Quantification of next-generation sequencing libraries using the QuantStudio Absolute Q Digital PCR System

Highlights

- Consistent digitization of reagents into >99% of available microchambers compared to 60.9% on droplet digital PCR platform
- Improved separation of positive and negative microchambers in two-dimensional threshold view

Introduction

Advances in next-generation sequencing (NGS) technologies have accelerated the discovery of actionable genomic targets. Accurate quantification of the final library products is critical to maximizing both data quality and output. However, conventional quantitative PCR (qPCR) methods commonly used to assess NGS library concentrations do not evaluate the concentration of complete library fragments. Moreover, in some cases, the amount of final library product is limited, and the input requirement for consistent qPCR quantification can become a hindrance.

Multiple studies have demonstrated that utilizing digital PCR (dPCR) as a quantification tool for NGS libraries before sequencing helps optimize sequencing run performance, data generation, and data quality [1,2]. dPCR, which gives absolute rather than relative quantification, is more accurate and precise at low concentrations than qPCR. By leveraging multiplexed dPCR, users can identify and quantify specific library fragments representing sequenceable molecules (Figure 1).

In this technical note, the Applied Biosystems™ QuantStudio™ Absolute Q™ Digital PCR System was used to perform NGS library quantification of sequenceable library molecules. Four NGS libraries with various insert sequence lengths were quantified in this study.

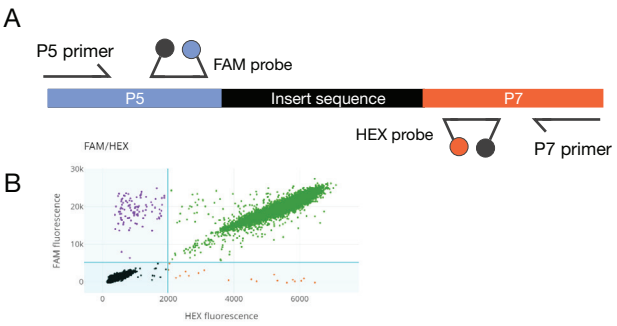

Figure 1. dPCR assay for NGS library quantification. (A) The duplex assay amplifies only libraries that have P5 and P7 adapters. **(B)** Using dPCR, absolute quantification of sequenceable library fragments is possible by counting the total number of double-positive microchambers.

Materials and methods

Library preparation, separation, and dilution

A single mixed-size ATAC-seq library was separated based on fragment length using the BluePippin™ system (Sage Science, Inc.) to create four separate NGS libraries with average fragment lengths of 300, 500, 700, and 1,000 base pairs. After size separation, each library was amplified using Q5™ High-Fidelity DNA Polymerase (New England Biolabs, Inc.) and cleaned up using the SPRIselect™ Reagent Kit (Beckman Coulter, Inc.). The amplified libraries were then diluted 1:200,000 to create “Dilution 1” and then subsequently diluted 1:4 serially for a total of 6 dilutions for each of the 4 fragment lengths.

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dPCR reagent preparation

dPCR quantification of the NGS libraries was performed using 5 µL of each serial dilution of the NGS library per reaction. The dPCR reagents are listed in Table 1. After preparing the dPCR mix, 9 µL of the reaction mixture was loaded into a well of the Applied Biosystems™ QuantStudio™ MAP16 plate (MAP = microfluidic array plate), followed by an overlay of 15 µL of isolation buffer. The prepared QuantStudio MAP16 plate was then loaded onto the QuantStudio Absolute Q Digital PCR System. The following thermal parameters were used for each digital PCR run: 96°C hold for 10 min, followed by 45 cycles of 95°C denaturation for 5 sec and 61°C annealing and extension for 30 sec. Data were collected in the channels for the FAM™ and HEX™ dyes.

Table 1. Preparation of dPCR reagents for NGS library quantification on the QuantStudio Absolute Q dPCR system.

Reagent	Volume per reaction (final concentration)
Digital PCR master mix (5X)	1.8 µL (1X)
Forward primer	(900 nM)
Reverse primer	(900 nM)
P5 FAM probe	(250 nM)
P7 HEX probe	(250 nM)
Diluted library	5 µL
Nuclease-free water	Fill to 9 µL

Analysis on QuantStudio Absolute Q software

Applied Biosystems™ QuantStudio™ Absolute Q™ Analysis Software was used to calculate the concentrations of the NGS libraries for each dilution series. The software reports the concentrations of targets that are positive for the FAM dye–labeled probe, positive for the HEX dye–labeled probe, and double-positive for both probes. By design, microchambers containing complete and sequenceable library fragments are positive for both the P5 (FAM) and P7 (HEX) probes. The double positives are represented as a cluster in the upper-right quadrant of the 2D fluorescence scatter view (Figure 1B, green dots). The concentration of double positives was then used in Equations 1 and 2 (below) to determine the concentrations of the original library stock for each dilution point.

Results

Quantification of sequenceable library fragments

The four libraries, which vary in fragment length, were each quantified across six 4-fold serial dilutions. The reported concentrations (in copies/µL (cp/µL)) from the QuantStudio Absolute Q dPCR system, for the dilution series for each library with specific fragment size, are shown in Figure 2. The observed concentrations decreased as would be expected for 4-fold dilution series, across all conditions. Using the reported concentration and Equations 1 and 2, the original concentrations of each NGS library were calculated to be 2.65 nM, 3.06 nM, 4.63 nM, and 16.98 nM for the 300 bp, 500 bp, 700 bp, and 1,000 bp NGS libraries, respectively (Table 2).

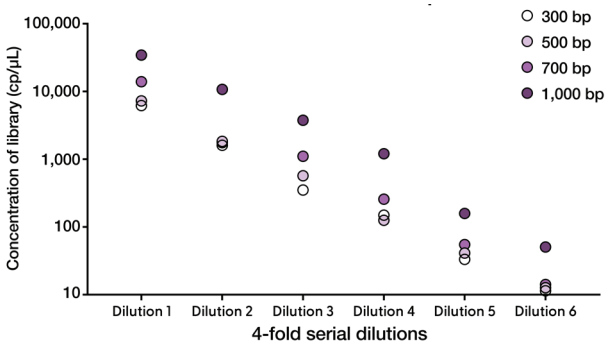

Figure 2. Concentrations of sequenceable NGS library fragments reported by the QuantStudio Absolute Q dPCR system. Quantitation is in cp/µL for the four size-separated NGS libraries of 300 bp, 500 bp, 700 bp, and 1,000 bp. Each size was quantified across 6 dilutions [3].

Table 2. Calculated stock library concentration for four size-separated NGS libraries at each point of 6-point dilution series [3].

Library	300 bp	500 bp	700 bp	1,000 bp
Dilution 1	2.06	2.41	4.62	11.44
4-fold	2.13	2.44	2.33	14.26
16-fold	1.86	3.02	5.86	19.93
64-fold	3.17	2.67	5.45	25.64
256-fold	2.82	3.50	4.67	13.44
1,024-fold	3.87	4.31	4.81	17.18
Average	2.65	3.06	4.63	16.98
Standard dev.	0.78	0.74	1.22	5.18

$$\text{Stock library concentration (cp/µL)} = \frac{\text{Concentration* (cp/µL)} \times 9\ \text{µL dPCR reaction}}{5\ \text{µL diluted library input}} \times 2 \times 10^5 \times \text{additional dilution} \quad \textbf{(Equation 1)}$$

$$\text{Stock library concentration (M)} = \text{Stock library concentration (cp/µL)} \times \frac{1 \times 10^6\ \text{µL}}{6.02 \times 10^{23}\ \text{cp/mol}} \quad \textbf{(Equation 2)}$$

* Concentration as reported by QuantStudio Absolute Q software.

Advantages of MAPs for consistent reagent digitization

An additional benefit of using MAP technology to perform dPCR is the robustness of reagent digitization. MAP technology leverages a micromolded plastic dPCR plate with fixed-volume arrays and ensures that distribution across >95% of the available microchambers is achieved for all dPCR reactions.

To compare the distribution or digitization efficiency between MAP technology–based dPCR and droplet-based dPCR, the four NGS libraries were quantified using droplet-based dPCR in parallel with the QuantStudio Absolute Q dPCR system. To quantify the NGS libraries, 46 reactions were run on both systems. Out of a total of 20,480 fixed microchambers per MAP technology–based dPCR reaction array, the mean accepted microchamber count (and standard deviation (SD)) for reactions run on the QuantStudio Absolute Q dPCR system was 20,412 (SD 127). Out of the 20,000 droplets expected per reaction, the mean accepted droplet count for the droplet-based dPCR reactions was 12,138 (SD 1,267). For this dataset, the minimum number of accepted microchambers per reaction for the QuantStudio Absolute Q dPCR system was 19,645 and the minimum number of accepted droplets for the droplet-based dPCR reactions was 8,629 (Figure 3).

Finally, a comparison between the representative 2D microchamber fluorescence plots of the droplet-based dPCR (Figure 3B) and QuantStudio Absolute Q dPCR system (Figure 3C) highlights the improved signal separation between the resulting double-positive microchambers and negative microchambers using the QuantStudio Absolute Q dPCR system. The improved separation aids in consistent thresholding of dPCR data and thus gives more robust and reproducible quantification results.

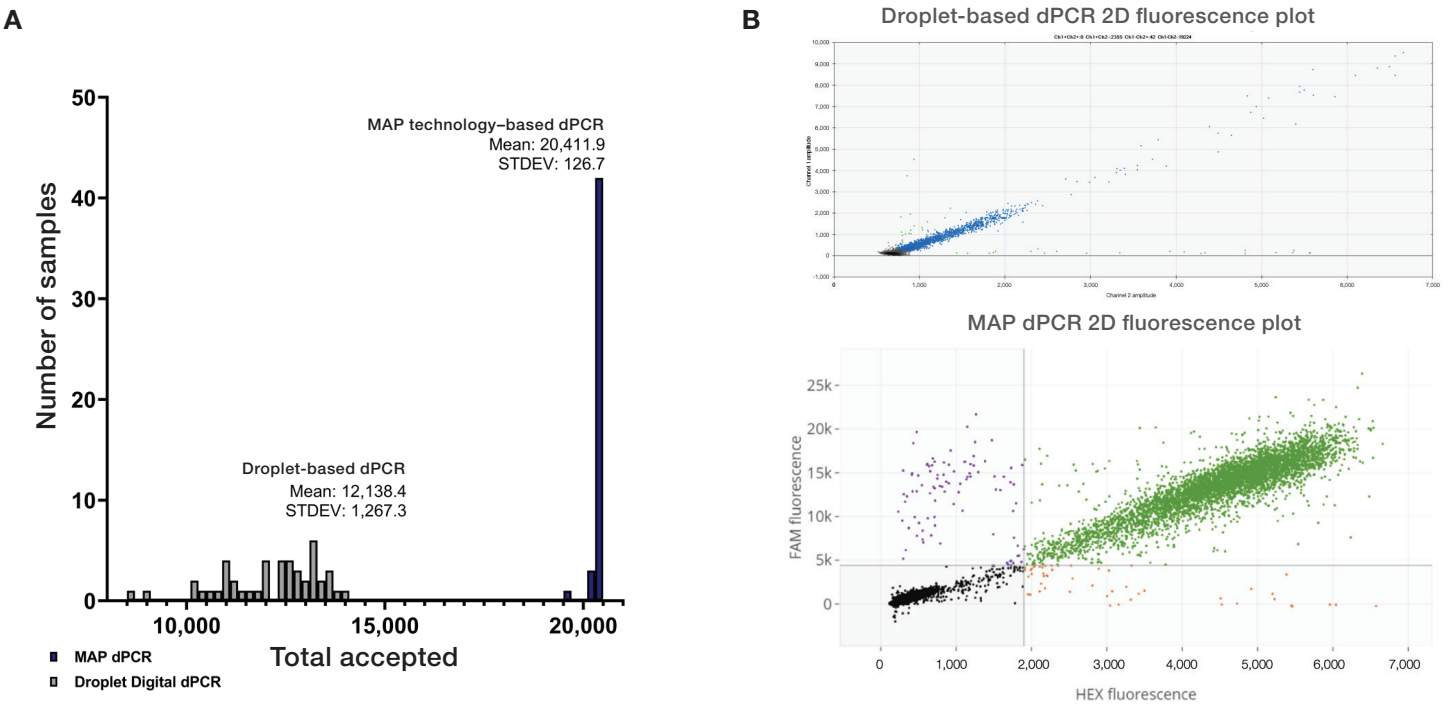

Figure 3. Superior reagent digitization and signal separation for MAP technology–based dPCR. (A) Frequency distribution of total accepted microchambers or droplets per dPCR reaction for 46 samples run in parallel on the QuantStudio Absolute Q dPCR system using MAP technology–based dPCR (dark blue) and a droplet-based dPCR platform (gray). **(B)** Two-dimensional fluorescence plots of a 1,000 bp library, from the droplet-based dPCR platform (top) and the QuantStudio Absolute Q Digital PCR System (bottom) [3].

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Summary

Quantification of sequenceable library fragments

Quantification of NGS libraries by dPCR is advantageous because it is possible to distinguish complete sequenceable library molecules as well as perform absolute quantification. Given that the final concentrations of NGS libraries can vary based on the preparation method and performance of the method used, it is critical for each dPCR reaction to be as consistent as possible to maintain robust quantification across a wide range of input concentrations.

The highest level of precision for dPCR quantification occurs when an average of 1.59 copies of target per microchamber is present in the reaction volume [4]. This means that for high-concentration samples, the total number of accepted microchambers is critical because more analyzed microchambers can improve precision by bringing the average closer to 1.59. In the case of NGS library quantification, because the sample consists of amplified nucleic acids, the concentrations of the target are usually very high. The consistently high numbers of analyzed microchambers with the QuantStudio Absolute Q dPCR system ensure high precision even at high concentrations of the target, ensuring high-quality NGS library quantification.

Here we have demonstrated the capabilities of the MAP technology to facilitate robust NGS library quantification across a wide range of fragment sizes and concentrations, while achieving an exceptionally high number of accepted microchambers per dPCR reaction.

References

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