Fully Automated Library Quantification for Illumina® Sequencing on the NGS STAR™

Introduction

Hamilton Robotics, an industry leader in liquid handling and laboratory automation equipment, has partnered with Kapa Biosystems to automate multiple aspects of the next-generation sequencing (NGS) workflow. This includes quantitative PCR (qPCR) based library quantification using the NGS STAR™ liquid handling instrument.

NGS STAR with optional 96 multi-probe head

Accurate quantification of libraries for NGS is an essential step in any successful sequencing workflow. qPCR is the gold standard for NGS library quantification, as it is the only method that accurately measures the number of molecules to serve as templates during cluster amplification and sequencing reactions. The sensitivity and broad dynamic range of qPCR enables accurate quantification of libraries from a wide range of applications including PCR-free WGS, Target Capture, ChIP-Seq, and RNA-Seq. However, performing an accurate and precise qPCR assay in a high-throughput, manual setting is both challenging and time consuming. In this application note, we describe a newly developed automated method for the quantification of Illumina NGS libraries using the KAPA Library Quantification Kit on the NGS STAR.

Reliable qPCR-based library quantification depends on three factors: (i) the efficiency and reproducibility of the standard curve used for absolute quantification, (ii) the ability of the DNA polymerase to amplify all adapter ligated molecules with similar efficiency, and (iii) accurate and precise liquid handling. All three factors are accounted for in the partnership between Kapa Biosystems and Hamilton Robotics.

i KAPA Library Quantification Kits contain all reagents required for quantification including a 2X KAPA SYBR® FAST qPCR Master Mix, 10X Primer Premix which include universal primers based on the Illumina P5 and P7 sequences, and a pre-diluted set of DNA Standards that are used to generate a highly reproducible standard curve. KAPA also supplies a Library Quantification Dilution Control (Kit Code: KK4906). This positive control is a 200 pM solution of the same linear, 452 bp dsDNA fragment comprising the DNA Standards and is also referred to as DNA Standard 0.

ii The kits contain the novel KAPA SYBR® FAST DNA Polymerase, which has been engineered through a process of directed evolution for high-performance SYBR® Green I-based qPCR. This enzyme has the ability to amplify diverse DNA fragments with similar efficiency, enabling the use of a universal standard for the reliable quantification of all Illumina libraries with an average fragment length of up to 1 kb, irrespective of library type or GC content.

iii Hamilton Microlab® STAR liquid handling instruments can accurately pipette volumes ranging from 0.5 µL to 1000 µL, with as little as 0.75% variation. The use of the Hamilton NGS STAR to dilute libraries, controls and set up qPCR reactions, especially in a high throughput fashion, provides the greatest consistency both within individual assays as well as amongst assays performed over extended periods of time with various users.

Experimental Design

The terms “accuracy” and “precision” are often used interchangeably, but it is important to note the differences between the two. Accuracy is the measurement of how true or correct a value is compared to a known or positive control. Precision is the measurement of how reproducible or variable a result can be repeated. It is possible for a process to be highly accurate, but imprecise. It is also possible for a process to be inaccurate, yet highly precise. For reliable qPCR reactions, it is imperative that the process be both highly accurate and highly precise. To determine the accuracy and precision of the KAPA Library Quantification application, we have designed an experiment to asses both the sample dilutions and qPCR reaction setups.

Two standard curves were set up in triplicate for each of the 6 DNA Standards provided with the KAPA Library Quantification Kit. The first of these standard curves was generated by using the NGS STAR, and the second was generated manually, assessing the accuracy of the automated qPCR setup process.

Four samples, including Standard 0, were processed in quadruplicate. Each of these initial four replicates were then set up for qPCR in triplicate for a total of 12 reactions per sample to assess the precision of the automated sample dilution process.

Finally, one sample (Standard 0) was diluted manually in duplicate. Each of these two initial replicates were then set up for qPCR in triplicate for a total of 6 reactions. The manual and automated dilutions of Standard 0 were compared to assess the accuracy of the automated sample dilution process. All samples were diluted to 1:10,000 and each reaction volume for qPCR totaled 20 µL.

Results

The ABI Step One Plus was used for initial data analysis with the default analysis settings for SYBR® Green reactions, including the use of ROX as a passive optical control. The C_t values for each reaction were calculated, and no wells were removed from statistical calculations as outliers.

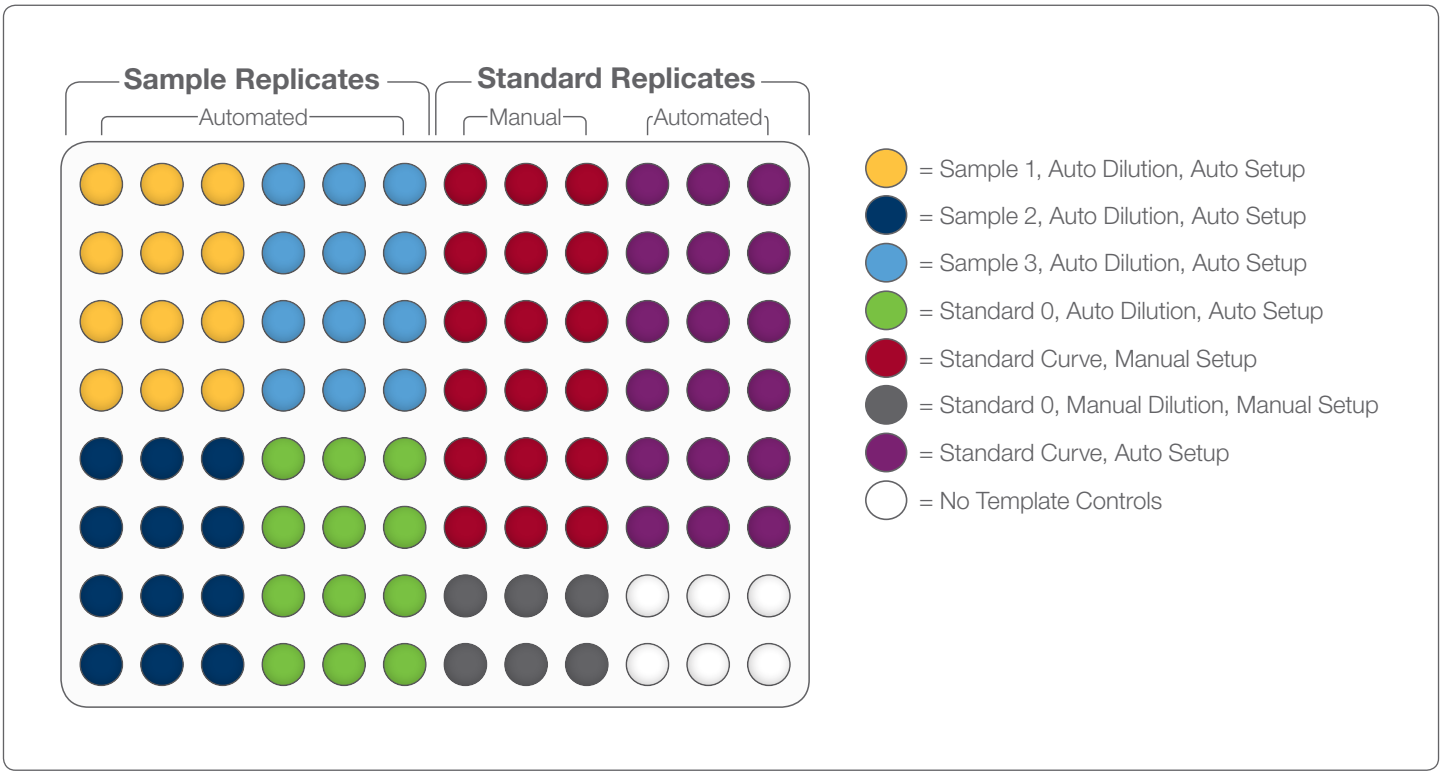
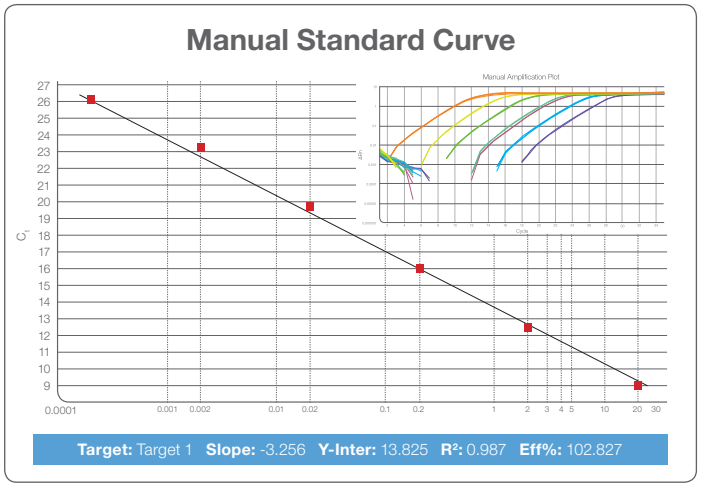


Figure 1: qPCR Plate Layout

Manual v. Automated Standard Curves: Assessing the Precision and Accuracy of Automated qPCR setup

The standard curve resulting from automated qPCR setup (R^2 value of 0.999) shows less variability and higher linear correlation compared to the standard curve prepared manually (R^2 value of 0.987), proving the automated qPCR setup to be both accurate and precise.



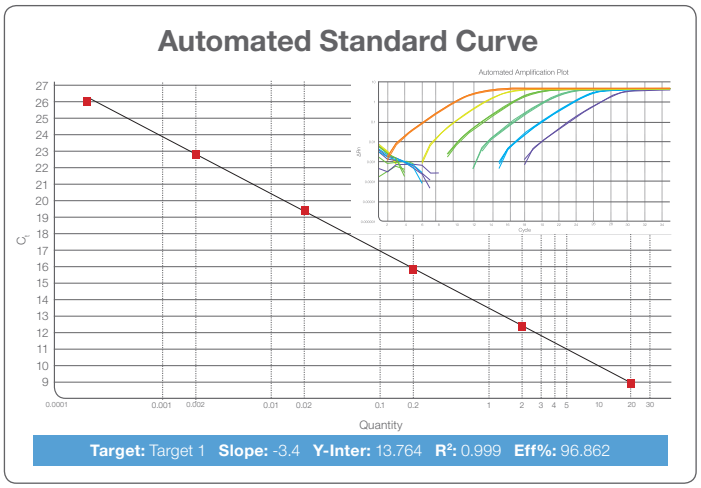


Figure 2: Comparison of manual (top) vs automated (bottom) qPCR reactions for standard curve

Comparison of Sample Replicates: Assessing the Precision of Automated Sample Dilutions

Four unique sample libraries were each distributed into 4 separate aliquots. Each of the 4 sample aliquots were then diluted to 1:10,000 and setup in triplicate for qPCR, resulting in 12 replicates for each of the 4 unique sample libraries. Based on both the standard curve and calculated C_t values, concentrations were determined for each of the 12 replicates per initial sample to determine the process variability in respect to both sample dilution and qPCR. Within all 4 sample aliquots, the standard deviation of the 12 replicate C_t values varied by less than 0.07, which proves the automated sample dilution process to be precise.

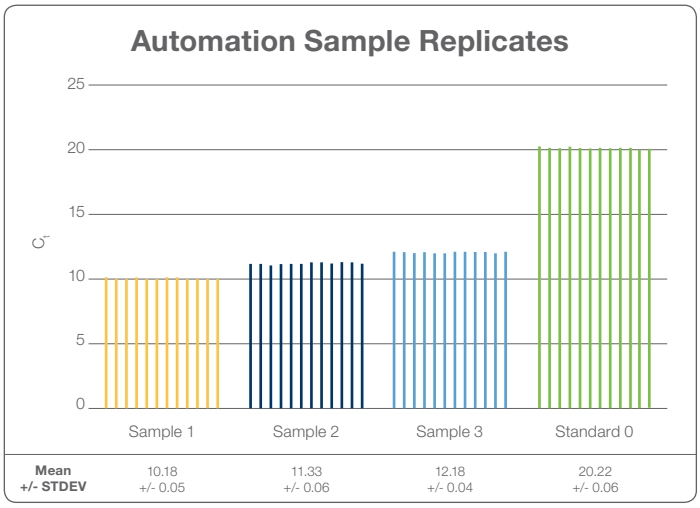


Figure 3: Comparison of replicate dilutions and qPCR reactions for 4 sample libraries

Manual v. Automated Standard 0 Quantification: Assessing the Accuracy of Automated Sample Dilutions

Two aliquots of Standard 0 were manually diluted to 1:10,000 with qPCR performed in triplicate, resulting in 6 total replicates. The average C_t values of the manual Standard 0 reactions were compared to the Standard 0 reactions that were diluted and setup by the Hamilton STAR. The average C_t value of the automated Standard 0 reactions (20.22) was nearly identical (>99%) to the average C_t value of the manual Standard 0 reactions (20.24), proving the automated sample dilution process to be accurate.

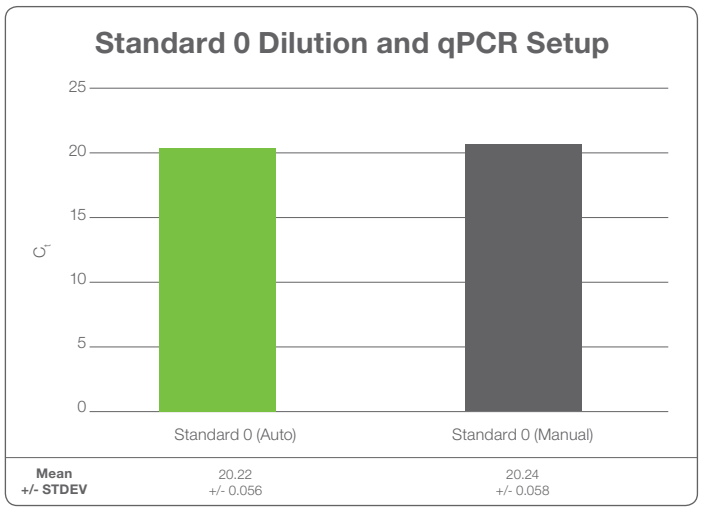


Figure 4: Comparison of replicate dilutions and qPCR reactions for Standard 0, Automated v. Manual

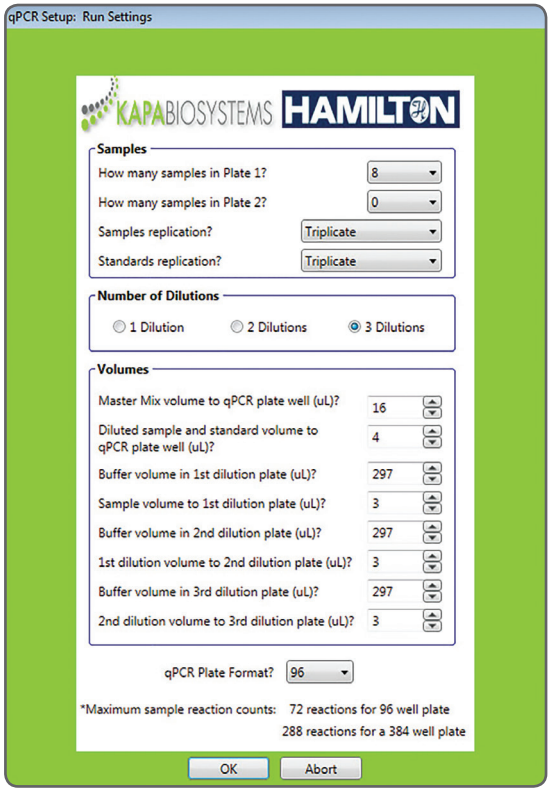
Conclusion

NGS library preparation has become standard practice for most genomics laboratories, however meeting the constant demands of increased sample throughput can become a challenge. In high-throughput settings, inaccurate quantification, normalization, and pooling all upstream to sequencing can become driving factors for process variation.

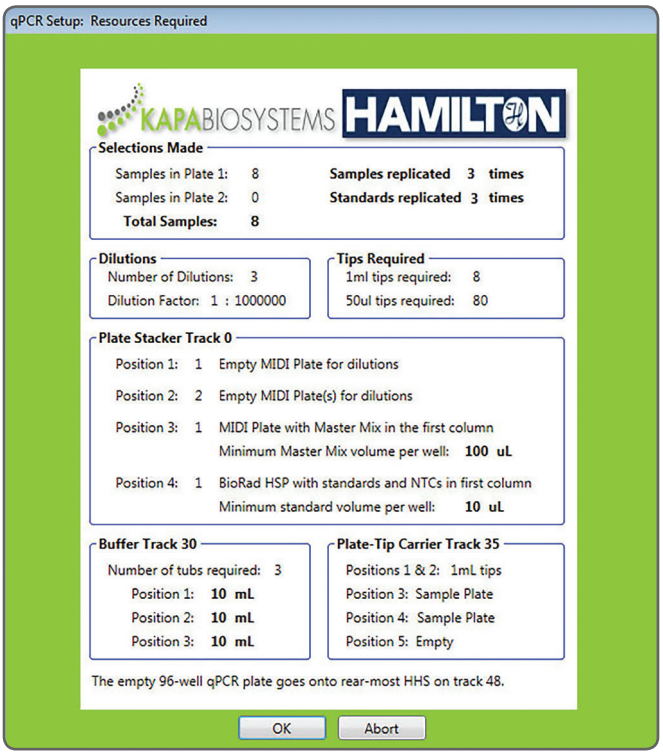
Accurate quantification of libraries for NGS by qPCR is a critical step in assessing library prep performance by quantifying only the material that can undergo flow-cell cluster formation. With accurate library concentrations, laboratories are able to optimize flow-cell loading procedures and maximize their sequencing capacities. This experimental data shows that the KAPA Library Quantification application developed on the NGS STAR produces a robust, flexible, accurate, precise, and fully automated solution for the quantification of libraries for NGS workflows.

Application Features

- Process samples from up to 2 source plates
- Process up to 96 samples per run
- Configurable dilution factors
- Variable number of qPCR replicates for samples and standards
- Variable volumes for qPCR master mix, samples and standards
- qPCR setup in either 96-well or 384-well plate format
- Barcode scanner confirms correct tip loading positions, sample plate barcodes, and automatically tracks all pipette transfer/intervention steps
- Heater shakers allow uniform and efficient mixing without cross-contamination
- Walk-away error handling allows for easy and seamless error recovery

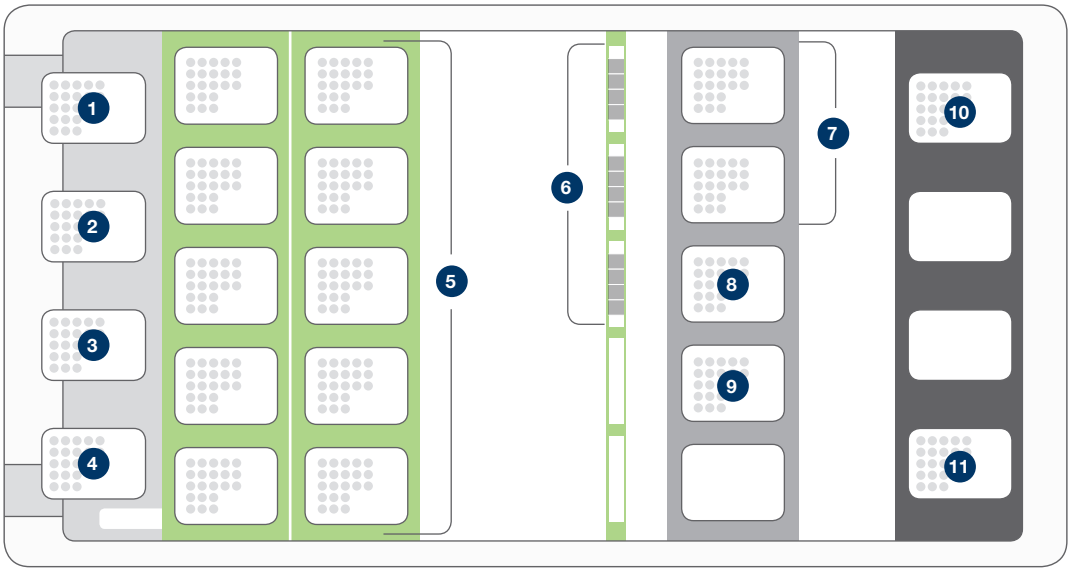


User interface for run parameters



User interface for consumable requirements

Deck Layout



NGS STAR Deck Layout

1 = Dilution Plate 1 (MIDI)

2 = Dilution Plate 2 and 3 (MIDI)

3 = Master Mix Plate (MIDI)

4 = Standard Plate (HSP)

5 = 50 µLTips

6 = Dilution Buffer in 50mL tubs

7 = 1 mL Tips

8 = Sample Plate 1

9 = Sample Plate 2

10 = 96-well qPCR Plate

11 = 384-well qPCR Plate

Table 1: Kapa Library Quantification Consumable Recommendations

Consumables	Vendor	P/N	Qty	Utility
Illumina Library Quantification Kit with Standards	Kapa Biosystems	Instrument Compatible*	1	qPCR Master Mix, primers, and standards
Dilution Buffer (10 mM TrisHCl pH 8)	General	N/A	Variable	Dilution of sample prior to qPCR setup
Standard 0	Kapa Biosystems	KK4906	1	Positive control for reaction setup
96 Deep well plates (MIDI)	Fisher Scientific	AB0859	2-4	Sample Dilution and Master Mix preparation
96-well PCR plates (HSP)	BioRad	HSP9601	2-3	Sample and standard input
96- or 384-well qPCR plate	Various	Instrument Compatible**	1	Setup of qPCR reaction

** Library Quantification kits with or without reference dyes are available for different qPCR instruments.*
*** User must select qPCR plate that is compatible with qPCR instrument.*

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