GENE-π

MEMO – How to calculate the limit of Blank (LoB) and the limit of detection (LoD)

For automated computation, see the statistical tools available on www.gene-pi.com

In this memo, we describe a method to calculate the LoB and the LoD of a Crystal Digital PCR™ assay adapted from the Clinical and Laboratory Standards Institute (CLSI) EP17-A2 standard (Protocols for Determination of Limits of Detection and Limits of Quantitation; Approved Guideline).

How to calculate the LoB

a) Definition: the LoB with a confidence level $(1-\alpha)$ is defined as the maximum concentration expected in a well with a probability of 1-α in a sample containing no target sequence. In other words, the LoB is the maximum concentration that is plausible with a $1-\alpha$ probability (typically 95% for $\alpha = 5\%$).

The sample used to calculate the LoB, known as a negative control or blank sample, in most cases contains no sequence for any given target. However, the blank sample should be representative of the nature of the samples - for example if the sample to be tested with the assay is circulating tumor (ctDNA) extracted from plasma or FFPE DNA, the blank sample should be a sample with no mutant sequences but with a background of fragmented wildtype DNA such as ctDNA extracted from a wild-type plasma sample.

b) Calculation method:

1) Perform Crystal Digital PCR™ on the naica® system on $N \geq 30$ negative control replicates to reach a confidence level of 95%. For higher confidence levels, more blank samples should be analyzed.
2) Using the Crystal Miner software, export the results (chamber concentrations in copies/µL) and order them in ascending concentration order (Rank 1 to N) for each target independently.
3) $P_{LoB,}$ the probability to be a true negative P_{LOB} is equal to 1- α. (If α = 0.05, P_{LoB} = 1-α = 0.95).
   The rank position X corresponding to this P_{LoB} equals to:
   $X = 0.5 + (N \times P_{LoB})$
4) To calculate the LoB, we need to determine the ranks flanking X and their corresponding concentrations C1 and C2.
   LoB = C1 + Y *(C2 - C1)
   C1 = concentration for rank X1 (X1 = Rank below rank X)
   C2 = concentration for rank X2 (X2 = Rank above rank X)
   Y = 0.Z; Z corresponds to the digit after the decimal point of X. (For example, if X=40.4, Y=0.4 or if X= 38.0, Y=0).
   If Y=0, X1 =X and **LoB = C1** (concentration for rank X)

How to calculate the LoD

a) Definition: the LoD with a confidence level $(1-\beta)$ is defined as the minimum concentration for which detecting the target sequence in a well is possible with a probability of $1-\beta$.
In other words, this is the minimum concentration that can be said to be non-zero and

statistically higher than the limit of blank LOB with a 1-β probability (typically 95% for $\beta = 5\%$).

b) Calculation method:

1. Perform Crystal Digital PCR™ on the naica® system on a minimum of five independently prepared Low-Level (LL) samples (LL1, LL2, LL3, LL4 and LL5), performing at least 6 replicates per sample. The LL samples used to calculate the LoD should be representative positive samples or a representative sample matrix with spiked-in target concentrations within a range of one to five times higher than the calculated LoB.
2. Determine the standard deviation SD_i for each group of replicates (SD1, SD2, SD3, SD4 and SD5). The quantification variability between LL samples should not be significantly different. The significance of this difference should be checked with a statistical method (Cochran's test, for example). A significant difference in quantification variability between LL samples suggests either an instability of the reaction or a concentration range too large for the selected LL samples (in which case, repeat the study with more appropriate samples).
3. Calculate the global SD (**SD_L**)

$$\mathrm{SD_L} = \sqrt{\left(\sum_{i=1}^{J}(ni-1)SDi^2\right) \Big/ \left(\sum_{i=1}^{J}(ni-1)\right)}$$

SD_i = SD of all results for the *i*th low level sample
n_i = the number of results for the ith low level sample
J = number of LL samples

Note that if all the LL samples contain the same number of replicates, SD_L can be calculated with the following formula:

$$\mathrm{SD_L} = \sqrt{(\sum({SD_i}^2)) / J}$$

4. Calculate **Cp** = coefficient to give the 95th percentile of a normal distribution

L = total of samples (J x n replicates, n ≥ 6)

$$c_P = \frac{1{,}645}{1 - \left(\frac{1}{4(L-J)}\right)}$$

The value 1.645 represents the 95th percentile from the normal distribution for β = 0.05. If a different β value is chosen, this multiplier will need to change accordingly.

$LoD = LoB + Cp*SD_L$

How to determine if a target is quantifiable or detectable

Once the LoB and LoD are calculated for each target of a given assay, use the following decision table to determine if the target concentration obtained for a given real-life sample can be considered as detectable or quantifiable:

Target X concentration value	Interpretation
C[X] ≤ LoB	Target not detected
LoB < C[X] < LoD	Target detected but not quantifiable (rerun assay with more sample volume if possible)
C[X] ≥ LoD	Target detected and quantifiable

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