Precise quantification of Ion Torrent libraries on the QuantStudio 3D Digital PCR System

Introduction

The template preparation step in the next-generation sequencing (NGS) workflow is critical to obtaining optimal sequencing yields on the Ion PGM™ and Ion Proton™ platforms and is highly dependent on the library input amount. Library concentrations that are too high or too low lead to reduced total reads and reduce the overall throughput of the system. As a result, an accurate and precise method for quantifying high-quality libraries prior to template preparation is critical to maximizing throughput from a sequencing run.

Digital PCR (dPCR) offers a highly precise quantification approach without the need for a reference or standard curve. In combination with a prevalidated assay for quantification of Ion Torrent™ libraries, the chip-based Applied Biosystems™ QuantStudio™ 3D Digital PCR System is perfectly suited for this application.

The Applied Biosystems™ QuantStudio™ 3D Digital PCR Chip works by distributing a standard PCR reaction mix into 20,000 individual PCR reactions. Up-front sample dilution ensures that a portion of these partitions contain the target molecule, while other partitions do not, leading to positive and negative reactions. Following amplification on a dual flat block thermal cycler, the fraction of negative reactions is used to quantify the number of target molecules in the sample, all without reference to standards or controls.

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, which may lead to cost savings when the consequences of suboptimal sequencing runs are considered

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

This application note describes a simple workflow for quantifying Ion Torrent™ NGS libraries using the QuantStudio 3D Digital PCR System. This workflow enables consistent sequencing data on the Ion PGM™ or Ion Proton™ System, resulting in savings in both time and cost when there is no need to repeat the template bead preparation step.

Library quantification in the Ion Torrent NGS workflow

The NGS workflow is illustrated in Figure 1. In summary, once a high-quality library has been constructed, the QuantStudio 3D Digital PCR System is used to quantify the number of sequenceable molecules. Based upon this result, the library is diluted to a level consistent with template bead preparation recommendations using the Ion OneTouch 2 System. This is followed by sequencing on the Ion PGM or Ion Proton System. Finally, the sequenced reads are analyzed on the Torrent Suite™ Server.

QuantStudio 3D Digital PCR System for Ion Torrent library quantification—assay design

Precise quantification of NGS libraries is critical for maximizing template bead preparation efficiency. An underestimation or overestimation of the library concentration leads to decreased data yields from a sequencing run. Current detection methods for quantifying NGS libraries, such as the Agilent Bioanalyzer™ instrument 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 concentration.

Using Applied Biosystems™ TaqMan™ Assay chemistry for library quantification alleviates this problem as the assay is designed to span both the P1 and A adapters (Figure 2). This approach limits quantification to constructs containing both adapter sequences. TaqMan Assays may be individually designed to quantify other NGS

Figure 2. TaqMan Assay design for Ion Torrent library quantification on the QuantStudio 3D Digital PCR System. The assay is composed of a forward primer (FP), reverse primer (RP), and TaqMan™ probe all complementary to the library P1 and A adapter sequences.

Figure 3. The QuantStudio 3D Digital PCR System workflow for NGS library quantification. The QuantStudio 3D Digital PCR System workflow for NGS library quantification begins with diluting the library such that it falls within the digital range. Then, the reaction mix is formulated by mixing the diluted sample with the Applied Biosystems™ QuantStudio™ 3D Digital PCR Master Mix and the TaqMan Assay. The reaction mix is then loaded onto the chip using the Applied Biosystems™ QuantStudio™ 3D Chip Loader. The sealed chip is then amplified on a dual flat block thermal cycler and results read directly on the QuantStudio™ 3D Digital PCR System. Applied Biosystems™ QuantStudio™ 3D AnalysisSuite™ Cloud Software is used to visualize the data and display the target concentration.

libraries, provided the probe and primer sequences are all complementary to the appropriate adapter sequences on the library. Once the assay has been designed, it is used directly in the QuantStudio™ 3D digital PCR workflow.

QuantStudio 3D Digital PCR System workflow

The QuantStudio 3D Digital PCR System system workflow for quantifying NGS library concentration is a simple process (Figure 3).

The first step in library quantification using dPCR is to dilute the library such that it falls within the digital range. This is necessary to ensure that, on average across the chip, there are between 0.1 and 1.7 copies per reaction (Figure 4). If the dilution is outside of this range, the level of precision around the measurement will be diminished. As a general guideline, we recommend starting with a 1 to 200,000 dilution of the library in 1X TE in order to be within this range. Based on this initial reading, dilutions may be adjusted above or below the 1 to 200,000 dilution, should the resulting value not fall within the digital range for a particular library.

The reaction mix is formulated by mixing diluted sample with QuantStudio 3D Digital PCR Master Mix and the TaqMan Assay (Table 1). The reaction mix is loaded on a Applied Biosystems™ QuantStudio 3D Digital PCR System 20K Chip and the chip is assembled. The sealed chip is amplified on a dual flat block thermal cycler and the results

Sample	Target	Dilution	Chip	Copies/Rxn
HSMy2-2 re...	FAM	2 to 1.5E6	B14XGK_131115_160639.eds	0.303
L7872 #2	FAM	2 to 2.354E6	B14TAA_131106_154632.eds	0.685
L7872 #1	FAM	2 to 2.354E6	B1FEEC_131106_154529.eds	0.67
CCP1	FAM	1 to 1.5E6	B14KRJ_131113_105452.eds	0.485
CCP2 repeat	FAM	2 to 3E6	B1FC95_131115_160738.eds	0.225
Exome 2	FAM	2 to 3E6	B1FE31_131112_120551.eds	0.329
L6054	FAM	2 to 3E6	B1F7A2_131112_120013.eds	0.285

Figure 4. QuantStudio 3D AnalysisSuite Software depicting the copies per reaction (circled) for each sample.

Table 1. Volumes of reagents necessary to set up your digital PCR experiment.

Component	Volume (µL)
2X master mix	7.50
20X assay	0.75
1X TE	4.75
Diluted sample	2.00
Total	**15.00**

are read on a QuantStudio 3D Digital PCR Instrument. Lastly, the data is visualized using the Applied Biosystems™ QuantStudio™ 3D AnalysisSuite™ Cloud Software, which displays the target concentration.

For a detailed description of running a digital PCR experiment, please refer to the QuantStudio 3D Digital PCR System User Guide (Pub. No. MAN0007720). See "Ordering information" for the list of products used for NGS library quantification with the QuantStudio 3D Digital PCR System.

Interpretation of results

Four different library types were tested in this study: Ion DNA Fragment, Ion AmpliSeq™ Exome, Ion AmpliSeq™ Comprehensive Cancer Panel, and Ion AmpliSeq™ Cancer HotSpot Panel Version 2. Library sizes ranged from ~100 to ~700 bp as determined by an Agilent 2100 Bioanalyzer™ instrument. The same library quantification workflow, as documented in this application note, was used for all libraries tested.

Data QC

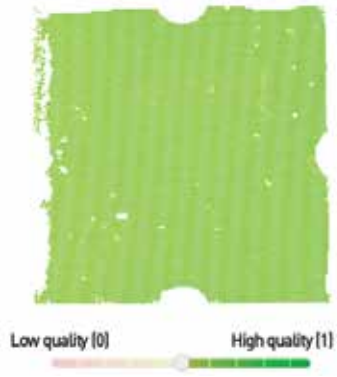
Integral to the QuantStudio 3D Digital PCR System workflow is an assessment of data quality coming from the instrument, where lower-quality data is automatically flagged on the Applied Biosystems™ QuantStudio™ 3D Digital PCR Instrument itself. Data can be further reviewed in the QuantStudio 3D AnalysisSuite Cloud Software to confirm final quality in two ways:

- The "Chip view" functionality, found on the "Review Quality" tab, is used to inspect the chip image for artifacts. The chip view can be toggled between "Color by Quality" (Figure 5A) and "Color by Call" (Figure 5B). The "Color by Quality" view confirms loading uniformity and image quality. Wells with lower quality scores are automatically filtered out in the chip image heat map. Note that, although not recommended for most chips, the quality score threshold applied can be manually adjusted to increase or decrease the number of wells filtered from the analysis. The "Color by Call" chip view confirms a random distribution of amplified and nonamplified wells across the chip.
- The "Histogram view" (Figure 5C) shows the discrimination between the nonamplified and amplified populations and confirms that the threshold separating the two populations has been correctly set by the software. If not, this can be manually adjusted by the user.

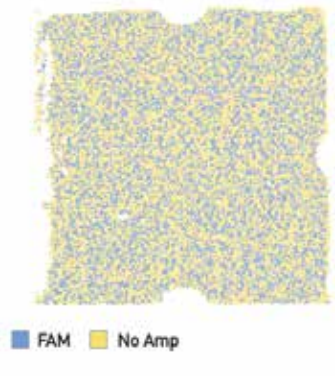
The images shown in Figure 5 are for a successful experiment in which all QC metrics meet the desired outcome.

Quantification

Chip view (A)



Chip view (B)



Chip view (C)

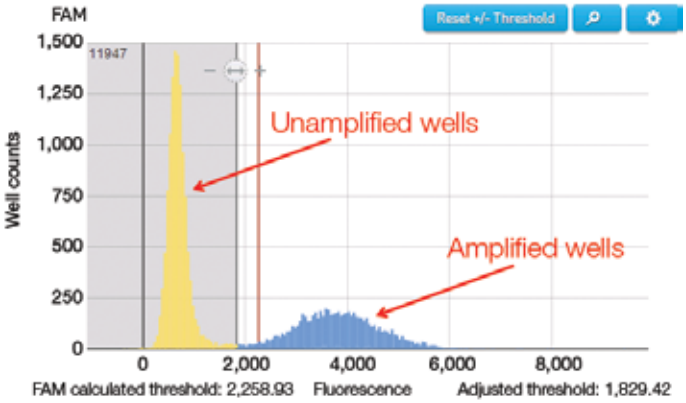


Figure 5. Alternative views in the Absolute Quantification application module of the QuantStudio 3D AnalysisSuite Software. (A) Image of the chip view depicting color by quality, which indicates the quality of loading on a chip. This particular chip is classified as high-quality loading due to the uniformity 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™ signal (amplified) and the unamplified 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 unamplified wells with a lower fluorescence, and the smaller blue population corresponds to the amplified wells with a significantly higher fluorescence. The separation between unamplified and amplified populations is indicative of good discrimination.

Prior to determining the final library concentration, data must be adjusted to take into consideration the degree of sample dilution made prior to chip loading. This process is simplified in QuantStudio 3D AnalysisSuite Software by entering the dilution factor for each sample in the "Define Chips" tab. To correctly calculate the dilution factor for each sample, two components must be taken into account: (1) sample dilution prior to preparing the reaction mix, and (2) dilution that occurs when the reaction mix is formulated (Figure 6).

For example, if your sample was originally diluted 1 to 200,000 and 2 µL of the diluted sample was added to a total of 15 µL, then a dilution factor of 1 to 1,500,000 should be entered into the software (Figures 6 and 7).

QuantStudio 3D AnalysisSuite Software will calculate a concentration in copies/µL and graphically represent the data (Figure 8). The graphical representation of the concentration is located in the "Results" section of the "See Results" tab. The software provides a 95% confidence range for each sample concentration as calculated by Poisson statistics. As a test of the reproducibility of the system, two replicates were performed for sample L7872. Comparison of the results confirms high reproducibility, as highlighted by the red oval in Figure 8.

The final step in the quantification process is to convert the copies/µL provided by the QuantStudio 3D Digital PCR System to a molar concentration as required for the template preparation step. Figure 9 shows how to convert copies/µL to nM so that you may proceed to the template preparation step of the NGS workflow.

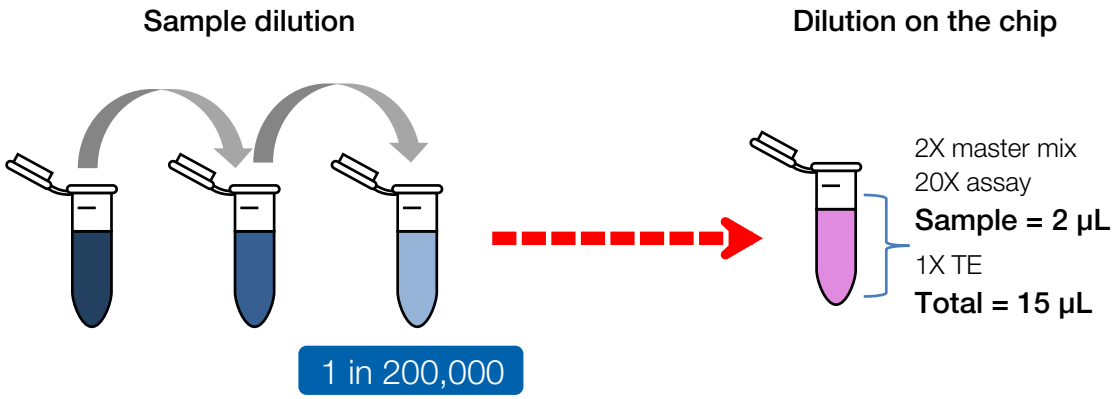


Figure 6. Sample calculation of a dilution factor.

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 12500(		FAM	1 to 9.375E6

Figure 7. QuantStudio 3D AnalysisSuite Software interface. The red oval indicates the column where the dilution factor is added for each sample in the "Define Chips" tab.

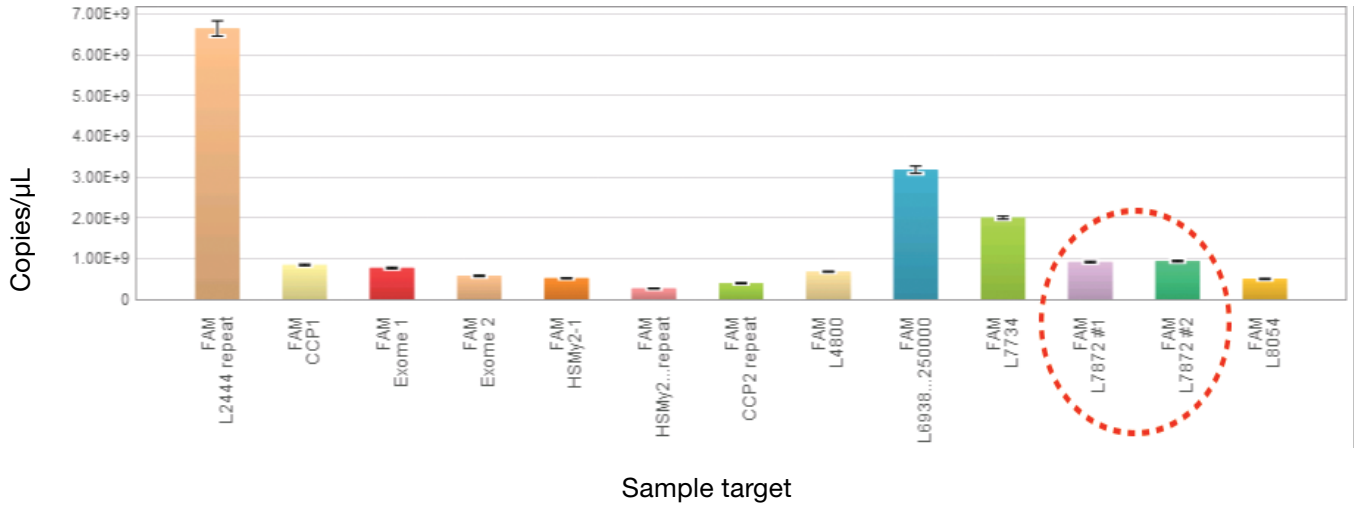


Figure 8. Calculated copies/µL of various library types by QuantStudio 3D AnalysisSuite Software. The software takes into account the dilution factor used when defining chips. Error bars indicate 95% confidence level for each sample. The red oval indicates replicate chips for sample L7872.

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. The correlation between the two methods is excellent, with an R^2 value of 0.9245 (Figure 10). These data indicate that both library size and type do not have an impact on library quantification with the QuantStudio 3D Digital PCR System.

Correlation between dPCR quantification and template bead preparation

Based on the calculated concentration (nM), an appropriate dilution of the library is necessary for template bead preparation using the Ion OneTouch 2 System. For a detailed description of using the Ion OneTouch 2 System, please refer to the Ion PGM™ Template OT2 200 Kit user guide (Pub. No. MAN0007220), the Ion PGM™ Template OT2 400 Kit user guide (Pub. No. MAN0007218), or the Ion PI™ Template OT2 200 Kit v3 user guide (Pub. No. MAN0009133). After template bead preparation, a quality control step is necessary to assess the quality of the templated beads generated in a sample prior to enrichment using either a flow cytometer or the Invitrogen™ Qubit™ 2.0 Fluorometer. The optimal library quantification corresponds to the library dilution that results in 10–30% of beads containing amplified template. Samples that fall within the recommended range generally produce higher quality data. Samples that contain templated beads below 10% have an insufficient number of beads to achieve optimal loading density on the Ion Torrent™ chip, which leads to decreased sequencing yields. Samples that contain templated beads above 30% will yield multi-templated beads or mixed reads, which helps to reduce the throughput due to an increase in filtered reads.

Step 1: Convert library size into ng/copy

Genome/template mass in Mb | 1 bp mass | Convert Mb to bases | Convert g to pg

(0.000696 Mb/copy) x (1.096 x 10^{-21} g/bp) x (1 x 10^{6} bp/Mb) x (1 x 10^{12} pg/g) = 7.63 x 10^{-7} pg/copy or 7.63 x 10^{-10} ng/copy

Step 2: Convert copies/µL from digital PCR to ng/µL

Copies/µL from digital PCR | ng/copy

(922,000,000 copies/µL) x (7.63 x 10^{-10} ng/copy) = 0.703 ng/µL

Step 3: Convert ng/µL to nM

Calculated ng/µL | Convert µL to L | Average MW of 1 nucleotide | Fragment size in bp

(0.703 ng/µL) x (10^{6} µL/L) x (1 nmol/660 ng) x (1/696) = 1.53 nM

Figure 9. Calculations for converting the values in copies/µL generated by the QuantStudion 3D AnalysisSuite Software to concentration in nM.

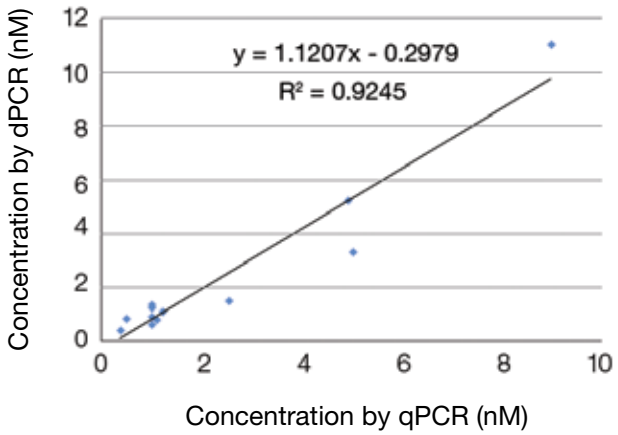


Figure 10. Correlation between the concentration (nM) as determined by qPCR and dPCR. An R^2 value of 0.9245 indicates high correlation.

The digital PCR concentrations obtained were compared to the Ion OneTouch™ 2 template bead preparation results for three of the libraries that were used in this study (Table 2). As a result of incorporating the prequantification step using the QuantStudio 3D Digital PCR System, all three libraries showed a tight templated bead range between 10% and 14% as determined by flow cytometry. This confirms that the QuantStudio 3D Digital PCR System measurement is sufficient for adjusting library concentration prior to the critical template bead preparation step of the Ion Torrent™ NGS workflow. The precise quantification that the QuantStudio 3D Digital PCR System provides can help reduce the need to repeat Ion OneTouch™ 2 reactions, which can lead to a significant cost and time savings.

Conclusions

Precise library quantification is a critical step prior to template bead preparation. Inclusion of too much or too little library into the template preparation step impacts the clonal expansion step, leading to suboptimal 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 Ion Torrent NGS libraries for the following reasons:

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

By replacing the Ion Torrent™–specific assay design described here with a 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.

Table 2. Correlation of QuantStudio 3D Digital PCR data with Ion OneTouch 2 data. These quality control data were generated using flow cytometry for three Ion Torrent libraries prior to enrichment.

Sample name	Library type (size)	Digital PCR concentration	Templated beads preenrichment
L7734	Fragment (276 bp)	3.32 nM	12.1%
L4800	Fragment (489 bp)	1.12 nM	13.6%
L2444	Fragment (322 bp)	11.03 nM	10.0%

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Ordering information

Product	Cat. No.
TaqMan Quantification Assay for Ion Torrent NGS Libraries, 20X, Assay ID: Ac04347676_a1	4331182
QuantStudio 3D Digital PCR System Package v2—includes:*	A29154**
QuantStudio 3D Digital PCR Instrument with Power Cord	4489084
ProFlex 2 x Flat PCR System	4484078
QuantStudio 3D Digital PCR Chip Adapter Kit for Flat Block Thermal Cycler	4485513
QuantStudio 3D Digital PCR Master Mix v2	A26358
QuantStudio 3D Digital PCR Chip Loader	4482592
QuantStudio 3D Digital PCR 20K Chip v2	A26316

* Catalog numbers listed in bundle are for individual components.
**Cat. No. A29154 is for all regions except Europe, the Middle East, and Africa (EMEA). Please use Cat. No. A29737 or A29738 for customers residing in EMEA. Package components are slightly different. Please check with your regional sales representative for details.

Reference

1. Pohl G, Shih IM (2004) Principle and applications of digital PCR. *Expert Rev Mol Diagn* 4:41–47.

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