Precise Quantification of Next Generation Sequencing Ion Torrent™ Libraries with the QuantStudio™ 3D Digital PCR Platform

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ABSTRACT

The template preparation step in the Ion Torrent™ Next-Generation Sequencing (NGS) workflow is critical to obtaining optimal sequencing yields on the Ion Torrent PGM™ and Ion Torrent Proton™ platforms and is highly dependent on the library input amount. Current detection methods for quantifying NGS libraries, such as a Bioanalyzer or spectrophotometer readings, are not able to specifically measure only those fragments that have incorporated both library adapters, which commonly results in an overestimation of library concentration. Using TaqMan® chemistry on the QuantStudio™ 3D Digital PCR System (QS3D) for the Ion library quantification application alleviates this problem as the TaqMan® assay is designed to span both the P1 and A adapters. This design approach limits quantification to constructs containing both adapter sequences. Thus, the need to precisely quantify sequenceable libraries prior to the critical template preparation step in the Ion Torrent workflow is achieved by the QS3D. Compared to alternative methods, the QS3D approach is particularly attractive to precisely quantify libraries due to a simple workflow and eliminating the need for standard curves as required by traditional qPCR methods. The concentration obtained from the digital platform was correlated to the percent of template beads (pre-enriched) for the Ion Torrent™ libraries and showed a tight range between 10-14% as determined by flow cytometry. This abstract describes a simple workflow for quantifying NGS libraries using the QuantStudio™ 3D Digital PCR Platform.

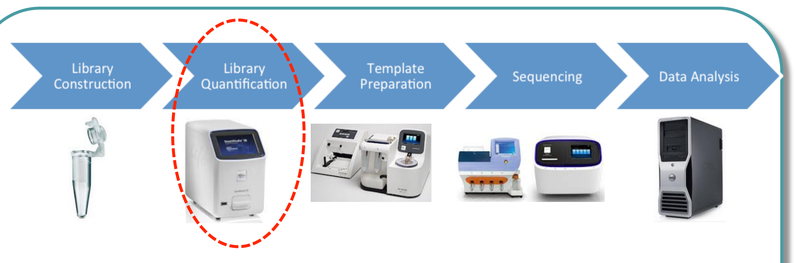

Figure 1. NGS workflow incorporating the QuantStudio™ 3D to accurately quantify NGS libraries prior to template preparation on the Ion OneTouch™ 2 System.

INTRODUCTION

Real-time PCR is an effective approach for NGS library quantification; however it requires a reference sample and the need to generate a standard curve. In contrast to RT-PCR, digital PCR[1] offers an alternative and highly precise approach without the need for a reference or standard curve. In combination with a pre-validated assay for quantification of Ion Torrent™ libraries, the chip based QuantStudio™ 3D Digital PCR System is perfectly suited for this application.

The QuantStudio™ 3D Digital PCR 20K Chip works by partitioning a standard PCR reaction mix into 20,000 individual PCR reactions. Upfront sample dilution ensures that a portion of these partitions contain the target molecule, while other partitions do not, leading to positive and negative reactions respectively. Following amplification on a dual flat-block thermal cycler, the fraction of negative reactions is used to generate an absolute count of the number of target molecules in the sample, all without reference to standards or controls. In a higher throughput setting, a laboratory may be sequencing different library types in parallel. If using real-time PCR for library quantification, the complexity of having to generate multiple standard curves can lead to increased cost, time and potential errors.
The key advantages of the QuantStudio™ 3D Digital PCR System for quantifying NGS libraries include the following:

- Highly precise and reproducible quantification without the need for a standard curve or reference sample
- Minimal sample handling reducing hands-on time
- Sealed chip-based consumable minimizing contamination from amplicons or other contaminating nucleic acids
- Highly affordable system and consumables leading to cost savings when considering the consequences of suboptimal sequencing runs

Figure 6.

MATERIALS AND METHODS

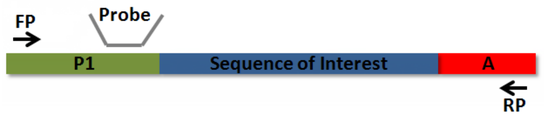

Figure 2. Schematic showing the TaqMan® assay design for Ion library quantification on the QuantStudio™ 3D Digital PCR system. The assay is composed of a forward primer, reverse primer and TaqMan® probe all complementary to the Ion library P1 and A adapter sequences.

Figure 3. The QuantStudio™ 3D PCR system workflow for NGS library quantification.

Component	Volume (uL)
2X Digital Master Mix	7.5
20X Assay	0.75
1X TE (or NFW)	4.75
Diluted Library	2
Total	15

Table 1. Digital PCR reaction set up.

Chip	Sample	Target (VIC)	Target (FAM)	Dilution
B14KF7_131112_120318.eds	L7734		FAM	1 to 1.5E6
B14KRJ_131113_105452.eds	CCP1		FAM	1 to 1.5E6
B14KUL_131115_160536.eds	L6938 1 in 1250000		FAM	1 to 9.375E6

Figure 6. QuantSudio™ 3D AnalysisSuite™ software interface. The red oval indicates the column where the dilution factor in added for each sample in the "define chips" tab.

RESULTS

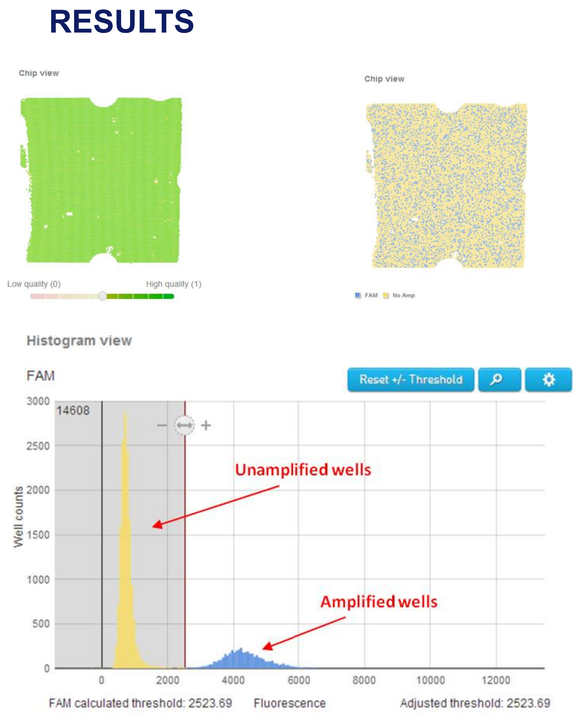

Figure 4: Alternative views in the Absolute Quantification application module of the QuantStudio™ 3D AnalysisSuite™ software. **(A)** Image of the chip view depicting color by quality, which indicates the quality of loading on a chip. This particular chip is classified as high quality loading due to the uniformity of the filled wells across the chip. **(B)** Example of a chip view depicting color by calls, which shows the distribution of both the FAM (amplified) and the non-amplified wells. Note that a random distribution of FAM signal across the chip is ideal. Any other signal patterns could indicate possible loading issues or leaking of the immersion fluid from the chip. **(C)** The histogram view has two populations, the larger yellow population corresponds to the non-amplified wells with a lower fluorescence and the smaller blue population corresponds to the amplified wells with a significantly higher fluorescence. The separation between non-amplified and amplified populations is indicative of good discrimination.

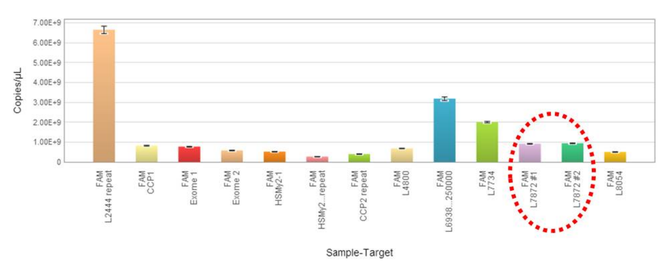

Figure 7. Calculated copies per µL of various library types by QuantStudio™ AnalysisSuite™ software. The software takes into account the dilution factor used when defining chips. Error bars indicate 95% confidence level for each sample. The small RNA samples above represent three replicate chips.

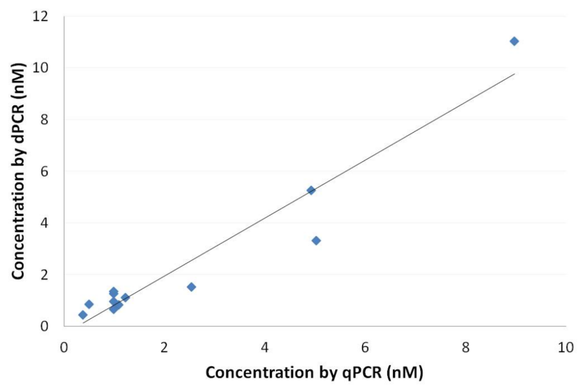

Figure 8. To measure the efficacy of quantification by dPCR to that of qPCR, we compared the concentrations of several different libraries as measured by these two methods. Correlation between the concentration (nM) as determined by qPCR and dPCR. An R^2 value of 0.9245 indicates high correlation.

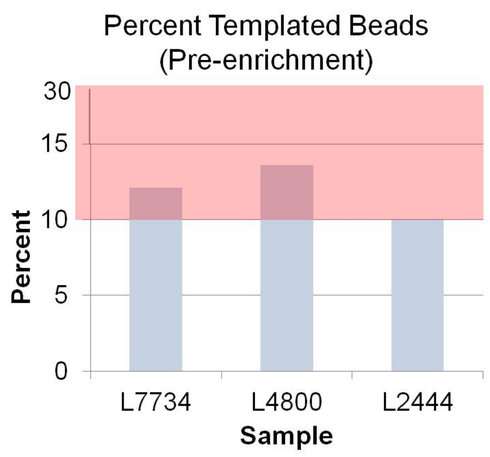

Figure 9. Correlation of QuantStudio™ 3D Digital PCR data with that of the Ion OneTouch™ 2 data. As a result of incorporating the pre-quantification step using the QuantStudio™ Digital PCR System, all three libraries showed a tight percent templated bead range between 10% and 14% These quality control data were generated using flow cytometry for four Ion Torrent™ libraries prior to enrichment.

CONCLUSIONS

Precise library quantification is a critical step prior to the template bead preparation. Inclusion of too much or too little library into the template preparation step impacts the clonal expansion step leading to sub-optimal sequencing yields. This application note describes a simple digital PCR workflow combined with a carefully designed TaqMan® Assay that can be used to precisely and accurately quantify high quality libraries of varying size and type. The QuantStudio™ 3D Digital PCR System is ideally suited for the quantification of NGS Ion Torrent™ libraries for the following reasons:

- Precise quantification without the need for a reference
- Precise quantification that results in a greater success rate of the template bead preparation step and overall sequencing results
- Easy workflow with minimum hands-on time
- Affordable system and consumables

By replacing the Ion specific assay design with similarly designed assay specific to the adapter sequences in use, the approach described in this study can also be used to quantify libraries from other platforms.