regression for visual limit of detection (LOD) and limit of quantitation (LOQ).

Based on signal to noise

Signal to noise is used only when the analytical method exhibits background noise when nothing is in solution. Typically five to seven concentrations are used with six or more determinations for each concentration. Signal to noise is calculated at each concentration. Signal is the measurement at each concentration, and noise is the blank control from the plate or method. LOD is set at a signal to noise of two, and LOQ is set at a signal to noise of three. Nonlinear modeling using the log concentration and 4PL logistics are typically used to determine LOD and LOQ. EXP(Predicted(log concentration)) will back transform the log concentration to concentration from the curve (see Figure 5).

Fit Curve Material=Material A						
Model	AICc	BIC	SSE	MSE	RMSE	R-Square
Logistic 4	92.151831	104.97425	13.45664	0.1293908	0.3597093	0.9835483

LOD and LOQ SN Ratio



Specified SN/Ratio	Predicted Log Concentration	Lower 95%	Upper 95%	Concentration in Micrograms
2	-0.607041	-0.761928	-0.452155	0.545
3	0.121798	0.021159	0.222437	1.130

Figure 5: Signal to Noise Method for Setting LOD and LOQ

Conclusion

Care needs to be made to match the method of limit determination to the analytical method. Curve fitting and statistical methods linked to good dilution/concentration schemes are crucial to proper evaluation of the limits. Limit definition is required by the health authorities and adds to the overall understanding of the capabilities of any analytical method. Statistical tools greatly aid and simplify the calculation and reporting of the limits.

References

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