Bio-Rad's ddPCR™ Library Quantification Kit for Ion Torrent Enables Accurate Library Quantification and Excellent Balancing of Pooled Libraries

Tech Note

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Digital PCR

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Introduction

Advancements in low-cost next-generation sequencing (NGS) platforms have enabled sequencing to become routine practice in many laboratory settings, facilitating the analysis of large amounts of genetic information. As these platforms rapidly develop solutions to simplify library preparation, there is an increasing need to understand the quality of the library generated prior to sequencing. Accurate quantification of the library is essential to achieve optimal data and maximize sequencing throughput on these platforms.

The Ion AmpliSeq library kits are designed for rapid preparation of amplicon libraries from genomic target regions. The workflow requires quantification of the input library prior to the emulsion step where clonal amplification occurs. Precise quantification at this step is important for reducing polyclonal reads, which are unusable, and for ensuring proper balance of multiple libraries pooled together for a single sequencing run. Inadequate balancing of pooled libraries will result in overrepresentation of some libraries and underrepresentation of others. Current quantification methods using real-time quantitative PCR (qPCR) are laborious and have significant limitations that make the library calibration unreliable (Laurie et al. 2013).

Here we demonstrate that Bio-Rad's Droplet Digital™ PCR (ddPCR) system can be easily incorporated into the library preparation workflow to accurately quantify and balance libraries for the Ion Torrent sequencing platforms. ddPCR provides highly precise measurements to quantify Ion Torrent libraries without the need for standards. An added benefit of ddPCR is the ability to assess library quality by viewing droplet population data plots that discern features such as well-constructed library fragments from adapter-adapter dimers.

Materials and Methods

Ion Torrent Library Construction

The Ion AmpliSeq library kit 2.0 (Life Technologies Corporation) contains barcode adapters that are ligated onto the fragments to be sequenced. The library is constructed such that both the X (or A) and P1 adapters are added onto all fragments (Figure 1). After purification of the barcoded library products, library concentration is determined by ddPCR. Quantified libraries are templated onto Ion Sphere particles and enriched with the Ion OneTouch DL system (Life Technologies). This is followed by sequencing of the libraries on Ion Torrent platforms (Life Technologies).

For this study, eight uniquely barcoded Ion AmpliSeq libraries were created from 10 ng of eight individual human genomic DNAs (NA11994, NA12872, NA18507, NA18537, NA18852, NA19107, NA19108, and NA19314 from the Coriell Institute for Medical Research). The libraries were constructed according to the manufacturer's protocol using the Ion AmpliSeq cancer panel primer pool with 16 cycles of amplification.

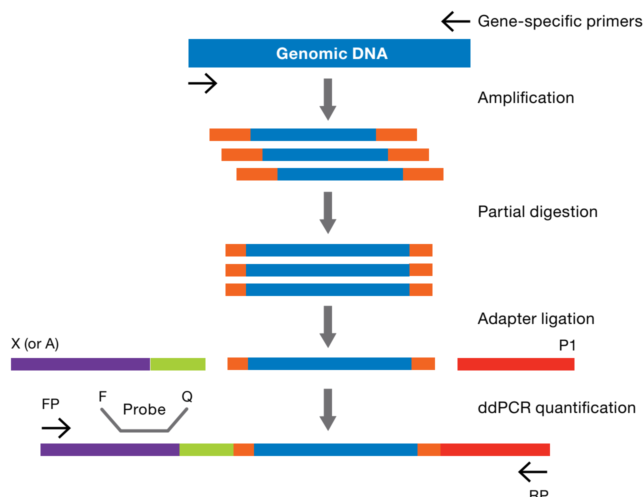

Fig. 1. Illustration of the Ion AmpliSeq workflow with the incorporation of ddPCR analysis to quantify library constructs. F, fluorophore; FP, forward primer; Q, quencher; RP, reverse primer.

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Ion Torrent Library Quantification by ddPCR
The ddPCR library quantification kit for Ion Torrent contains a FAM-labeled hydrolysis assay designed to target the X (or A) and P1 adapter sequences. Signal from the assay is used to confirm the formation of properly adapted library fragments from fluorescence amplitude plots generated by QuantaSoft™ software (Bio-Rad Laboratories, Inc.).

ddPCR quantification for each sample was performed by preparing serial dilutions of the purified library in TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). For quantification using the ddPCR library quantification kit, 20 µl mixtures composed of Bio-Rad's 2x ddPCR supermix for probes (no dUTP), 20x library quantification assay for Ion Torrent, and 4 µl of 10^{-4}-, 10^{-5}-, and 10^{-6}-fold library dilutions were made. Each mixture was converted into droplets in the QX100™ droplet generator then amplified by PCR using a C1000 Touch™ thermal cycler (Bio-Rad). Thermal cycling conditions were 95°C for 10 min (hot-start), followed by 40 cycles of 95°C for 30 sec then 56°C for 60 sec (with ramp rates of 2°C/sec for each temperature), then a 12°C infinity hold. Following amplification, droplets were analyzed with the QX100 droplet reader (Bio-Rad). Stock nanomolar concentrations were backcalculated from the resulting copies per microliter reported in the QuantaSoft software for each library. The barcoded libraries were diluted to input concentrations ranging from 2–10 pM and pooled together at each concentration for loading onto the Ion OneTouch DL system for clonal amplification. Downstream sequencing was performed on the Ion PGM system using the Ion 314 chip (Life Technologies).

Molarity Calculation from Copies per Droplet
To backcalculate the stock concentration of each library from ddPCR concentration values within the range of 100–5,000 copies/µl, account for the dilution factors, including the fivefold dilution of the ddPCR reaction mixture (4 µl of the dilution into 16 µl of supermix).

Example: A library at the 10^{-6} dilution yielded 2,000 copies/µl in the ddPCR reaction mixture. Multiply 2,000 by 10^6 and by 5 to account for the dilution ($2{,}000 \times 10^6 \times 5 = 10^{10}$ copies/µl of original stock library). To obtain nM: 10^{10} copies/µl x 10^6 µl/L/6.022 x 10^{23} copies/mole = 1.66×10^{-8} M or 16.6 nM.

Quantification Using Ion Library Equalizer Kit and KAPA qPCR
The manufacturer's recommended options for quantifying Ion AmpliSeq libraries prior to template preparation in the Ion OneTouch DL system were also evaluated in parallel with ddPCR quantification. The same barcoded libraries were processed with the Ion Library Equalizer kit (Life Technologies), which is designed to achieve equimolar libraries without quantification, and quantified by qPCR using the KAPA SYBR® FAST master mix Bio-Rad iCycler™ qPCR kit (Kapa Biosystems, Inc.). Each method followed the manufacturer's protocol, and the results were compared with ddPCR using sequencing metrics obtained from the Ion PGM post-run sequencing report. The metrics used for comparison included percentage filtered polyclonal reads, percentage final reads, and the distribution of reads for barcoded samples, which were balanced by ddPCR, the Ion Library Equalizer kit, or the KAPA qPCR kit.

Results and Discussion

In order to examine the accuracy of ddPCR quantification compared to the Ion Library Equalizer kit or the KAPA qPCR quantification method, the barcoded libraries generated from the Ion AmpliSeq library kit were normalized to be equimolar and sequenced on five separate Ion 314 chips according to the quantification methods studied. Accurate library quantification is essential for minimizing polyclonal reads and ensuring proper balance of multiple libraries combined into a single Ion Torrent sequencing run.

Using the concentrations determined by ddPCR quantification, the Ion Library Equalizer kit, and the KAPA qPCR kit, the eight independently barcoded libraries were diluted and combined at equimolar concentrations of 8 pM each, then loaded onto the Ion OneTouch DL system. The resulting percentage total reads mapped for each barcoded library were compared across the different quantification methods and the ideal representation from each library was calculated to be 12.5% of the total reads (Figure 2). The libraries measured with ddPCR quantification demonstrated excellent balance, with less than 2% difference in representation between all eight libraries pooled for the same sequencing run while the libraries normalized with the Ion Library Equalizer kit and quantified by KAPA qPCR demonstrated an imbalance of up to 16% and 45%, respectively. Sequencing results from ddPCR-quantified libraries were observed to be consistent and accurate in comparison to the results obtained from the other two quantification methods.

To determine the optimal library input as a function of percentage final reads and percentage filtered polyclonal reads, stock concentrations calculated from ddPCR quantification were diluted and balanced using different input concentrations ranging from 2–10 pM. The libraries were loaded onto the Ion OneTouch DL system for clonal amplification and sequenced on the Ion PGM system. The results show that percentage polyclonality and percentage final library reads are relatively unaffected by most ddPCR input concentrations from the Ion Torrent post-sequencing run data analysis (Figure 3). The 2 pM input has a slight decrease in percentage final library reads, likely due to underpopulating the Ion Sphere particles (ISPs) with template,

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while the 10 pM input shows an increase in polyclonal reads due to overloading the ISPs with multiple templates. The results obtained in this study suggest that loading library concentrations between 4–8 pM is within the acceptable range for the Ion OneTouch DL system and subsequent sequencing on an Ion 314 chip, while 6 pM is the optimal loading recommendation (calculated by ddPCR quantification). Emulsion generation and amplification in the Ion OneTouch DL system likely contribute to the quality of the sequence obtained from the Ion PGM system.

In addition to quantification, ddPCR is information rich and can provide qualitative analysis of libraries by plotting fluorescence amplitudes of droplets versus the droplet counts for each well. The observed population above the positive droplets is attributed to droplets that contain adapter-adapter dimers because these are the most efficiently amplified and thus have the highest fluorescence amplitude signal. Likewise, the population below the positive droplets is attributed to potential multiple insert ligations or larger fragments that are abnormal and likely due to inherent errors in the library preparation at the ligation step (Figure 4).

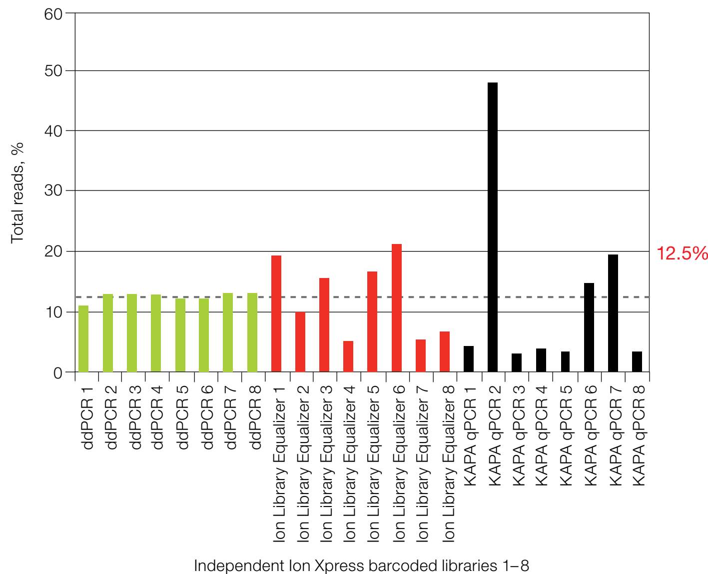

Fig. 2. Comparison of eight balanced Ion AmpliSeq cancer panel libraries using ddPCR, Ion Library Equalizer kit, and KAPA qPCR quantification. The same eight independently barcoded libraries were quantified by ddPCR, Ion Library Equalizer kit, and KAPA qPCR kit. Each library was diluted and pooled to 8 pM equimolar concentrations, as determined by the three quantification methods, and processed on three separate Ion 314 chips according to the different methods studied. Proper library balancing was assessed by the percentage total sequencing reads. The ddPCR balanced libraries (■) were within 2% difference of each other whereas the libraries normalized by the Ion Library Equalizer kit (■) were imbalanced by up to 16% difference and the libraries quantified by KAPA qPCR (■) were imbalanced by up to 45% difference. The ideal percentage total read was calculated as 12.5% representation of each barcoded library within each pooled run.

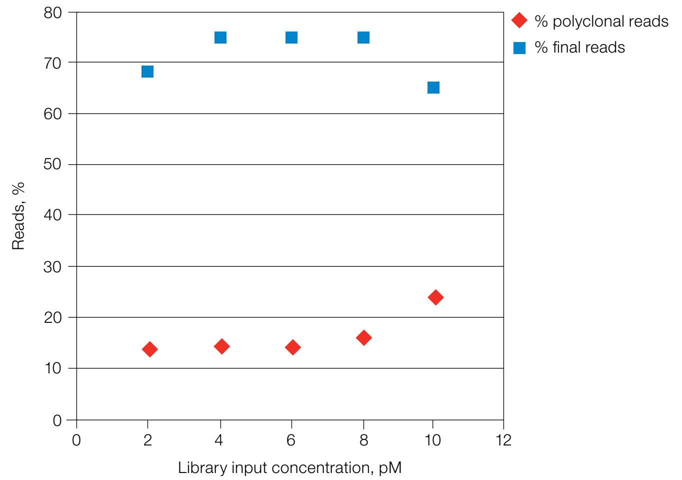

Fig. 3. Percentage filtered polyclonal reads and percentage final reads by library input based on ddPCR quantification. The percentage filtered polyclonality and the percentage final library reads are all relatively insensitive to changes in concentration for the range examined here (2–10 pM) across the same Ion AmpliSeq library. The ideal input range for this study falls between 4–8 pM. We found 6 pM to be the optimal loading concentration for the Ion OneTouch DL system based on ddPCR quantification.

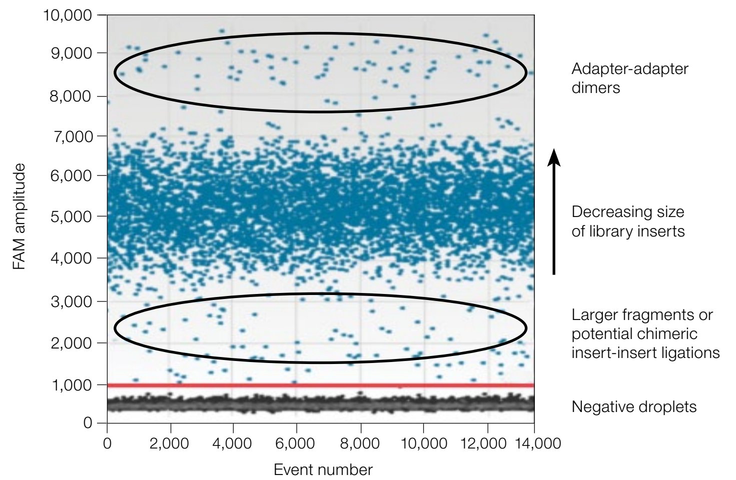

Fig. 4. Library quality assessment. 1-D fluorescence amplitude plot corresponds to an Ion AmpliSeq library quantified by ddPCR. The plot represents data from a single well in ddPCR showing separation of positive and negative droplets as determined by the pink threshold line. The population above the positive droplets in the plot represents adapter-adapter dimers with no insert while the population below the positive droplets likely arises from larger fragments or potential chimeric inserts ligated together. ddPCR provides a quantitative as well as qualitative measurement of the library.

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By quantifying potentially poorly formed library inserts, the user can get a sense of their library quality. If the library is considered to be of too poor quality, the user may choose to remake the adapted library prior to sequencing, thus saving time and money by not sequencing poorly constructed libraries. The combination of accurate quantification and qualitative measurements of library constructs is a unique feature of ddPCR.

Conclusions

Here we demonstrate an improvement in quantification and balancing of pooled libraries for Ion PGM sequencing runs. We explored the Ion AmpliSeq cancer panel primer pool using eight independently barcoded DNA samples to observe the performance in balancing across each library. We compared the use of ddPCR to quantify purified library versus the recommended methods of normalizing with the Ion Library Equalizer kit or quantifying by qPCR. Our results show that ddPCR was superior to qPCR in terms of the sequencing metrics measured because of optimally templated ISPs and demonstrated excellent balance between all libraries that were pooled for the same sequencing run.

The QX100 and QX200 Droplet Digital PCR systems complement NGS platforms by providing a means to measure absolute concentration of relevant molecular species without the need for standards. ddPCR enables accurate and precise balancing of pooled library samples and permits proper analysis to optimize the performance of NGS systems. Additional benefits of ddPCR include rich fluorescence amplitude data to assess library construction quality.

For more information, visit
www.bio-rad.com/web/ddPCRIonTorrentQuantKit.

Reference

Laurie MT et al. (2013). Simultaneous digital quantification and fluorescence-based size characterization of massively parallel sequencing libraries. Biotechniques 55, 61–67.

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