Current standard protocols for the Illumina Genome Analyzer sequencing platform employ laborious, costly, and unreliable methods for quantifying DNA libraries.

KAPA Library Quantification Kits combine a quality-controlled set of DNA quantification standards with the unmatched performance of KAPA SYBR® FAST qPCR reagents to provide a rapid, sensitive, and reliable method for quantifying PCR-amplifiable molecules in next-generation sequencing DNA libraries.

Introduction

Next-generation sequencing (NGS) workflows continue to evolve at an unrelenting pace, driven by users striving for greater reliability, throughput, and efficiency. Current standard protocols for all three major commercial platforms 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 reliable clonal amplification using either emulsion PCR (emPCR) or bridge PCR (bPCR) – underestimation results in non-clonality, while overestimation leads to inefficiency via poor yields of clonally amplified templates.

The standard methods for quantifying Illumina GA DNA libraries have a number of important disadvantages. First, electrophoresis and spectrophotometry measure **total** nucleic acid concentrations, whereas optimal cluster density depends on the appropriate concentration of **PCR-amplifiable** DNA molecules. Since the proportion of amplifiable DNA molecules in a library may vary with each sample, expensive and time-consuming bPCR titrations are required for reliable optimization. Second, these methods have low sensitivity, consuming nanograms of precious samples, or about 1000 times more molecules than are required for sequencing. Finally, electrophoresis and spectrophotometry are not suited to high-throughput of samples, requiring laborious and error-prone manual liquid handling.

In principle, quantitative PCR (qPCR) is inherently well-suited for NGS library quantification[1, 2], and overcomes many of the difficulties inherent in the standard approaches:

- qPCR quantifies only amplifiable library molecules;
- is accurate across an extremely large dynamic range;
- and is amenable to high sample throughput and automated liquid handling.

Moreover, because qPCR is extremely sensitive, it allows accurate quantification of very dilute libraries and consumes small amounts of sample. The ability to accurately obtain reliable cluster density from dilute library samples means that less PCR amplification is required, which minimizes the biases that can arise from variable amplification efficiencies.

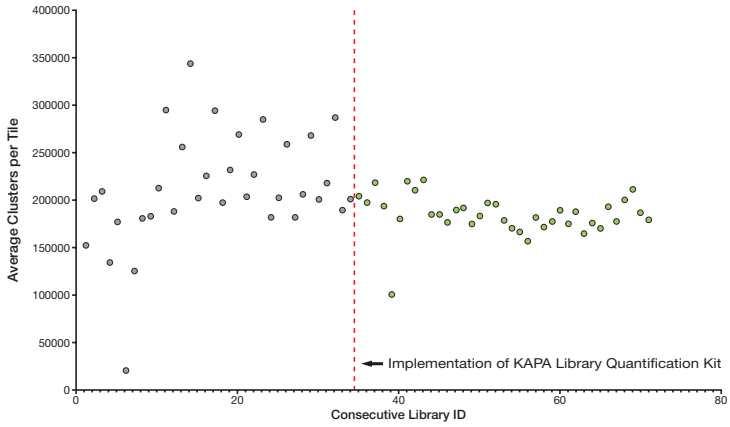

Fig.1 Cluster density before and after implementation of the KAPA Library Quantification Kit at the Broad Institute. The implementation of KAPA Library Quantification Kits into the Illumina GA sequencing workflow at the Broad Institute significantly reduced cluster density variability and eliminated the need for titrations. Average number of clusters per tile are shown for consecutive libraries.

Accurate and reproducible qPCR assays for NGS library quantitation require a reliable supply of well-defined DNA quantification standards, with minimal variation over time, and qPCR reagents capable of efficient amplification of long and complex templates. Traditional qPCR reagents are optimized for short amplification targets; longer targets, unbalanced GC-content, and problematic secondary structures may result in unreliable quantification of some library molecules. To address these requirements, Kapa Biosystems has developed a suite of qPCR-based NGS Library Quantification Kits comprising highly reproducible sets of serially diluted DNA standards and state-of-the-art qPCR reagents, which include a DNA polymerase specifically engineered for robust, SYBR® Green I-tolerant amplification of long and difficult templates. Here we describe the implementation of KAPA Library Quantification Kits for improving the workflow and reducing cluster density variability for sequencing on the Illumina GA IIx at the Broad Institute (**Figure 1**).

Methods

Before quantifying Illumina libraries by qPCR, approximate concentrations of the samples were determined using the Quant-iT™ Kit (Invitrogen). To avoid overloading the qPCR assay, samples with concentrations >20 ng/µl were diluted to ~15 ng/µl. Using the Bravo Automated Liquid Handling Platform (Agilent Technologies), four serial two-fold dilutions – 1:1000, 1:2000, 1:4000, and 1:8000 – of each library sample were prepared in triplicate, and loaded into 384-well optical assay plates. Using the automated liquid handling platform, 10 µl qPCR reactions were assembled according to the recommendations in the KAPA Library Quantification Kit for Illumina GA technical data sheet. Similarly, 10 µl qPCR reactions containing KAPA Library Quantification Illumina GA DNA Quantification Standards were assembled in triplicate. The reactions were run on a 384-well plate with the recommended thermocycling profile on an ABI 7900HT Fast Real-Time PCR instrument (**Figure 2**).

Analysis and calculations were performed as described in the KAPA Library Quantification Kit for Illumina GA technical data sheet. Briefly, concentrations (pM) of the DNA Quantification Standards were entered into the on-instrument analysis software, allowing standard curves to be generated and qPCR efficiencies to be calculated. Accordingly, the software returned calculated concentrations for each library sample dilution; obvious outliers (e.g. due to pipetting errors) were discarded, and the remaining data points from the triplicate dilution series were used to calculate qPCR efficiency for each library sample. 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. Triplicate data points were averaged, and the resulting concentrations were adjusted for size differences between the library fragments and the DNA Quantification Standards (452 bp). Finally, size-adjusted concentrations were multiplied by the relevant dilution factor to arrive at the concentration of the original library sample.

Results

Before qPCR was adopted for library DNA quantification, cluster density was very variable. Implementation of the KAPA Library Quantification Kit in sequencing workflows resulted in a significant reduction in variability across multiple libraries (**Figure 1**). qPCR library quantification negated the need for cluster amplification titration runs in the Illumina GA sequencing workflow. Moreover, greater accuracy in library quantification has led to much more consistent cluster density across multiple libraries and over an extended period of time.

Previous attempts to produce quantification standards in-house resulted in significant shifts in cluster density each time a new set of standards was introduced. Utilizing the quality-controlled DNA Quantification Standards from Kapa Biosystems eliminated this variability (**Figures 3 and 4**).

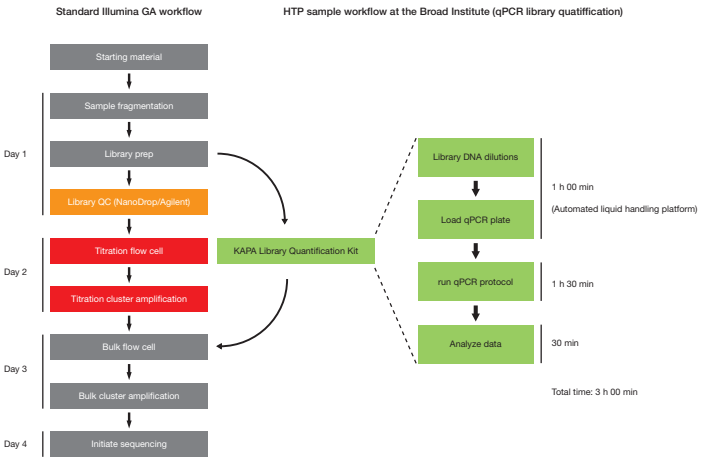

Fig 2. Streamlined workflow using qPCR library quantification. Performing the assay in a 384-well format allows for 96 samples to be processed along with DNA Quantification Standards in a single run. qPCR set-up time using the Bravo Automated Liquid Handler is approximately 1 hour. This, combined with a 1.5 hour qPCR assay, enables approximately 500 samples to be quantified by 1 full-time employee per day.

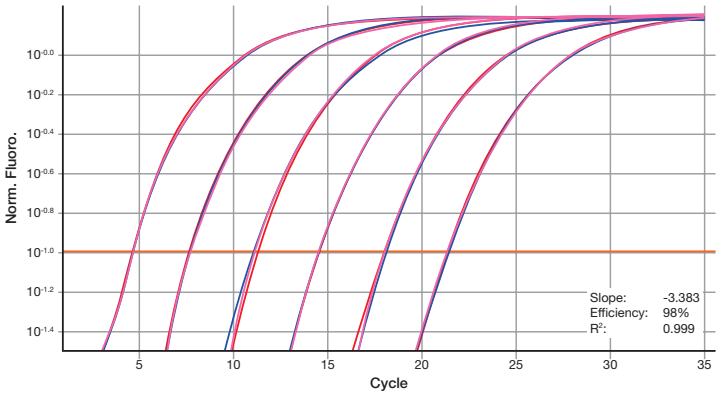

Fig 3. The lot-to-lot variability of the KAPA Library Quantification Kit optimized for the Illumina GA platform. Three distinct lots (red, pink, blue) were compared by analyzing amplification plots of each set of quantification standards. Triplicates of each data point were averaged.

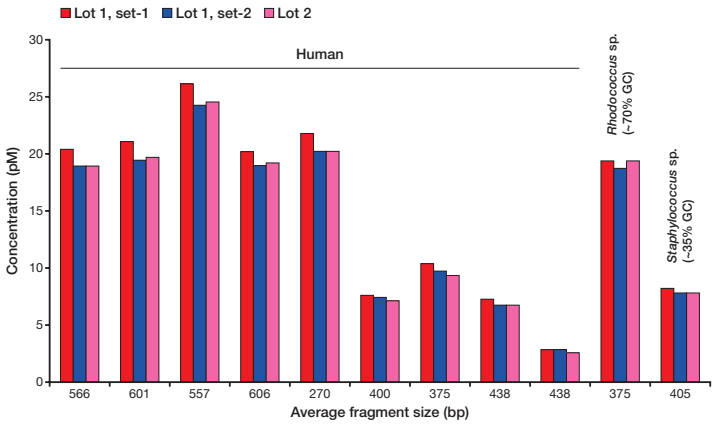

Fig 4. Minimal lot-to-lot and kit-to-kit variability. Nine human DNA libraries and two microbial DNA libraries were used to compare quantification results obtained with distinct lots ("Lot 1" and "Lot 2"), and distinct sets of reagents from the same lot ("set 1" and "set 2") of KAPA Illumina GA Library Quantification Kits.

Quantification of low concentration DNA libraries

The broad dynamic range and high sensitivity of qPCR allows for accurate quantification of low concentration DNA libraries. Low concentration Illumina GA libraries may have to be sequenced for a variety of reasons. In many cases, researchers prefer to minimize – or eliminate – library amplification in order to reduce or avoid amplification bias. Alternatively, a mishap in the laboratory or unreliable reagents may lead to unexpected low yields following library amplification and gel extraction. Such low concentration library samples may be impossible to quantify accurately using spectrophotometry or electrophoresis, making it difficult to obtain appropriate cluster density for efficient sequencing.

In the example presented here, the majority of the sample was lost during gel loading for size selection after adapter ligation, but library construction was nevertheless carried out as usual. The standard PCR amplification protocol yielded PCR products that were not detectable using the Quant-iT™ Kit (Invitrogen) because of the extremely low input sample concentration. To avoid amplification biases, it is preferable not to perform more than the standard number of PCR cycles when amplifying sequencing libraries. To avoid the time and expense of reconstructing this library, quantification was attempted by qPCR. Using the KAPA Library Quantification Kit, the concentration of the undiluted library was determined to be 0.3 nM; well within the dynamic range of the assay. Cluster densities for this library averaged 140,000 clusters/tile, and ~900 MB of sequence data per lane was obtained.

The engineered KAPA SYBR® DNA Polymerase efficiently amplifies a wide range of templates

Accurate qPCR relies on efficient amplification, and SYBR® Green is known to inhibit wild-type *Taq* polymerase[3,4]. Using directed *in vitro* evolution, Kapa Biosystems has engineered *Taq* polymerase to overcome inhibition by SYBR® Green I, enabling efficient qPCR amplification of targets that present a challenge for the wild-type enzyme. KAPA SYBR® FAST qPCR reagents are formulated with this engineered polymerase, and are supplied with KAPA Library Quantification Kits to ensure robust quantification of longer fragments, across a broad range of GC-content, as required for accurate DNA library quantification (**Figure 5**).

To test the reliability of qPCR quantification of diverse libraries, the KAPA Library Quantification Kit was used to determine the concentration of two Illumina GA libraries with unusually high (*Rhodococcus* sp.; ~70% GC) or low (*Staphylococcus* sp.; ~35%) GC content. Both libraries amplified with an efficiency of ~95% or higher (**Figure 6**).

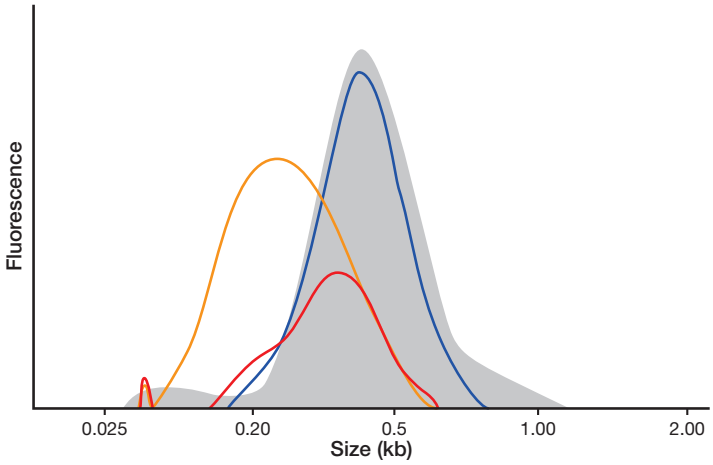

Fig 5. Fragment size distributions before and after qPCR. Fragment size distributions before (grey fill) and after qPCR amplification using three commercial qPCR Master Mixes (KAPA SYBR® FAST, blue; Stratagene Brilliant SYBR II, red; Finnzymes DyNAmo, orange). Reactions were performed with the following cycling protocol using a Stratagene MX4000 qPCR instrument: 95 °C for 10 min followed by 40 cycles of 95 °C for 10 sec and 60 °C for 45 sec.

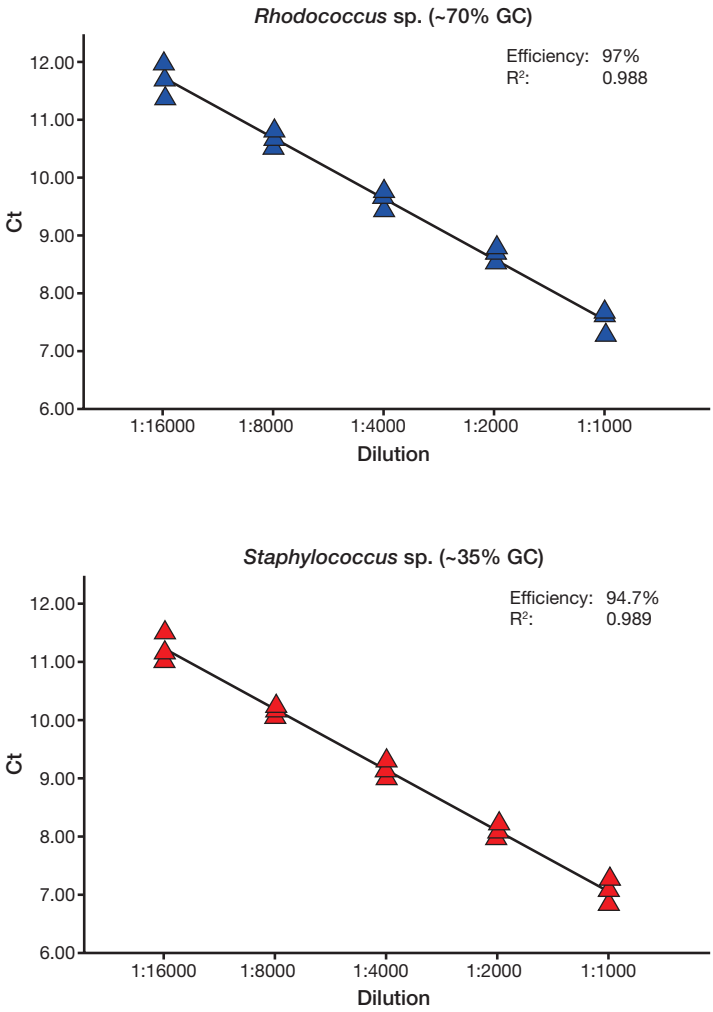

Fig 6. Amplification efficiency of libraries with a high and low-GC content. Two-fold dilution series (1:1000 through 1:16000) were prepared in triplicate, and qPCR performed according to the recommendations in the product technical data sheet.

References

1. Meyer, M., Briggs, A. W., Maricic, T., and Ho, B. (2007). From micrograms to picograms: quantitative PCR reduces the material demands of high-throughput sequencing. *Nucleic Acids Research* **36(1)**: e5.

2. Quail, M. A., Kozarewa, I., Smith, F., Scally, A., Stephens, P. J., Durbin, R., *et al*. (2008). A large genome center's improvements to the Illumina sequencing system. *Nature Methods*, **5(12)**: 1005-1010.

3. Arezi, B., Xing, W., Sorge, J.A., & Hogrefe, H.H. (2003). Amplification efficiency of thermostable DNA polymerases. *Anals of Biochemistry* **321(2)**: 226-35.

4. Kermekchiev, M.B., Kirilova, L.I., Vail, E.E., and Barnes, W.M. (2009). Mutants of *Taq* DNA polymerase resistant to PCR inhibitiors allow DNA amplification from whole blood and crude samples. *Nucleic Acids Research* **37(5)**: e40.