KAPABIOSYSTEMS

KAPA Library Quantification Kits coupled with the Illumina Eco™ Real-Time PCR System provide a rapid, sensitive, and reliable method for quantifying PCR-amplifiable molecules and reducing variability in cluster density.

Introduction

Accurate quantification of the number of amplifiable molecules in a library is critical for optimal sequencing using Illumina platforms. Current standard protocols employ laborious, costly, and unreliable methods for quantifying library DNA molecules prior to clonal amplification of sequencing templates. Accurate quantification of *bona fide*, PCR-competent sequencing templates is crucial for ensuring consistent, optimal cluster densities. Accurate library quantification is equally important when pooling indexed libraries for multiplexed sequencing to ensure equal representation of each library.

Standard methods for quantifying NGS libraries have a number of important disadvantages. Electrophoresis and spectrophotometry measure total nucleic acid concentrations, whereas optimal cluster density depends on the appropriate concentration of PCR-amplifiable DNA molecules. These methods also have low sensitivity, consuming nanograms of precious samples, and are not suitable for high-throughput workflows.

Quantitative PCR (qPCR) is widely regarded as the gold standard for accurate quantification of DNA libraries as it is the only technique capable of specifically measuring the number of amplifiable molecules. Furthermore, qPCR can accurately quantify extremely dilute libraries, such as unamplified array eluates from sequence capture experiments.

KAPA Library Quantification Kits comprise DNA Standards (six 10-fold dilutions) and 10X Primer Premix, paired with KAPA SYBR® FAST qPCR Kits. The 452 bp Illumina DNA Standard consists of a linear DNA fragment flanked by qPCR primer binding sites. Quantification is achieved by inference from a standard curve generated using the six DNA Standards. KAPA Library Quantification Kits are rigorously tested to ensure minimal lot-to-lot variation. In addition, KAPA SYBR® FAST qPCR Kits are designed for high performance, high-throughput, real-time PCR. The kit contains a novel DNA polymerase engineered via molecular evolution, resulting in a unique enzyme optimized for qPCR using SYBR® Green I dye chemistry. KAPA SYBR® FAST qPCR Kits are ideally suited for library quantification applications, because they enable high efficiency amplification of both AT- and GC-rich targets, and of fragments up to 1 kb in length.

Accurate qPCR-based library quantification depends on three factors: (i) the accuracy and reproducibility of the standards used, (ii) the ability of the DNA polymerase used in the qPCR to amplify all adaptor-flanked molecules with equal efficiency, and (iii) accurate and reproducible liquid handling. In addition, the specificity and efficiency of qPCR require precise temperature control during denaturation and annealing. For the highest quantification accuracy, the temperature must remain uniform across the entire heating block to ensure all samples are processed equally.

The Illumina Eco™ Real-Time PCR System utilizes a proprietary thermal system that enables extremely uniform temperature control and fast ramp rates. To achieve accurate temperature control, the Eco™ thermal system incorporates a precisely electroformed hollow silver block that is heated and cooled by a single Peltier device. During PCR cycling, conductive fluid circulates rapidly throughout the hollow chamber, transferring heat from the Peltier device evenly across the entire block. This unique design virtually eliminates thermal non-uniformity and block edge effects, providing a new level of thermal performance of ± 0.1 °C well-to-well uniformity across a 48-well plate. The result is higher qPCR performance, which includes tighter replicates, improved reaction efficiency and specificity and ultimately more accurate quantification.

Methods

Library construction. Genomic DNA (gDNA) from *Mycobacterium tuberculosis* H37Rv (Genbank ID:AL123456; 65% GC) or *Escherichia coli* DH10B (Genebank ID:NC_010473; 51% GC) was sheared using a nebulizer to generate 200 bp - 500 bp fragments, and sheared DNA was purified using a QiaQuick PCR Cleanup Kit (Qiagen). Indexed Illumina libraries were prepared from sheared gDNA using the Illumina TruSeq™ DNA Sample Prep Kit (Cat. No#PE-940-2001) following the Illumina TruSeq™ Low-throughput (LT) Protocol. Adaptor-ligated products 380 bp - 460 bp in length were selected and purified using the Pippin Prep™ apparatus with a 2% agarose gel cassette (Sage Science). Enrichment amplification was performed using the KAPA Library Amplification Kit (KK2611); the thermocycling protocol for the library enrichment PCR was as follows: initial denaturation at 98 °C for 15 sec, followed by 14 cycles of 98 °C for 15 sec, 60 °C for 30 sec and 72 °C for 30 sec.

qPCR library quantification. Library quantification by qPCR was performed using the KAPA Library Quantification Kit for Illumina according to the recommendations in the Technical Data Sheet. Briefly, 1 µl of each library sample was diluted into 999 µl Dilution Buffer (10 mM Tris-HCl, 0.05% Tween20, pH 8.0); three additional two-fold serial dilutions (100 µl transferred into 100 µl) were made to generate 1:1000, 1:2000, 1:4000, and 1:8000 dilutions of each library. 20 µl or 10 µl qPCR reactions were assembled (Table 1) in a 48-well Eco™ plate according to the low-throughput plate layout (Figure 1, top panel) and sealed with an optical Eco™ adhesive seal. Reactions were run using the cycling protocol shown in Table 2.

Table 1: qPCR reaction setup for library and DNA standards

Reaction component	Final conc.	Per 20 µl reaction[1]	Per 10 µl reaction
PCR grade water	–	4 µl	-
2X KAPA SYBR® FAST qPCR Master Mix	1X	10 µl	5 µl
10X Primer Premix	10X	2 µl	1 µl
Template (DNA standard 1- 6 or library)	–	4 µl	4 µl

[1] Reaction volume of 20 µl is recommended for the Illumina Eco™. 10 µl reactions were also validated using this protocol.

Table 2: KAPA Library Quantification Kit cycling parameters for the Illumina Eco™

Cycling step		Temperature and time	
Initial denaturation		5 min at 95 °C	Hold
Denaturation		30 sec at 95 °C	x 35 cycles
Annealing/Extension[2]		45 sec at 60 °C	
Melt[1]	Melt	95 °C	15 sec
	Anneal	60 °C	15 sec
	Melt	95 °C	15 sec

[1] Dissociation (melt curve) analysis is optional and in certain circumstances may provide a useful indication of possible primer and/or adaptor dimer contamination of libraries.

Recommended Eco™ plate layouts. Depending on the required throughput, we suggest two alternative sample layouts for the 48-well Eco™ plate (Figure 1). The preferred layout ("Low-throughput") makes allowance for a two-fold dilution series of each library, which provides the following advantages: (1) the qPCR amplification efficiency of the unknown samples can be calculated; (2) additional dilutions help to accommodate libraries with unexpectedly high concentrations that might otherwise fall outside the bounds of the standard curve; (3) the additional datapoints are helpful in troubleshooting unexpected or inconsistent results.

Notwithstanding the usefulness of the two-fold sample dilutions, they may be omitted for the sake of higher sample throughput. In many high-throughput scenarios, sample and library preparation protocols are relatively reproducible, resulting in libraries of fairly uniform quality and concentration. Moreover, there should usually be an opportunity for optimizing and validating the sample preparation and sequencing pipeline, including library quantification. In such situations, the need for greater sample throughput may outweigh the advantages of quantifying the two-fold sample dilutions. A "High-throughtput" layout for the 48-well Eco™ plate which maximizes the number of libraries quantified per plate, is also illustrated in Figure 1.

Low-throughput Eco™ plate layout

High-throughput Eco™ plate layout

Figure 1: Recommended plate layouts for the Eco™ instrument. The low-throughput plate layout allows for the quantification of 4 libraries (top panel). 6 DNA Standards (columns 1 and 2) and a 2-fold serial dilution (1:1000, 1:2000, 1:4000, and 1:8000) of each library (1 - 4) are set-up in duplicate. 4 no-template control (NTC) reactions are also included. The high-throughput plate layout allows for the quantification of 18 libraries (bottom panel). 6 DNA Standards (columns 1 and 2) and a single 1:1000 dilution of each library (1 - 18) are set-up in duplicate. NTC reactions are not included.

Analysis. Analysis and calculations were performed as described in the KAPA Library Quantification Kit for Illumina Technical Data Sheet. Concentrations (pM) of the DNA Standards were entered into the Illumina Eco™ analysis software, allowing standard curves to be generated and qPCR efficiencies to be calculated. The analysis software returned calculated concentrations for each library sample dilution. Obvious outliers (e.g. due to pipetting errors) were discarded. To minimize the effects of systematic pipetting inaccuracies or imperfect qPCR efficiency, the most concentrated library dilution falling within the range of the standard curve was used to calculate the concentration of each undiluted library sample. Triplicates were averaged, and the resulting concentrations were adjusted to account for size differences between the library fragments (average ~420 bp) and the DNA Standard (452 bp). Finally, the size-adjusted concentration was multiplied by the relevant dilution factor to arrive at the concentration of the original library sample (Table 3).

Average Cq scores and dilution factors of the two-fold sample dilutions were used to calculate qPCR efficiencies for each library sample.

Results

Both of the libraries tested (*E. coli*, 51% GC; *M. tuberculosis*, 65% GC) were successfully quantified using the Illumina Eco™ Real-Time PCR System and the KAPA Library Quantification Kit (Figure 2). The standard curve showed a calculated amplification efficiency of 95%, with R^2=0.999. The concentrations of the *E. coli* and *M. tuberculosis* libraries were determined to be 1359 pM and 1413 pM respectively.

Calculated amplification efficiencies for the *E. coli* and *M. tuberculosis* libraries were 106% (R^2 = 1.000) and 95% (R^2 = 0.998) respectively, indicating that the high GC content of the *M. tuberculosis* library had a negligible effect on qPCR amplification efficiency.

The libraries were pooled in equimolar concentrations and loaded onto a single lane of a flowcell at a final concentration of 10 pM for cluster amplification, yielding 273,456 clusters/tile. Paired-end sequencing (2 x 75 bp) was carried out on an Illumina GAII; 58% of filter-passed mapped reads were assigned to the *E. coli* genome, with the remaining 42% of reads corresponding to *M. tuberculosis* genomic sequences.

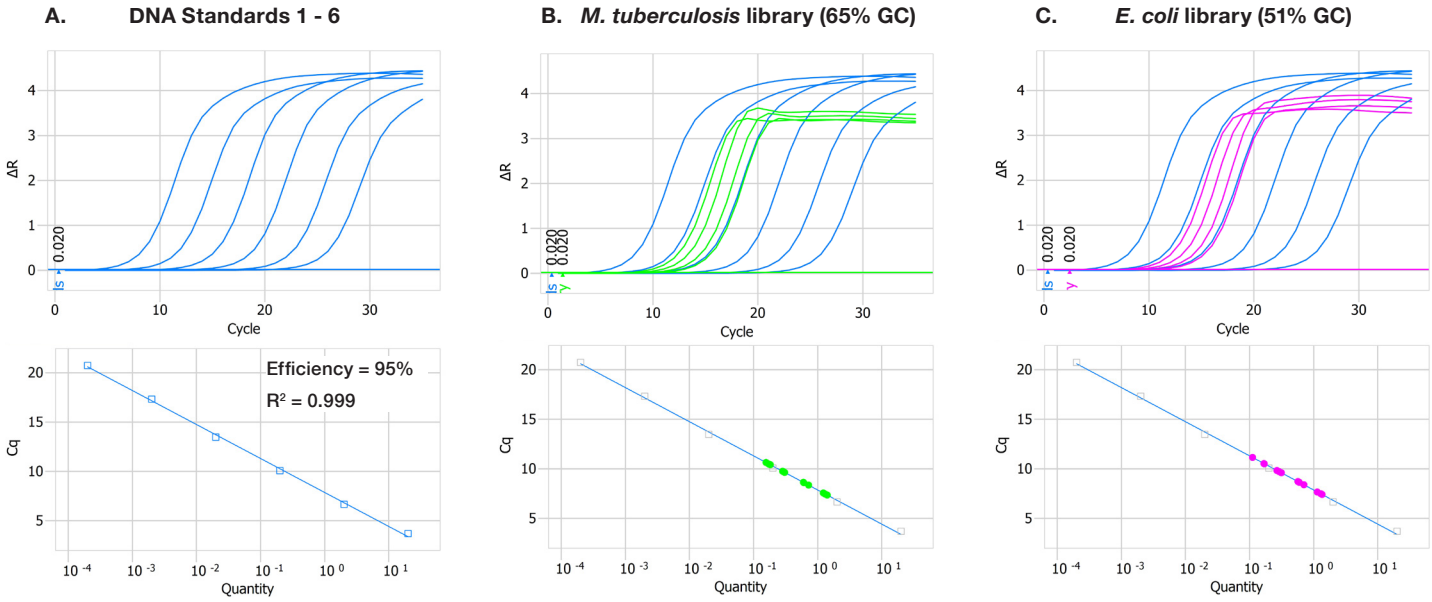

Figure 2: Amplification plots, linear regressions, and calculated amplification efficiencies for DNA Standards and library dilutions. Amplification plots (top) and standard curve (bottom) are shown for DNA Standards 1 - 6 (blue, column A), and for 2-fold serial dilutions of the *M. tuberculosis* (green, column B) and *E. coli* (pink, column C) libraries. Calculated amplification efficiencies for the *E. coli* and *M. tuberculosis* libraries were 106% (R^2 = 1.000) and 95% (R^2 = 0.998) respectively. The 95% reaction efficiency of the GC-rich (65%) *M. tuberculosis* library indicates that the KAPA Library Quantification Kit is capable of consistent quantification across a broad range of library types.

Table 3: Calculation used to determine the concentration of undiluted library

Library name	Dilution factor	Conc. in pM calculated by qPCR instrument (triplicate data points)			Avg. conc. (pM)	Size adjusted conc. (pM)	Conc. of undiluted library (pM)
Library	1:1000	A1	A2	A3	A	$A \times \frac{452}{\text{Avg. fragment length}} = W$	W x 1000
M. tuberculosis	1:1000	1.22	1.41	1.31	1.31	1.41	1413
E. coli	1:1000	1.36	1.14	1.29	1.26	1.36	1359

Ordering information

KAPA Library Quantification Kit - Illumina
Product code: KK4824

Kits contain 5 mL KAPA SYBR® FAST qPCR Universal Master Mix (2X) - 1 mL Primer Premix, and 6 x 80 µL DNA Quantification Standards. Kits contain primers, DNA standards, and qPCR reagents specific for the Illumina sequencing platform and Eco™ qPCR instrument. Primer Premix and DNA Quantification Standards are also sold separately.

For ordering information please contact: **sales@kapabiosystems.com** or visit **www.kapabiosystems.com**.

For more technical support please contact: **support@kapabiosystems.com**.

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