Transitioning from Quantitative PCR to Droplet Digital PCR for *Mycoplasma* Detection

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Abstract

The following application note provides a guide for those who want to transition from quantitative PCR (qPCR) to Droplet Digital PCR (ddPCR) for *Mycoplasma* testing. The qPCR and ddPCR formats are illustrated, and their interpretations are contrasted. Compared to qPCR, Droplet Digital PCR provides higher sensitivity with a quantitative readout that reports in both genome copies (GC)/reaction and colony forming units (CFU)/ml. The ddPCR assay also demonstrates high reproducibility across operators, instruments, lots, and days.

Introduction

Mycoplasma is a common cell culture contaminant (Uphoff and Drexler 2014). Due to its small size (2–3 µm), *Mycoplasma* cannot be detected by standard light microscopy. In addition, *Mycoplasma* is gram negative and therefore resistant to the beta-lactam antibiotics routinely used in cell culture. When manufacturing cell-based vaccines, biologics, and gene therapies, *Mycoplasma*-free batches are critical for patient safety. If *Mycoplasma* is detected, the entire contaminated production batch must be discarded, which can lead to both supply and related financial losses. To avoid this, false-positive results should be minimized during *Mycoplasma* testing. A simple, rapid, accurate *Mycoplasma* testing solution is a must for ensuring safe and reliable biomanufacturing and minimizing production costs.

Traditional culture-based *Mycoplasma* detection tests require more than 28 days to complete. For many applications, the ability to detect *Mycoplasma* contamination more quickly is important to avoid the need for late-stage mitigation strategies. Quantitative PCR–based tests are replacing bacterial culture–based *Mycoplasma* testing because they shorten the time to results (Jean et al. 2017). However, the most widely used qPCR *Mycoplasma* assay uses SYBR® chemistry, which can generate nonspecific signals and poor resolution at very low input DNA range (<10 copies). The qPCR readout is quantification cycle (Cq), defined as the cycle number where the fluorescence intensity crosses a preset threshold. The lower the Cq, the higher the input DNA concentration. The *Mycoplasma* GC:CFU ratio is used to distinguish free-floating genomic DNA (gDNA) from that of live *Mycoplasma*. The GC:CFU ratio for each culture varies due to growth rate and culture condition variability, so accurate determination of a GC:CFU ratio requires the absolute quantification of *Mycoplasma* GC/well. This cannot be done with qPCR because Cq measurements are relative without a standard curve.

A major advantage of ddPCR technology over qPCR is its ability to partition the bulk PCR reaction into tens of thousands of water-in-oil droplets, turning each droplet into an individual nanoliter bioreactor containing 0, 1, 2… *n* copies of DNA templates (Figure 1). At the end of thermocycling, each partition is identified as either “0,” with no DNA template, or “1” with ≥1 copies of template. The number of input DNA templates is calculated using the simplified Poisson distribution formula $C \propto -ln(E)$, where C is the copy number of input DNA, and E is the percentage of empty droplets. Poisson distribution statistics provide an easy way to calculate the absolute number of DNA molecules in the initial bulk PCR reaction with precision and accuracy and without a standard curve (Basu 2017). The ddPCR readout is copies/µl, and the digital nature of Droplet Digital PCR removes amplification efficiency and instrument variability from copy number calculations. Absolute GC/reaction quantification by Droplet Digital PCR also enables the direct calculation of the GC:CFU ratio for each *Mycoplasma* culture.

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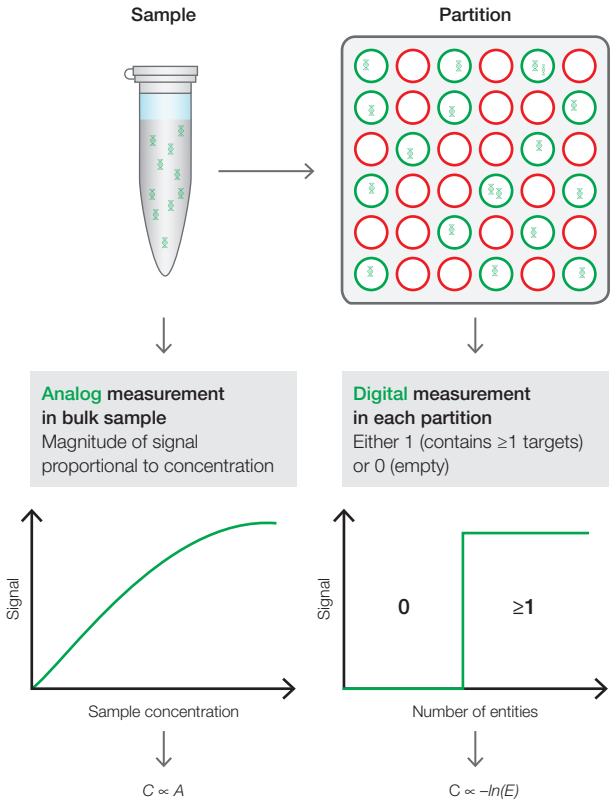
Figure 2 illustrates the qPCR and ddPCR assay readouts. For the qPCR assay (Figure 2A), the Cq is calculated as the cycle number at which the amplification curve crosses the threshold (shown as a horizontal line). For the ddPCR assay (Figure 2B), an orthogonal droplet cluster occupies each quadrant of the FAM (channel 1) vs. HEX (channel 2) 2-D plot. The *Mycoplasma* or positive control copies/µl is calculated using all FAM-positive droplets.



C = concentration; *A* = signal; *E* = % of empty droplets.

Fig. 1. Comparison of qPCR and ddPCR technologies. Quantitative PCR is a bulk measurement with an analog signal output that is proportional to the concentration of input DNA. Droplet Digital PCR uses partitioning to provide a digital signal of 0 or 1 for each partition. The percentage of empty partitions is used to calculate the copies of input DNA (Basu 2017).

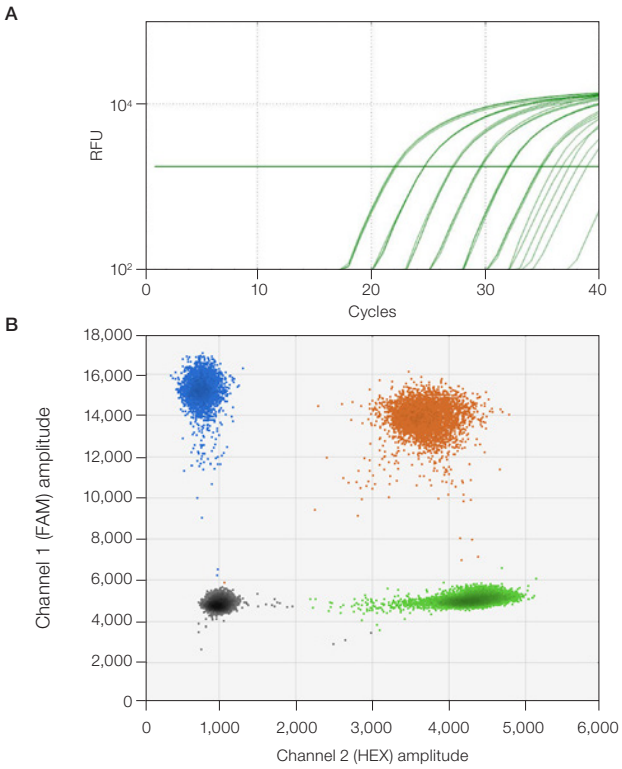
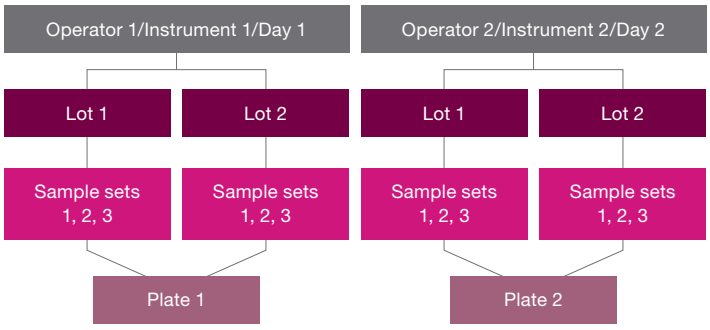


Fig. 2. Readouts for qPCR and Droplet Digital PCR. A, the amplification plot of a titration curve using serially diluted *M. pneumoniae* gDNA. Positive calls for qPCR are determined by the Cq at which the amplification curve crosses a predetermined threshold, indicated as the horizontal line. **B**, 2-D plot of the ddPCR assay readout. Droplet clusters appear in each quadrant. The identification for each cluster is determined by the locations of clusters from the positive control. *Mycoplasma* positive control cluster (FAM) (■); empty droplet cluster (background fluorescence only) (■); *Mycoplasma* and internal control double-positive cluster (FAM + HEX) (■); internal control cluster (HEX) (■). RFU, relative fluorescence units.

Materials and Methods

The ddPCR study design is shown in Figure 3.



Sample Set	Sample ID
1	10 CFU/ml, 1 CFU/ml *Acholeplasma laidlawii* extracted using QIAamp DNA Mini Kit
2	*M. hyorhinis* gDNA at 35, 10, 5, 3 GC/well
3	*M. pneumoniae* gDNA 1:5 titration from 100,000–1.28 GC/well

Fig. 3. Study design. A ddPCR study was performed on three sample sets by two operators using two reagent lots and two instruments on two different days. The qPCR test was performed with two operators using two reagent lots and one instrument on two different days, using the same sample set as the ddPCR study.

Sample Preparation

Purified *M. pneumoniae* gDNA was purchased from American Type Culture Collection (ATCC) (#qCRM-15531D) and prepared as fivefold serial dilutions for both Droplet Digital PCR and qPCR analysis. Purified *M. hyorhinis* gDNA was also purchased from ATCC (#qCRM-17981D) and diluted to 35, 10, 5, and 3 GC/well. Heat-inactivated *A. laidlawii* CFU standards (Minerva Biolabs GmbH, #103-8003) were resuspended in cell culture media containing 10% fetal bovine serum (FBS) at concentrations of 1 and 10 CFU/ml. The resuspended *A. laidlawii* samples were extracted with the QIAamp DNA Mini Kit (QIAGEN, #51306) and eluted with 100 µl of elution buffer.

qPCR and ddPCR Protocol

A commercially available, SYBR® based qPCR assay was run side-by-side with the Vericheck ddPCR *Mycoplasma* Detection Kit (Bio-Rad Laboratories, Inc., catalog #12013126). The CFX96 Touch Real-Time PCR System (Bio-Rad, #1855195) was used for qPCR, and the QX200 AutoDG Droplet Digital PCR System (Bio-Rad, #1864100) was used for Droplet Digital PCR. The qPCR data were analyzed using CFX Maestro Software (Bio-Rad, #12013758), and the threshold was set with the software's automatic thresholding function. The cutoff for a qPCR positive call was set at Cq = 36.23, with melting temperature (T_m) between 75–82°C. The ddPCR data were analyzed using QX Manager Software, Regulatory Edition (Bio-Rad #12012172), and thresholds were set using positive control–based automatic thresholding. Wells containing ≥1 positive droplets were considered positive. A detailed ddPCR operation protocol can be found in the Vericheck ddPCR *Mycoplasma* Detection Kit.

Results

Assay Sensitivity

Assay sensitivity was tested for each assay by comparing 10 CFU/ml and 1 CFU/ml *A. laidlawii* standards (Figure 4). Quantitative PCR detected the latter at an average Cq of 36.87. These samples were considered negative because they exceeded the 36.23 Cq cutoff. The same samples tested positive by Droplet Digital PCR (Figure 2B). The ddPCR measurements corresponded well with expected values. The limit of detection (LOD) of the ddPCR assay was at least tenfold lower than that of the qPCR assay. The GC:CFU ratio was calculated using the formula:

GC:CFU ratio = (GC/well × eluate volume) / (9 × CFU/ml)

The eluate volume was 100 µl, and each reaction used 9 µl of extracted sample. As such, the GC:CFU ratio of this batch of *A. laidlawi*i CFU standards was between 464.68 and 478.56.

The GC/well sensitivity for each assay was assessed side-by-side using *M. hyorhinis* gDNA standards. Table 1 summarizes the GC LODs for each assay. Droplet Digital PCR detected *M. hyorhinis* gDNA in every sample with a mean of 6.19 GC/well, supporting the GC LOD of ≤6 GC/well. The qPCR kit claimed an LOD of 10 GC/well, which was supported by 100% positivity at 10 expected copies/well. Thus, Droplet Digital PCR provides higher sensitivity than qPCR in this application.

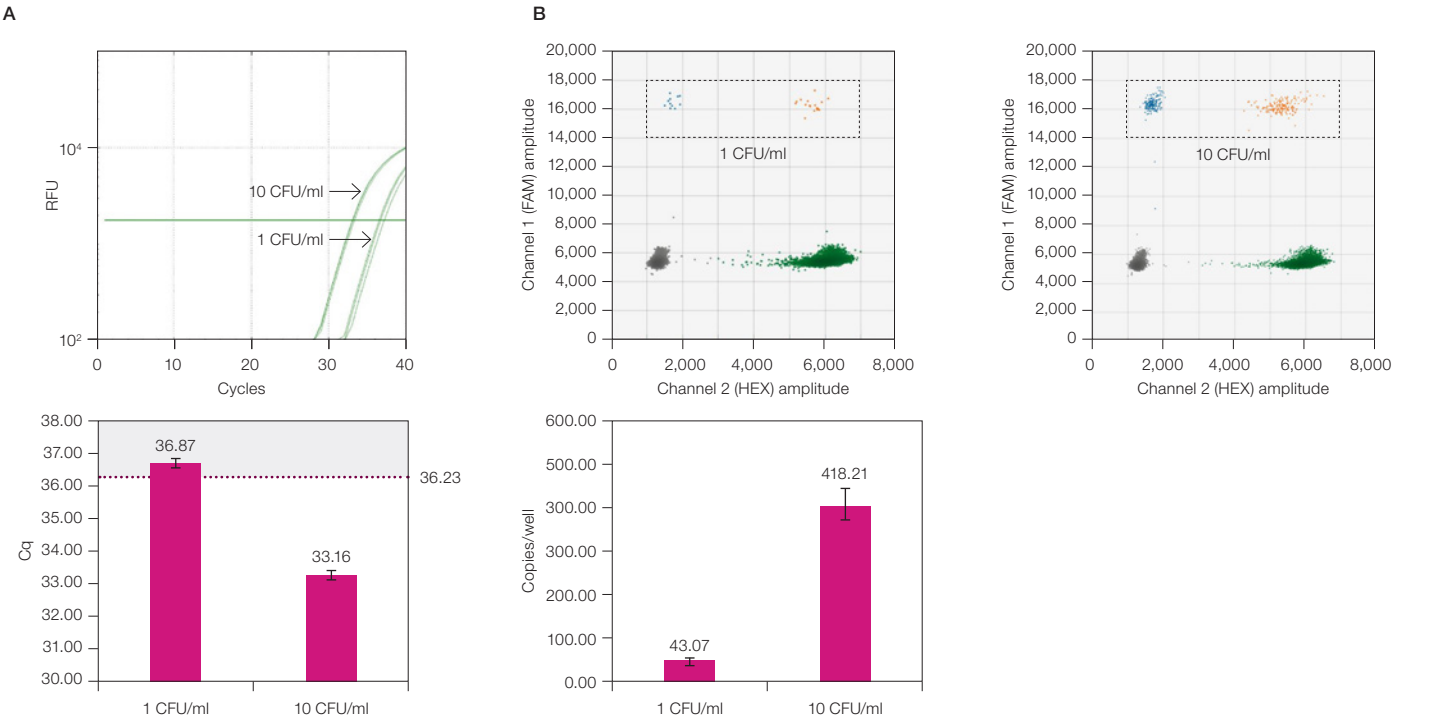


Fig. 4. Comparison of qPCR and ddPCR sensitivity for *A. laidlawii* standards. A, qPCR amplification curves (top) and average Cq values (bottom) for 1 and 10 CFU/ml *A. laidlawii* standards. The qPCR LOD was 10 CFU/ml because none of the 1 CFU/ml standards reached the 36.23 Cq cutoff for positive detection. **B**, ddPCR 2-D plots (top) with *A. laidlawii*–positive droplets highlighted in the black, dashed rectangles; average copies/well values (bottom) for 1 and 10 CFU/ml *A. laidlawii* standards. The ddPCR LOD was 1 CFU/ml because all ddPCR samples tested positive with ≥1 FAM-positive droplet/well. Error bars represent standard deviation, N = 12. Cq, quantification cycle; RFU, relative fluorescence units.

Table 1. Comparison of qPCR and ddPCR sensitivity for *M. hyorhinis* gDNA. Only positive qPCR wells were used for mean Cq calculation. The LOD is defined as lowest concentration that tests positive at 95%.

	qPCR LOD ~10 GC/Well			ddPCR LOD ~6.2 GC/Well		
Expected Copies/Well	Mean Cq	Number of Positives/n	Positive, %	Observed Mean Copies/Well	Number of Positives/n	Positive, %
35	33.00	12/12	100	45.75	12/12	100
10	34.55	12/12	100	16.54	12/12	100
5	35.38	43/48	90	9.55	48/48	100
3	36.54	17/40	43	6.19	40/40	100

Assay Reproducibility

The reproducibility of the ddPCR assay was assessed using fivefold serial dilutions of *M. pneumoniae* gDNA standards at 100,000–1.28 copies/well. The operator-to-operator variability was analyzed as a composite of lot-to-lot, instrument-to-instrument, and day-to-day results. The consistency between two operators was measured by the slope as well as the R^2 value of the linear regression fit for each group of data. The variation between the slopes and R^2 values was negligible, indicating excellent reproducibility across operators, instruments, lots, and days.

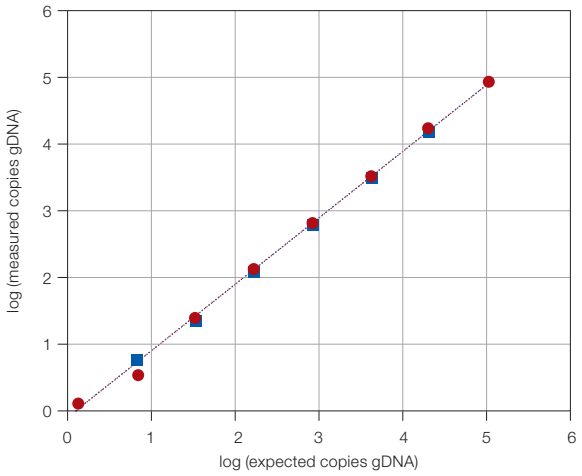


Fig. 5. Reproducibility of ddPCR assay. The assay was performed by two operators, each testing two lots with two instruments on two different days. The slope of the linearity plots differed by 1%, and the R^2 values differed by 0.1%. User 1 (■): R^2 = 0.9992; user 2 (●): R^2 = 0.9982.

Discussion

The purpose of this study was twofold: to illustrate the expected output data format for each assay and to compare their results using the same set of *Mycoplasma* samples. At least one assumption was made for this study. The qPCR assay was run on the CFX96 Touch System and automatic thresholding was done using CFX Maestro Software, but T_m and cut-off criteria were derived from a different instrument and different software.

Conclusion

This application note provides a starting point for transitioning from qPCR to Droplet Digital PCR for *Mycoplasma* testing. Droplet Digital PCR transforms a traditionally qualitative method into a quantitative one that reports data in copies per well. Its ability obtain an absolute number of genome copies enables calculation of the GC:CFU ratio in any given *Mycoplasma* culture without need for a standard curve. In addition, the ddPCR assay is more sensitive than qPCR at <10 copies/well and <10 CFU/ml. With a sensitivity of 1 CFU/ml, the ddPCR assay readily meets the <10 CFU/ml criteria set by the European (EDQM 2016), U.S. (U.S. Pharmacopeial Convention 2019), and Japanese (PMDA 2016) Phamacopeias. In conclusion, the Vericheck ddPCR *Mycoplasma* Detection Kit provides a quantitative, probe-based method for *Mycoplasma* detection with high precision, reproducibility, specificity, and sensitivity.

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