



Mastering Assay Validation Strategies for Quantitative PCR (qPCR) and Droplet Digital PCR (ddPCR™) in Regulated Environments

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Learn how to incorporate analytical sensitivity parameters into qPCR and ddPCR assay validation. This brief guide describes several real-world examples and accompanies the webinar.

Key Analytical Sensitivity Parameters in qPCR and ddPCR Assay Validation: Limit of Blank (LOB), Limit of Detection (LOD), and Limit of Quantification (LOQ)

LOB, LOD, and LOQ are analytical sensitivity parameters in qPCR and ddPCR assay validation. They help determine the lower limits

at which reliable quantification, detection, and differentiation from background noise can be achieved. These parameters are essential to assess the performance and reliability of the assay for accurate measurement of target analytes (Figures 1 and 2).

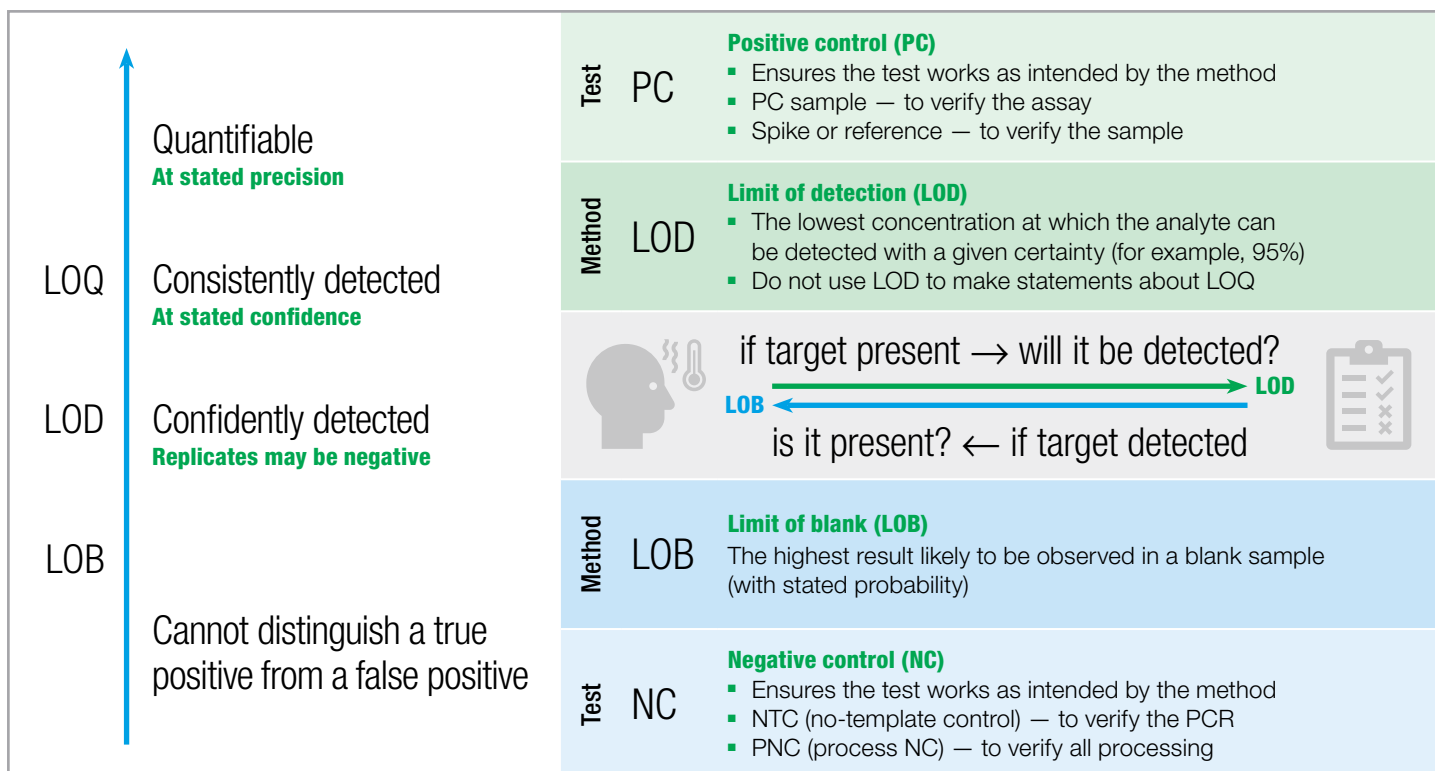


Fig. 1. The relationship between LOB, LOD, LOQ, and the detection sensitivity of an assay. Limit of blank (LOB), limit of detection (LOD), and limit of quantification (LOQ) are crucial parameters in assessing the detection sensitivity of qPCR or ddPCR assays. LOB represents the highest analyte concentration likely to be measured without the presence of the target analyte, whereas LOD is the lowest concentration reliably distinguished from LOB. LOQ, on the other hand, is the lowest concentration of the target analyte that can be quantitatively determined with acceptable precision and accuracy. Together, these parameters provide a comprehensive understanding of the assay's sensitivity, delineating the range within which reliable detection and quantification can occur.

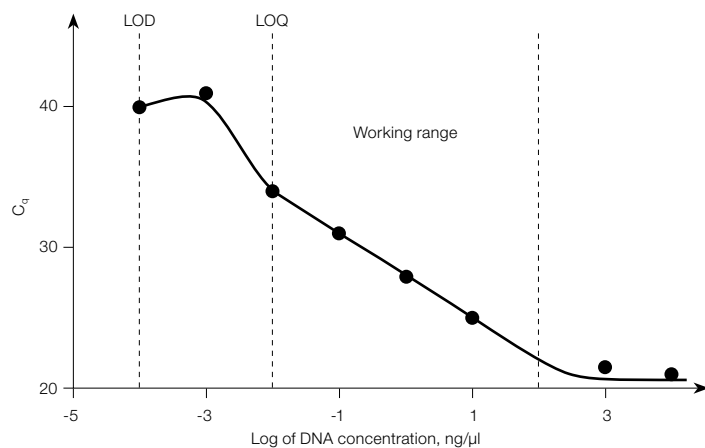


Fig. 2. LOD and LOQ in relation to the working detection range of an assay.
C_q, quantification cycle; LOD, limit of detection; LOQ, limit of quantification.

LOB Considerations

The assessment of LOB is integral to assay validation, as it involves analyzing blank or negative samples to identify potential sources of background noise. A good PCR assay should not have any background. Therefore, it is important to address issues that may cause background, including factors such as:

- Primer dimers (directly visible only when using DNA binding dye assays; can also be present in probe assays)
- Workflow contamination
- Cross-reactivity of variant probe(s) with wild-type template(s)

Researchers can effectively evaluate and mitigate background noise by implementing a set of blank, or negative, samples. Ideally, these samples represent the relevant matrix and cover the entire workflow from extraction to reverse transcription.

How many blank samples are necessary to assess LOB? Little guidance exists to answer this question. However, if you want to align with LOD/LOQ experiments, then at least ten should be used, according to the International Organization for Standardization (ISO 2019). Note that statistical quantification of LOB is meant to evaluate background noise levels, not the occasional contamination event.

To assess LOB, analyze a set of blank samples. To determine which samples qualify as blank, consider the following:

- Relevant matrix and workflow covered in the assay, including extraction and reverse transcription controls. For example, water samples processed throughout the entire workflow should be used as controls
- For variant analysis: wild-type samples

LOD: A Key Focus in ISO 20395 Guidelines

LOD signifies the lowest concentration of an analyte that can be reliably detected (but not necessarily quantified) with statistical confidence. ISO 20395 guidelines on LOD determination advocate performing at least ten replicates at each concentration and an extra sample to account for potential technical failures. This approach ensures a reliable and robust means of determining the lowest analyte concentration the assay can detect with a defined confidence level. Additionally, leveraging historical data from similar assays and insights from research use only (RUO)-level validations (specific to the assay) can assist in estimating the LOD range to test. Typically, LOD determination involves testing a concentration series above and below the expected LOD to establish accurate assay sensitivity.

LOQ: Another Key Focus in ISO 20395 Guidelines

LOQ is another critical assay validation parameter that denotes the lowest concentration of an analyte that can be reliably quantified at a defined accuracy and precision level. ISO 20395 guidelines for LOQ determination are similar to LOD in that they include the performance of at least ten replicates at each concentration and the consideration of historical data and RUO-level validations. This comprehensive approach ensures the robustness and reliability of the assay in accurately quantifying analytes at low concentrations. Moreover, testing a range of concentrations with small increments above and below the expected LOQ helps establish the assay's quantitative capabilities with precision and accuracy, ensuring compliance with regulatory standards and enabling reliable data interpretation in regulated environments.

The ISO 20395 guidelines on LOD and LOQ outline several key criteria. To incorporate these criteria into experimental design, we offer the following suggested approaches from ISO 20395:

“A minimum of 10 replicates should be performed at each concentration.” Include 11 samples (to accommodate a technical failure).

“With small increments in concentration, use 2 fold above and below the expected LOQ.”

- Look at historical data (other assays are a similar setup) and data from the RUO-level validation (for this specific assay) to estimate the range within which to test for LOD/LOQ
- Example range: 0, 2, 4, 8, 16, and 32 copies/reaction

“Estimation of LOD shall consider both the statistical distribution of true-positive samples and false-positive results.” (See the LOB Considerations section to determine background levels for the assay.)

Exceptions to Using LOD and LOQ to Quantify Input Concentrations

Using LOD and LOQ to quantify input concentration is useful, with some limitations noted below from the ISO 20395 guidelines on LOD and LOQ:

- Certified reference material is typically lacking for many assays
- ddPCR technology (as a counting-based method) may be used to establish such a reference sample. From ISO 20395:

“As an enumeration-based measurement procedure, ddPCR can form the basis of a primary reference measurement procedure, subject to establishment of selectivity and completeness of count and a statement of measurement uncertainty.”

- For artificial or purified templates, note that selectivity is not typically an issue
- Because of limited dependence on amplification efficiencies, completeness is easier to achieve, especially for singleplex analysis. (Multiplex has a risk of interference)

Practical approach:

- Dilute synthetic template to 1,000–10,000 copies/ μ l
- Quantify template using ddPCR technology
- Dilute and spike in at concentrations within the expected LOD/LOQ range

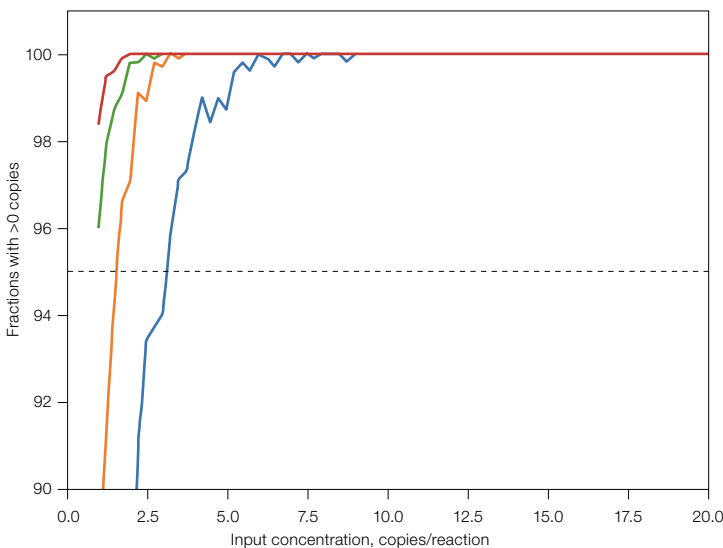
An Example of How to Use LOD and LOQ When Defining Expected Values

Now, let's examine an example of LOD and LOQ in a ddPCR assay (Figures 3 and 4 and Table 1). For the simulation of an LOD analysis shown in Figure 3, the assay is tested using dilutions of the target analyte, starting from a known concentration until the signal is no longer distinguishable from background noise. LOD is the concentration at which the signal becomes indistinguishable from noise (with a predefined confidence level), calculated using the Poisson distribution. Similarly, for LOQ shown on the right plot of Figure 3, the assay is tested using dilutions of the target analyte, and the lowest concentration at which the signal can be reliably quantified with acceptable precision and accuracy is determined. LOQ can then be set at a signal-to-noise ratio higher than that used for LOD determination, ensuring reliable quantification even at low analyte concentrations.

To summarize, when using LOD and LOQ for defining expected values in ddPCR, the lower end is defined by sampling noise, which is analyzed by Poisson statistics. (In a perfect scenario, the only limitation on sensitivity is sampling noise, which cannot be changed; thus, sampling noise sets the lower limit for LOD and LOQ.)

However, in practice, we expect to encounter higher LOD/LOQ values because of additional events that occur on top of sampling noise. These events may increase LOD/LOQ due to higher background signal (LOB) or because they act as additional sources of variation. Therefore, blanks are not 100% negative, and we typically see a higher LOB.

LOD Analysis Based Only on Poisson Variation



LOQ Analysis Based Only on Poisson Variation

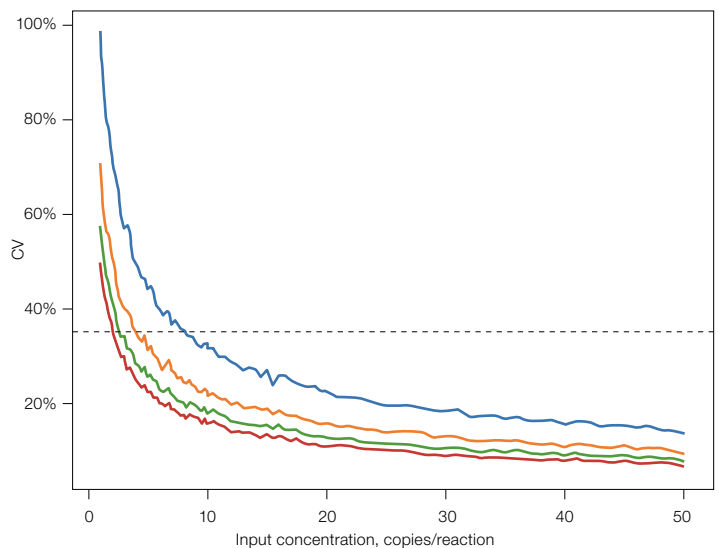


Fig. 3. An LOD and LOQ analysis of a ddPCR assay performed in simulation, based only on Poisson variation, found using the lowest detectable and quantifiable concentrations of the target analyte. In LOD analysis (left plot), dilutions of the target analyte are tested until signal noise is indistinguishable, determining the concentration at which the signal vanishes with a specified confidence level (calculated using Poisson distribution). For LOQ (right plot), dilutions are tested to identify the lowest concentration with reliable quantification, setting LOQ at a higher signal-to-noise ratio than LOD to ensure precise measurement (even at low analyte concentrations). CV, coefficient of variation; LOD, limit of detection; LOQ, limit of quantification. Left plot: LOD for one replicate (—), 3.25; LOD for two replicates (—), 1.75; LOD for three replicates (—), 1.0; LOD for four replicates (—), 1.0. Right plot: LOQ for one replicate (—), 8.25; LOQ for two replicates (—), 4.25; LOQ for three replicates (—), 2.75; LOQ for four replicates (—), 2.25.

Table 1. Example results for the Figure 3 scenario involving LOD and LOQ analysis of a ddPCR assay based only on Poisson variation. CV, coefficient of variation; DP, dilution point; DP1–6, samples one through six; LOD, limit of detection; LOQ, limit of quantification; NA, not applicable; NTC, no-template control.

	DP1	DP2	DP3	DP4	DP5	DP6	NTC
Target copies	32.00	24.00	16.00	8.00	4.00	2.00	0.00
Replicate 1	25.60	17.80	12.60	3.80	1.80	1.20	0.00
Replicate 2	26.60	14.60	9.60	4.80	4.60	3.80	0.00
Replicate 3	24.60	24.00	14.40	7.60	2.60	2.00	0.00
Replicate 4	35.60	20.60	16.20	5.00	2.00	1.20	0.00
Replicate 5	25.80	24.80	13.40	4.80	1.80	2.60	0.00
Replicate 6	20.20	20.40	14.60	6.60	2.80	2.20	0.00
Replicate 7	26.80	19.80	21.40	7.40	4.20	2.80	0.00
Replicate 8	22.40	18.80	13.60	5.40	1.60	2.40	0.00
Replicate 9	26.40	26.20	15.20	8.20	3.80	1.20	0.00
Replicate 10	28.40	18.20	19.00	8.60	2.80	2.80	0.00
Replicate 11	26.00	20.00	11.20	5.80	2.00	1.60	0.00
%CV	14.64	16.51	22.79	25.70	38.28	38.30	NA

← LOD between 0 (0% positive) and 2 (100% positive). →

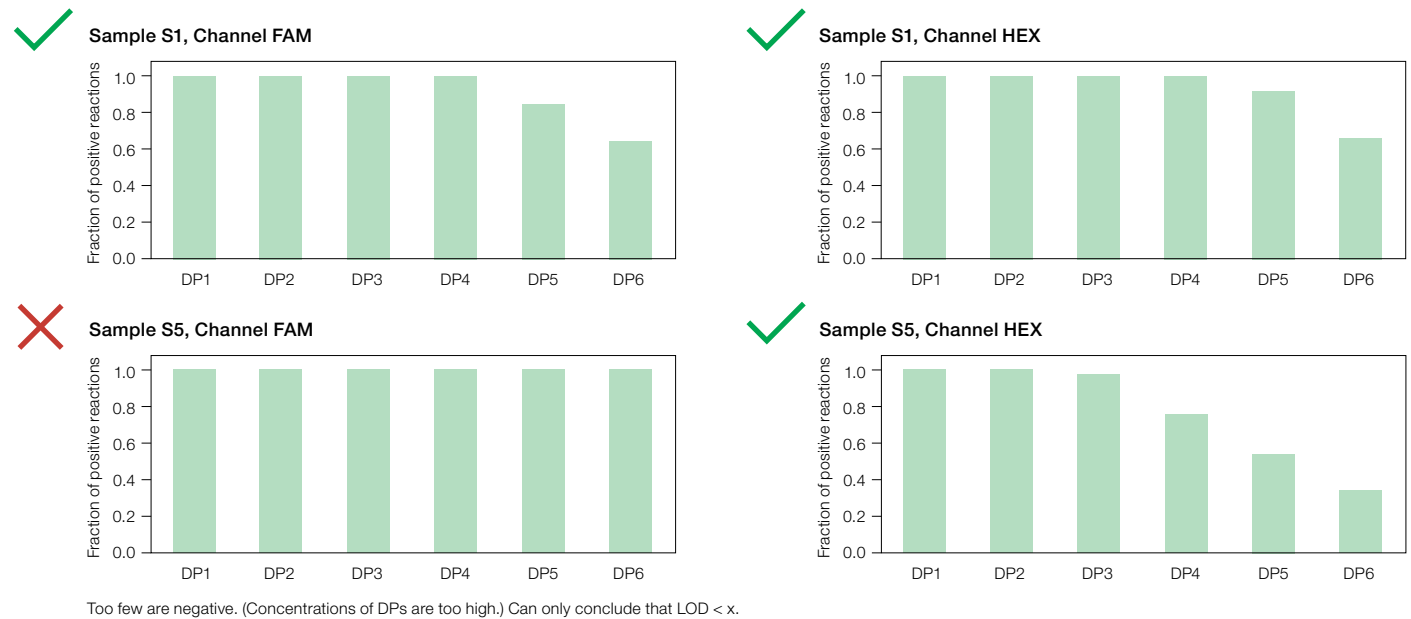


Fig. 4. LOD/LOQ example results continued for the Figure 3 scenario. Scrutinizing the dilution point (DP) results across a range of analyte concentrations helps to determine the best LOD range by identifying the lowest concentration at which reliable signal detection occurs. LOD should also represent the point at which the signal becomes distinguishable from background noise with a specified confidence level. In turn, LOD can ensure accurate and reliable detection of the target analyte in the ddPCR assay. As a result, in the bottom left scenario (x), the concentration of DPs is too high and should not be used for an LOD value. FAM and HEX indicate ddPCR detection channels. DP1–6, samples one through six; LOD, limit of detection; LOQ, limit of quantification.

How to Set Levels of LOD and LOQ Using Data Analysis

Statistical approaches like probit analysis or regression models can be used to estimate LOD. For example, probit analysis can be used to find the concentration of a 95% hit rate level, which would be the most suitable concentration to set LOD. Note that merging data reduces LOD (Figure 5).

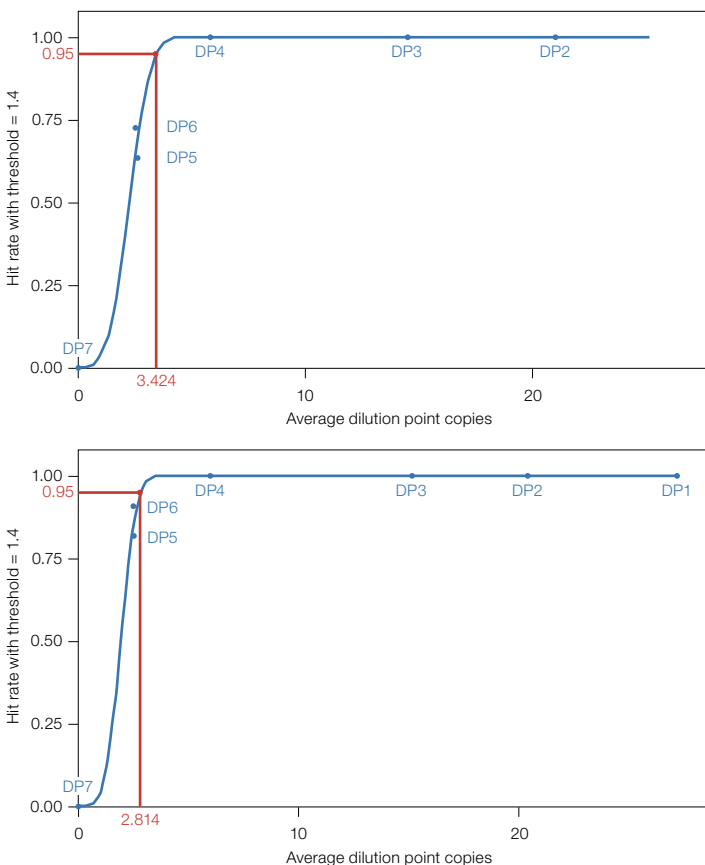


Fig. 5. Probit analysis of LOD to identify the concentration having a 95% hit rate. DP, dilution point; DP1–6, samples one through six; LOD, limit of detection.

Table 2. A second example dataset for setting LOD and LOQ. The fourth dilution point with eight copies is the lowest concentration with a CV <35%; therefore LOQ would be set at eight molecules. Target copies indicate target copies per reaction. CV, coefficient of variation; DP, dilution point; DP1–6, samples one through six; LOD, limit of detection; LOQ, limit of quantification; NA, not applicable; NTC, no-template control.

	DP1	DP2	DP3	DP4	DP5	DP6	NTC
Target copies	32.00	24.00	16.00	8.00	4.00	2.00	0.00
Replicate 1	25.60	17.80	12.60	3.80	1.80	1.20	0.00
Replicate 2	26.60	14.60	9.60	4.80	4.60	3.80	0.00
Replicate 3	24.60	24.00	14.40	7.60	2.60	2.00	0.00
Replicate 4	35.60	20.60	16.20	5.00	2.00	1.20	0.00
Replicate 5	25.80	24.80	13.40	4.80	1.80	2.60	0.00
Replicate 6	20.20	20.40	14.60	6.60	2.80	2.20	0.00
Replicate 7	26.80	19.80	21.40	7.40	4.20	2.80	0.00
Replicate 8	22.40	18.80	13.60	5.40	1.60	2.40	0.00
Replicate 9	26.40	26.20	15.20	8.20	3.80	1.20	0.00
Replicate 10	28.40	18.20	19.00	8.60	2.80	2.80	0.00
Replicate 11	26.00	20.00	11.20	5.80	2.00	1.60	0.00
%CV	14.64	16.51	22.79	25.70	38.28	38.30	NA

LOQ equals 8 copies per reaction (lowest concentration with CV <35%).

LOD < 2.

A mathematical equation listed below defines how to set the LOD and LOQ levels (Forootan et al. 2017) (Figure 6).

How to Define LOD and LOQ Levels

LOD

- Probit analysis to interpolate concentration having a 95% hit rate

LOQ

- Lowest concentration or copy number having a variability below the stated requirement
- Threshold to be set as a function of needs and expectations
- For example, CV <35%
- Note: it is incorrect to calculate the CV on C_q values as the SD/mean; instead use:

$$CV_{C_q} = \frac{2}{\sqrt{(1+E) \left(\frac{SD_{C_q}}{C_q} \right)^2 \times \ln(1+E) - 1}}$$

Fig. 6. Description of how to define LOD and LOQ levels, with the mathematical equation for LOQ defined (adapted from Forootan et al. 2017). CV, coefficient of variation; LOD, limit of detection; LOQ, limit of quantification.

A Second Example of How to Set LOD and LOQ

With an understanding of LOD, LOQ, and LOB, we have now defined the boundaries within which the assay can reliably detect, quantify, and differentiate target analytes from background noise (Table 2).

How to Assess Precision of an Assay

Precision of the assay must be ensured for accurate detection and quantification of target analytes while minimizing the impact of background noise and false positives. Precision is defined as shown in Figures 7 and 8.

Precision with Two Repeats

Three levels

1. Repeatability (within run variation).
2. Intermediate precision (within a laboratory, between run variations).
3. Reproducibility (between laboratories).

Designs

Minimal (two repeats).

Replicate	1	2	3
Run	1	1	2
Day	1	1	2
Operator	1	1	2
Instrument	1	1	2

Repeatability ←————→

Intermediate precision ←————→

Precision with Three Repeats

Three levels

1. Repeatability (within run variation).
2. Intermediate precision (within a laboratory, between run variations).
3. Reproducibility (between laboratories).

Designs

Minimal (three repeats).

Replicate	1	2	3	4	5
Run	1	1	1	2	3
Day	1	1	1	2	3
Operator	1	1	1	2	2
Instrument	1	1	1	1 or 2	1 or 2

Repeatability ←————→

Intermediate precision ←————→

Fig. 7. Precision of a qPCR or ddPCR assay using two repeats. The definition of precision in a qPCR or ddPCR assay involves its repeatability, precision within the lab, and reproducibility between different laboratories. For example, to achieve high precision, you need three replicates per run with two runs per day over two days, with two operators on two instruments.

Fig. 8. An additional method to setting precision with three repeats. The number of technical replicates impacts precision determination; increasing to three enhances statistical power and confidence, contingent on factors like assay variability, desired confidence level, and resource availability. Three repeats offer better variability estimation, robustness to outliers, improved statistical confidence, and enhanced systematic error detection, potentially correcting assay biases.

The Role of Clinical and Laboratory Standards in Precision

The Clinical and Laboratory Standards Institute (CLSI) plays a significant role in ensuring the precision of molecular assays by developing and sharing consensus-based standards and guidelines.

CLSI standards provide recommendations and best practices for designing, validating, and implementing molecular assays in clinical and laboratory settings. Standardized methodologies promote consistency in testing procedures and enhance the reliability and reproducibility of assay results.

Figure 9 illustrates an example of CLSI standards in our assay scenario.

Precision

Three levels

1. Repeatability (within run variation).
2. Intermediate precision (within a laboratory, between run variations).
3. Reproducibility (between laboratories).

Designs

- Minimal (3 repeats)
- CLSI

Samples

- Representative matrix
- Contain different concentrations of the target of interest, spanning the range of interest
- Examples for the vector copy number include extracting gDNA from 12 samples and spiking 4 high, 4 medium, and 4 low (just above LOD/LOQ) or mixing transduced cells into normal samples

Fig. 9. How to use CLSI standards to design an accurate molecular assay in the example scenario. By promoting standardized methodologies, CLSI ensures consistency in testing procedures across clinical and laboratory settings, bolstering the reliability and reproducibility of assay results. CLSI, Clinical and Laboratory Standards Institute; LOD, limit of detection; LOQ, limit of quantification.

Assay Precision versus Robustness

Assay precision assesses the consistency of results within conditions. Good precision indicates low variability and enhances confidence in assay accuracy. In contrast, assay robustness gauges the assay's resilience to minor variations in experimental conditions. A robust assay ensures stable and reliable performance, even under slight perturbations.

Robustness examines the assay's performance under various conditions to ensure its reliability in real-world scenarios. The process involves evaluating how the assay responds to factors such as RNA degradation, temperature variations, or different operators performing the assay. As described in Figure 10, analyzing the assay's results under diverse conditions enables the researcher to determine its robustness and identify potential vulnerabilities or limitations that may impact its performance in practical settings.

Robustness

Definition

"Evaluate the effect of small deviations in relevant method parameters on the method performance and the measurement results." (CLSI 2019)

Examples

- Concentration and source (manufacturer) of primers and probes — test different lots
- Composition of PCR reagents — test deviating concentrations and/or volumes
- Thermal cycler and thermal cycling parameters — test deviating annealing temperatures

Design

Similar to precision analysis but differs in selecting variables deliberately.

Fig. 10. Robustness evaluates performance under varying conditions. Analyzing the assay's results under diverse conditions that deviate from the standard primer/probe concentrations or annealing temperatures enables the researcher to determine its robustness. These results can then be used to identify any potential vulnerabilities or limitations that may impact its performance in practical settings. CLSI, Clinical and Laboratory Standards Institute.

An Example of a Robustness Evaluation against RNA Degradation

Let's take a look at an assay robustness scenario that involves determining how well the assay can withstand degraded input RNA (Figure 11).

1. High-quality RNA initiates the process.
2. RNA is artificially degraded (for example, by using heat) to variable degrees.
3. RNA integrity number (RIN) and the percentage of RNA fragments >200 nucleotides (DV200) are measured. Expect higher C_q (quantification cycle) values for more degraded RNA. Require ΔC_q values to remain constant across the degradation series. (See Figure 12 for reference on trueness of fold changes [TOI].)

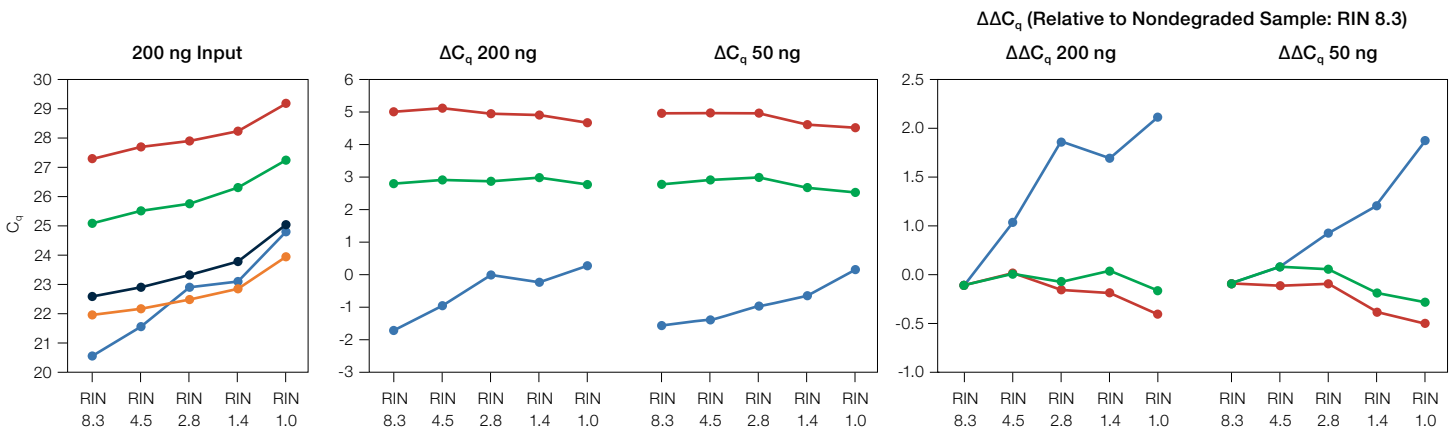


Fig. 11. Example of robustness against RNA degradation. A high-quality RNA sample was subjected to varying durations of heat treatment to yield RNA samples of varying quality (integrity). As expected, the more the RNA was degraded, the higher the observed C_q values. If the assay performs well, then reference genes should compensate for this effect so that after normalization, ΔC_q values should be constant (not affected by RIN values of the RNA). To facilitate interpretation, the right plot shows $\Delta\Delta C_q$ values relative to the intact sample. A perfect assay should show zero values across all tested RNA integrities. C_q , quantification cycle; RIN, RNA integrity number. Results of the three candidate assays for the gene of interest (→), (←), (←); results of the reference genes for normalizing the data (←), (←).

While assay robustness ensures consistent performance under varying conditions, you also want to evaluate assay trueness, guaranteeing the accuracy of results compared to known references. This is another attribute that reinforces the integrity and credibility of assay findings across diverse settings.

Trueness of an Assay

Trueness refers to the closeness of agreement between the average value obtained from a large series of test results and the true value. Assessing trueness involves determining how accurately the assay measures target analytes compared to a known reference or standard.

Approaches like the MicroArray Quality Control (MAQC) study utilize sample mixtures as built-in standards to assess trueness (Web Collection 2021). By comparing assay results to these reference standards, researchers can ascertain the assay’s accuracy and reliability in quantifying target analytes, thus ensuring the validity of their experimental outcomes. Note that this type of study can only assess the trueness of relative quantities, not absolute quantities.

The MAQC study invented the approach with sample mixtures as built-in truth. Figure 12 provides an example.

Trueness

ISO 20395

Three general approaches are used to obtain a suitable reference value:

1. Use of certified reference materials of a similar matrix to that of the test sample and a target copy number concentration within the same range as the routine samples.
2. Recovery experiments using spiked samples.
3. Comparison with results obtained from another method, such as Droplet Digital PCR.

Expression

Evaluate trueness of fold changes in expression — easy to establish.

Mixture	Fractions	TOI
I	100% A	1.000
II	75% A + 25% B	0.750
III	50% A + 50% B	0.501
IV	25% A + 75% B	0.251
V	100% B	0.001

Fig. 12. Example of assay trueness. To determine assay trueness, it is recommended that certified reference materials be used within a matrix similar to that of the test sample and at a concentration range similar to routinely tested samples. As shown in the expression example, using the certified reference material makes it simple to establish fold changes in gene expression. ISO, International Organization for Standardization; TOI, trueness of fold changes.

Applying Assay Trueness to Gene Expression Levels

When we apply trueness to our analysis of the expression levels of a target, we can plot the results as shown in Figure 13.

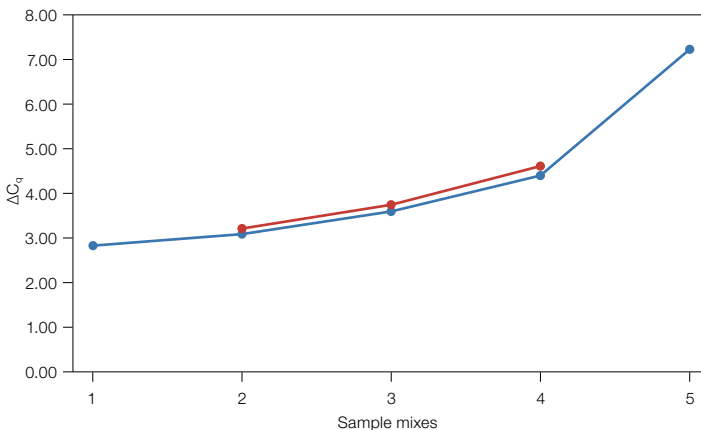


Fig. 13. Comparing assay results to the theoretical C_q for the assay as a measure of trueness. The experimental expression levels of the target, plotted in blue, are compared to the theoretical values for the assay, plotted in red, and provide a measure of assay trueness. C_q , quantification cycle; empirical ΔC_q (—); theoretical ΔC_q (—).

Summary: Assay Validation Benefits

Validation of qPCR and ddPCR assays in regulated environments requires careful consideration of analytical sensitivity parameters, data analysis, and interpretation to ensure the assay's reliability, accuracy, and compliance with regulatory standards.

Mastering assay validation strategies provides several key benefits, including:

- Enhanced confidence in the accuracy and reliability of assay results and more reproducibility in experimental outcomes
- Validity of experimental findings, strengthening credibility of scientific research publications and the scientific community as a whole
- Improved compliance with regulatory requirements and industry standards and greater ability to adapt to changes in these guidelines
- Increased efficiency in troubleshooting assay-related issues, facilitating adoption of new methods and technologies
- Reduced risk of data misinterpretation or misrepresentation

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