Validation of Droplet Digital PCR for High-Throughput Molecular Quantification

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SUMMARY

Droplet digital PCR (ddPCR) enables highly sensitive and precise absolute nucleic acid quantification without reliance on standard curves, supporting high-throughput molecular workflows. In this study, we performed analytical validation of **61 ddPCR assays**, including **27 bacterial/fungal and 34 viral control targets**, using a CLSI-aligned framework.

Validation employed 10-fold serial dilution curves spanning approximately **10^2–10^7 copies per reaction**, with replicate testing across multiple runs and operators. Performance characteristics evaluated included reportable range, optimal testing range, precision, limit of detection (LoD), and lower limit of quantification (LLOQ) using predefined acceptance criteria ($R^2 \geq 0.95$, ≤25% RSD, ≤30% uncertainty).

All assays demonstrated linear quantification across a **five-log dynamic range**, with strong regression performance ($R^2 \geq 0.95$). Precision analysis showed that **~87% of measurements within 10^4–10^6 copies met ≤20% RSD**, confirming this interval as the optimal quantification range. At higher concentrations, RSD values were typically **<10%**, while increased variability (**30–60% RSD**) was observed at ≤10^3 copies due to stochastic partitioning effects.

LoD, defined as the lowest concentration detected with 95% confidence, ranged from **1.3×10^2 to 5.6×10^3** copies across the validated assay panel. Most assays achieved LoD values between **5×10^2 and 1.5×10^3** copies. Sigmoidal detection probability modeling further demonstrated that most assays achieved the 95% positivity threshold at approximately **10^3** copies, confirming the robustness of ddPCR assays.

LLoQ values were typically **5×10^3–1×10^4 copies**, with uncertainty ≤30% for nearly all assays. These results demonstrate that ddPCR provides **robust, reproducible, and scalable quantification**, supporting its application in high-throughput molecular assay validation and quality control.

METHODS

Study Design and Validation Framework: A total of **61 ddPCR assays** (27 bacterial /fungal and 34 viral/particle-like targets) were analytically validated using a CLSI-aligned framework. Performance characteristics evaluated included reportable range, optimal testing range, precision, limit of detection (LoD), and lower limit of quantification (LLoQ) with predefined acceptance criteria ($R^2 \geq 0.95$, ≤25% RSD, ≤30% uncertainty).

Validation curves were generated using 10-fold serial dilutions (~10^7 to 10^2 copies) across a minimum of five concentration levels, each tested in triplicate across multiple runs by multiple operators.

Sample Preparation and Extraction: Bacterial, viral, and control materials were extracted using commercially available kits (Qiagen, ThermoFisher, Roche), with standardized input/output volumes. Extracted nucleic acids were diluted in TE buffer for downstream analysis.

ddPCR Workflow: Reactions (25 µL) were prepared using Bio-Rad EvaGreen or probe-based chemistries, with 5 µL template input. Droplets were generated using the **AutoDG**, amplified on **PTC Tempo/C1000 thermal cyclers**, and analyzed using the **QX200 Droplet Reader**.

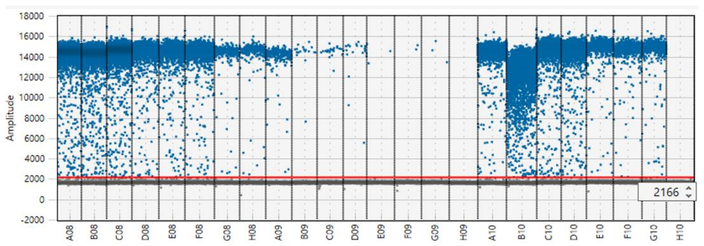
Data Analysis: Absolute quantification was determined using Poisson statistics. Linearity ($R^2 \geq 0.95$), precision (≤25% RSD), LoD (95% confidence), and LLoQ (≤30% uncertainty) were calculated across dilution series and assay panels

CONCLUSIONS

- Analytical validation of 61 ddPCR assays demonstrated robust performance across bacterial, fungal and viral control targets.
- Assays exhibited a broad reportable range **(10^2–10^7 copies)** with strong linearity (**$R^2 \geq 0.95$**).
- The optimal quantification range **(~10^4–10^6 copies)** demonstrated high precision, with ~87% measurements achieving **≤20% RSD.**
- LoD values were predominantly within the $5x10^2$ - $1.5x10^3$ copy range, with **≥95%** detection typically achieved at **~ 10^3 copies**.
- Most assays achieved LLoQ values between **5×10^3 and 1×10^4** copies while maintaining ≤20% measurement uncertainty.
- These results support ddPCR as a sensitive, reproducible, and scalable platform for high-throughput molecular assay validation and quality control applications.

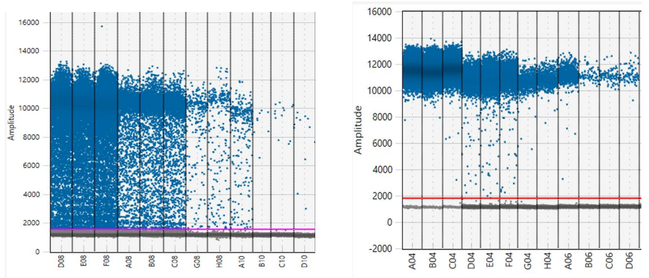
RESULTS

Figure 1. Optimized ddPCR Assay Showing Clear Cluster Separation



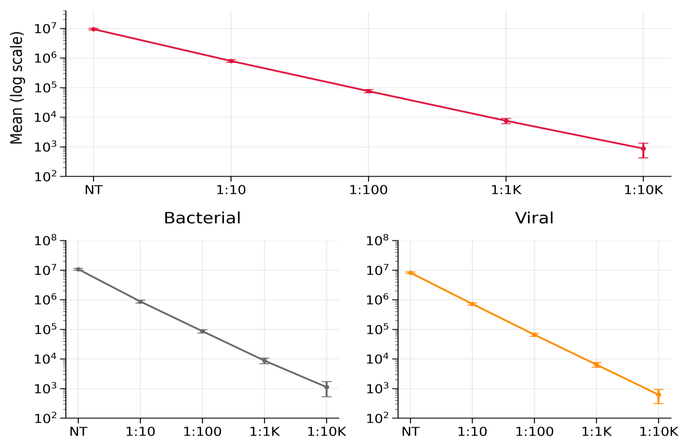
Most assays consistently achieved >16,000 accepted droplets, <2% rain, and >8,000 amplitude units of positive/negative cluster separation. In this representative assay, the distinct positive and negative droplet populations support reliable thresholding and precise target quantification.

Figure 2. Restriction Enzyme Treatment Improves Droplet Cluster Separation



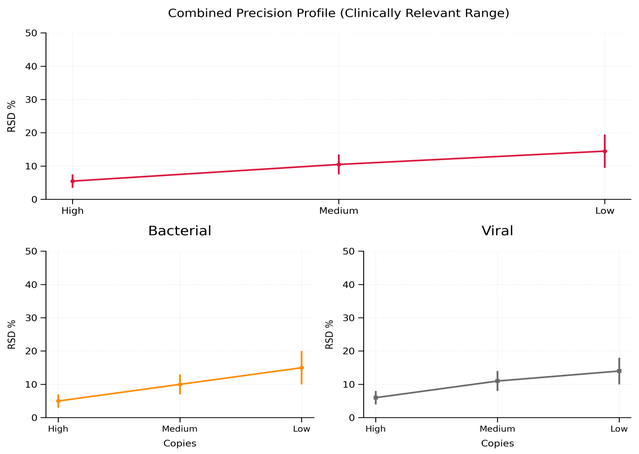
In a small subset of assays, elevated background fluorescence and droplet rain were observed (left). Restriction enzyme digestion reduced background signal and improved positive/negative cluster separation (right), resulting in more reliable quantitative measurements.

Figure 3. Validated Reportable Range (10^3–10^7 copies) Across ddPCR Assays



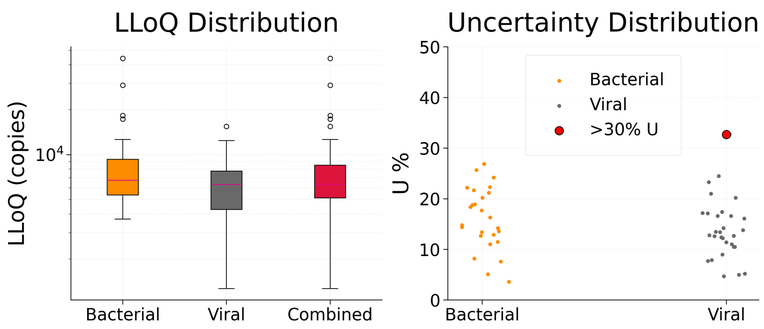
Log-linear dilution curves demonstrating assay performance across combined (crimson), bacterial (dark gray), and viral (dark orange) panels. The combined panel (top) highlights overall assay performance, while individual panels confirm comparable linearity across target classes. Error bars represent standard deviation.

Figure 4. Precision Profile Across the Clinically Relevant Quantification Range



Precision (RSD%) across clinically relevant concentration ranges for combined (crimson), bacterial (dark orange), and viral (dark gray) panels. Precision remained ≤15% across **high (5%-6%), medium (10%-11%)** and **low (14%-15%)** input levels. Error bars represent standard deviation. Variability increases with decreasing input concentration, consistent with Poisson-driven partitioning effects in digital PCR.

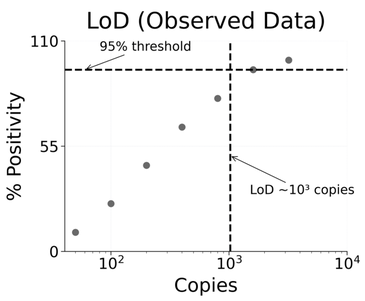
Figure 5. LLoQ and Measurement Uncertainty Across ddPCR Assays



LLoQ is the lowest concentration that can be quantified with acceptable precision and uncertainty. Most bacterial and viral assays achieved reliable quantification between **~5×10^3 and 1×10^4** copies (Box plot – Distribution of Assays LLoQ values), while **~ 80%** of assays demonstrated **<20%** measurement uncertainty (Cluster image – Uncertainty distribution of LLoQ). These results demonstrate robust and reproducible ddPCR quantification across diverse molecular targets.

Figure 6. LoD Performance (Table) and Detection Probability Modeling

Panel	Mean	Range
Bacterial	1.27×10^3	1.93×10^2 – 5.07×10^3
Viral	9.75×10^2	1.31×10^2 – 5.60×10^3
Combined	1.11×10^3	1.31×10^2 – 5.60×10^3



Across the validated assay panel, LoD values ranged from **1.31×10^2 to 5.60×10^3** copies, reflecting the high analytical sensitivity of the ddPCR platform. The representative sigmoid curve demonstrates the expected increase in detection probability with increasing target concentration and shows that most assays achieved the **95%** detection threshold at approximately **10^3** copies. The strong agreement between observed data and model predictions supports the robustness of the LoD estimates and highlights the reliability of ddPCR for low-abundance target detection and quantification.

REFERENCES

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