ddPCR™ Quanti□cation and Quality Control of Ion Torrent Sequencing Libraries for Optimal Loading and Multiplex Balancing onto the Ion PGM

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Introduction

A ddPCR method was developed to accurately quantify and quality control Ion Torrent AmpliSeq sequencing libraries. A FAM assay probe was designed to bind the universal region of Ion Torrent's adapter sequence. ddPCR 1-D plots allow assessment of the quality of the libraries generated, as adapter dimer and potential chimeric species are in populations distinct from those of the correctly constructed libraries. Accurate ddPCR quanti□cations allow the user to load the optimal amount of libraries such that passing percent-templated Ion Sphere particles (ISPs) are achieved, and maximal useable sequencing reads are generated while minimizing □ltered polyclonal reads. qPCR quanti□cations using KAPA Bioscience's kit yielded failed percent-templated ISPs, which caused low □nal library reads, high □ltered polyclonal reads, and highly variable reads between multiplexed libraries. Performing "equalization" per Life Technologies' protocol caused up to a fourfold difference in reads.

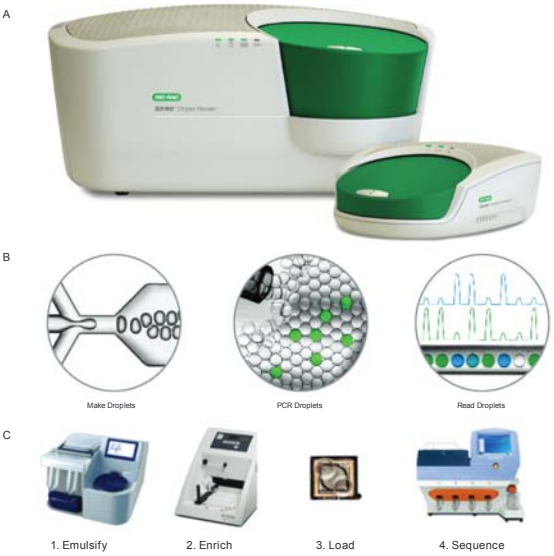

Fig. 1. A, QX100 Droplet Digital™ PCR system; B, ddPCR work□ow; C, Ion Torrent sequencing work□ow.

Materials and Methods

Genomic DNA (Coriell), Ion AmpliSeq library preparation manual (Life Technologies cat #MAN0006735), Ion AmpliSeq library kit 2.0 (Life Technologies cat #4475345), Ion AmpliSeq Cancer Hotspot panel v2 (Life Technologies cat #4475346), Ion Xpress barcode adapters 1–16 kit (Life Technologies cat #4471250), Ion library equalizer kit (Life Technologies cat #4482298), Library quanti□cation kit for Bio-Rad® iCycler™ thermal cycler (KAPA Bio cat #KK4847), C1000 Touch™ thermal cycler with 96 deep wells (Bio-Rad cat #185-1197), CFX96 Deep Well™ optical reaction module (Bio-Rad cat #184-4096), ddPCR™ NGS library quanti□cation kit for Ion Torrent (Bio-Rad cat #186-3041), QX100™ Droplet Digital™ PCR system (Bio-Rad cat #186-3001), Ion OneTouch, Ion 314 chip, and Ion PGM system (Life Technologies cat #4474779, #4462923, and #4462921)

Ion Torrent DNA sequencing libraries were constructed from eight individual Coriell DNAs using the Ion AmpliSeq Cancer Hotspot panel, library kit, and Ion Xpress barcode adapters. Libraries were prepared for ddPCR or qPCR quanti□cations or "equalized" using Life Technologies' kit. An attempt was made to normalize the eight libraries to an 8 pM total concentration using ddPCR- or qPCR-determined concentrations or "equalization," and then pooled. For ddPCR, input concentrations were titrated from 2–10 pM in search of an optimum sequencing load amount. The pooled libraries were templated on Ion Sphere particles (ISPs) and enriched using the Ion One Touch DL systems. After loading onto Ion 314 chips, the libraries were sequenced on the Ion PGM.

Library Preparation

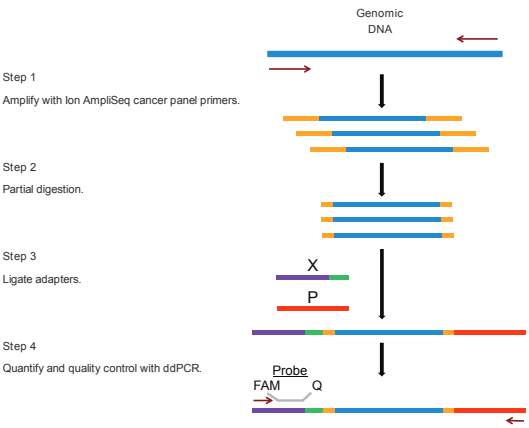

Fig. 2. Library preparation work□ow.

ddPCR Results

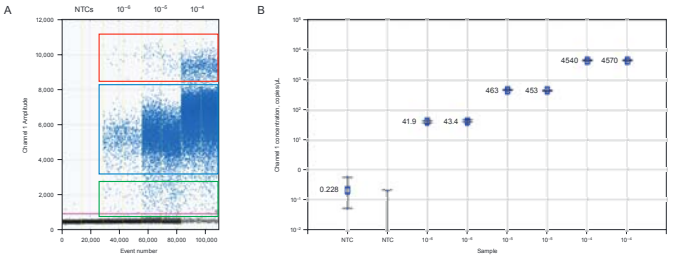

Fig. 3. A, 1-D FAM amplitude plot of a dilution series of an Ion Torrent library: NTC, 10^{-6}, 10^{-5}, and 10^{-4}-fold dilutions. The red box shows a population of Adapter-Dimers, blue box the Sequencing Library, and green box the Potential Chimeric Products; B, library concentrations measured by Droplet Digital PCR yield a linear curve.

qPCR Results

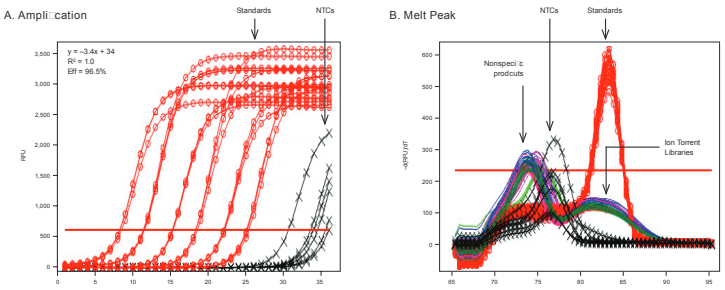

Fig. 4. A, the KAPA kit's ampli□cation plot standard curve is linear with high PCR ef□ciency. NTCs are apparently yielding nonspeci□c product; B, it may be the case that KAPA's qPCR assay is poorly designed. The substantial nonspeci□c products cause KAPA's SYBR®-based qPCR kit to overestimate the true concentrations.

Sequencing Results

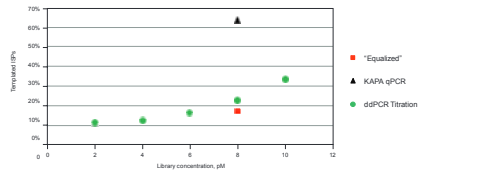

Fig. 5A. % Templated ISPs (unenriched). The concentration of libraries to load onto Ion Sphere particles (ISPs) was titrated per ddPCR concentrations; most resulting templated ISPs were within Ion Torrent's optimal range of 10–30%. Note that percent templated ISPs failed per [KAPA qPCR]s.

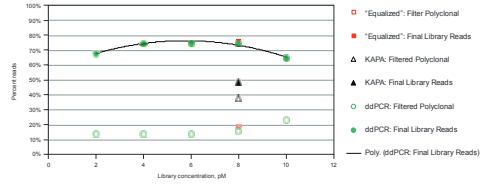

Fig. 5B. Sequencing metrics. Ion Torrent recommends 8 pM libraries be loaded when preparing template-positive Ion Sphere particles. We found that 4–8 pM, and optimally 6 pM, are the best concentrations to load per ddPCR. Note the severe failure per KAPA qPCR.

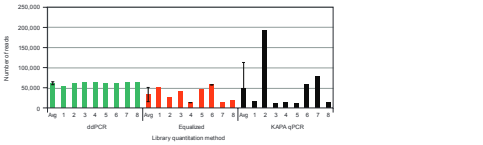

Fig. 5C. Multiplex library balancing. The number of reads per library for an 8-plex sequencing run were well-balanced when utilizing concentrations measured by Droplet Digital PCR. There was signi□cantly more variation between the indexed libraries with the other methods.

Conclusions

- ddPCR accurately quanti□es Ion Torrent sequencing libraries
- ddPCR allows for a quality control assessment of the libraries
- If the ddPCR QC shows signi□cant nonspeci□c products, a researcher might choose to not perform a sequencing run, saving time and money
- KAPA qPCR generated secondary products that yielded inaccurate library concentration determinations. This caused failed templated ISPs, low □nal reads, high polyclonality, and highly variable reads
- The Equalizer kit caused reads to be up to 4-fold variable for an 8-plex
- A load range of 4–8 pM, and optimally 6 pM, per ddPCR was found
- The 8-plex of barcoded libraries was exceptionally balanced per ddPCR
- Maximal □nal and minimal polyclonal reads were established when using ddPCR, which saves signi□cant time and money when sequencing

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