KAPA Library Quantification Technical Guide

KAPA Library Quantification Kits for Illumina® platforms

v3.20

Contents

Introduction

Accurate quantification of the number of adapter-ligated molecules in a library prepared for Illumina next-generation sequencing is desirable at various stages of sequencing workflows, including:

- After adapter ligation, to determine the amount of input material converted to adapter-ligated molecules (conversion rate) and/or the quantity of template used for library amplification.
- After library amplification, to determine whether a sufficient amount of each library has been generated and/or to ensure equal representation of indexed libraries pooled for target capture or cluster amplification.
- Prior to cluster amplification, to confirm that individual libraries or sample pools are diluted to the optimal concentration for flow cell loading. Overestimation of library concentration may result in a lower than desired cluster density. Underestimation of library concentration may result in higher than desired cluster density, which can lead to poor cluster resolution. Both scenarios result in suboptimal utilization of sequencing capacity.

qPCR is widely regarded as the gold standard for NGS library quantification, as it accurately measures the number of molecules that can serve as templates during library and cluster amplification. Furthermore, the sensitivity and broad dynamic range of qPCR enables accurate quantification of very dilute libraries.

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 reproducible liquid handling.

KAPA Library Quantification Kits provide an industry-leading solution for qPCR-based quantification of NGS libraries. The kit contains the KAPA SYBR® FAST DNA Polymerase, engineered through a process of directed evolution for high-performance qPCR. The ability of the engineered polymerase to amplify diverse DNA fragments with similar efficiency renders it uniquely suitable for library quantification, and enables the use of a universal DNA standard for the accurate and reliable quantification of all Illumina libraries – irrespective of library type, GC content or fragment length. The pre-diluted set of DNA Standards included in the kit are rigorously tested to ensure minimal lot-to-lot variation and consistent performance over time.

Effective date: September 2020

Product Description

KAPA Library Quantification Kits for Illumina® platforms provide all the reagents needed for absolute, qPCR-based quantification of Illumina libraries flanked by the P5 and P7 flow cell oligo sequences. Kits contain:

- Library Quantification DNA Standards 1 – 6 (a 10-fold dilution series of a linear, 452 bp template),
- Library Quantification Primer Premix (10X), containing the following primers:

  Primer 1: 5'-AAT GAT ACG GCG ACC ACC GA-3'
  Primer 2: 5'-CAA GCA GAA GAC GGC ATA CGA-3'

- KAPA SYBR® FAST qPCR Master Mix (2X), available with various passive reference dyes.

Library quantification is performed by amplifying the set of six pre-diluted DNA Standards and appropriately diluted library samples by qPCR, using the KAPA SYBR FAST qPCR Master Mix and primers based on the Illumina P5 and P7 flow cell oligo sequences. The average Cq value for each DNA Standard is plotted against $\log_{10}$(concentration in pM) to generate a standard curve, which is used to calculate library concentrations (Figure 1).

Kits are subjected to stringent functional quality control to ensure consistent performance and minimal lot-to-lot variation. All components are free of detectable exo- and endonuclease activities, and meet strict requirements with respect to DNA contamination. Please contact sequencing.roche.com/support for more information.

Product Applications

KAPA Library Quantification Kits for Illumina platforms are designed for the accurate and reproducible quantification of libraries prepared for Illumina sequencing. Any library with a concentration >0.0002 pM that contains sequences complementary to the primers contained in the 10X Primer Premix can be quantified with the kit – irrespective of the library type, how it was constructed, or on which Illumina instrument it will be sequenced. The kit supports quantification of libraries across a range of GC contents, and average fragment lengths up to 1 kb.

In addition to NGS library quantification, the kit can also be used to detect library contamination in work spaces used during the preparation of Illumina libraries.

KAPA Library Quantification Kits for libraries constructed for sequencing on other NGS platforms are also available. Please contact sequencing.roche.com/support for details.

The KAPA Library Quantification assay consists of repetitive pipetting steps, which can easily be automated. The use of an automated liquid handling system is highly recommended for high-throughput NGS pipelines. Please refer to **Assay automation** in the **Important Parameters** section for more information.

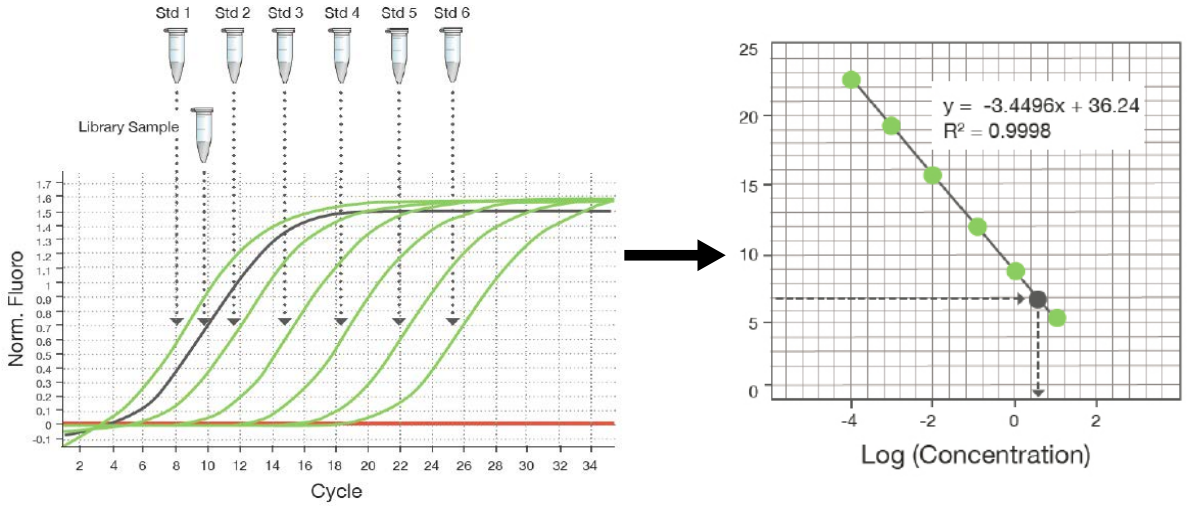

Figure 1. Principle of the KAPA Library Quantification assay. A set of six pre-diluted DNA Standards (representing a 10-fold dilution series of a linear, 452 bp dsDNA fragment), as well as appropriately diluted library samples, are amplified by qPCR, using the KAPA SYBR FAST qPCR Master Mix and primers based on the Illumina P5 and P7 flow cell oligo sequences. The average Cq value for each DNA Standard is plotted against $\log_{10}$(concentration in pM) to generate a standard curve. The standard curve is used to convert the average Cq values for diluted library samples to concentration. This is followed by a size-adjustment calculation, to compensate for differences between the length of the DNA Standard and the average fragment length of the libraries. Finally, the undiluted or working concentration of each library is calculated. Please refer to the **KAPA Library Quantification Kit Technical Data Sheet (KR0405)** for a detailed protocol and data analysis guidelines.

Safety Information

Precautions

- Handle all samples as if potentially infectious, using safe laboratory procedures. As the sensitivity and titer of potential pathogens in the sample material can vary, the operator must optimize pathogen inactivation and follow the appropriate measures according to local safety regulations.
- Do not eat, drink, or smoke in the laboratory area.
- Do not pipette by mouth.
- Wear protective disposable gloves, laboratory coats, and eye protection, when handling samples and kit reagents.
- Wash hands thoroughly after handling samples and reagents.

Waste handling

- Discard unused reagents and waste in accordance with country, federal, state, and local regulations.
- Safety Data Sheets (SDS) are available online on dialog.roche.com, or upon request from the local Roche office.

Important Parameters

KAPA Library Quantification Kits enable the accurate and reproducible qPCR-based quantification of libraries prepared for Illumina® sequencing. Successful implementation of the assay in NGS workflows depends on a number of factors, which are discussed below.

Product and sample handling

Observe all product storage and handling guidelines, as outlined in the **KAPA Library Quantification Kit Technical Data Sheet**.

Dilute DNA solutions (such as NGS libraries) are sensitive to degradation during freezing and thawing. This should be kept in mind when designing the most appropriate long-term storage strategy for your libraries and assay controls.

Since dilute DNA quickly degrades in an unbuffered environment:

- Libraries and controls must always be stored and diluted in a weak buffer (10 mM Tris-HCl, pH 8.0 at 25°C). Tween® 20 buffer may be included in the **dilution** buffer at a final concentration of 0.05% to improve pipetting accuracy and reduce DNA adsorption to plastic tubes and pipette tips.
- Do not store diluted libraries and controls at room temperature, or for long periods of time prior to reaction setup. Dilutions must be prepared fresh for each assay and kept on ice or at 4°C while qPCRs are set up. Calculated library concentrations may be highly variable and/or inaccurate if diluted samples are stored at room temperature, or for long periods of time (even at 4°C) prior to reaction setup. If a sample has to be re-assayed, fresh dilutions must be prepared for the repeat assay.

Consumables and equipment

Always use high-quality, low DNA-binding PCR tubes/plates and pipette tips. Ensure that qPCR instruments and pipettes are properly maintained and regularly calibrated according to the manufacturers' recommendations.

Accurate liquid handling

Since qPCR is a very sensitive technique, and the dynamic range of this assay extends to very low template copy numbers, the reliability of results is highly dependent on accurate liquid handling. Care must be taken to ensure the highest degree of accuracy when executing this protocol. This can be achieved as follows:

- Always ensure that reagents and samples are fully thawed and thoroughly mixed before use. After thawing and mixing, briefly centrifuge tubes to remove any droplets from tube walls.
- Concentrated solutions of DNA may be viscous, making it difficult to accurately dispense small volumes for analysis. Avoid making extremely large dilutions during sample preparation. If samples require very large dilutions to fall within the dynamic range of the assay, it is preferable to perform serial dilutions (e.g. make two consecutive 1/100 dilutions instead of a single 1/10 000 dilution).
- If possible, avoid the use of multi-channel pipettes.
- Use a new pipette tip for each pipetting step, especially when dispensing the DNA Standards and when multiple dilutions of the same sample are prepared. Cross-contamination between standards and/or samples will affect the accuracy of quantification.
- Avoid placing the pipette tip too far under the reagent surface during aspiration, as this may result in liquid adhering to the outside of the tip.
- After aspirating the desired volume of any reaction component, examine the pipette tip before dispensing to ensure that the correct volume is being transferred.
- Always try to dispense reaction components as close as possible to the bottom of the tube or well.
- Flush/rinse pipette tips by pipetting up and down 2 – 3 times after dispensing.
- Ensure that no residual liquid remains in the tip after dispensing.
- Use an automated liquid handling system to dilute libraries and controls and set up reactions, especially when large numbers of samples are processed. Automated liquid handling provides the greatest consistency, both within assays, as well as among assays performed over extended periods of time.

Reaction volume

While the standard protocol for this assay specifies 20 µL reactions, the reaction volume can be scaled down to 10 µL, particularly if the assay is performed in 384-well format and/or for reasons of economy. Accurate liquid handling is particularly important when using 10 µL qPCR volumes.

When scaling down to 10 µL reaction volumes, the volume of template (DNA Standard, diluted library or control) may also be halved (to 2 µL per reaction), or kept at the 4 µL used for 20 µL reactions. The latter is recommended, as it obviates the need to dispense very small template volumes (i.e. facilitates accuracy). If the template volume is not halved for 10 µL reactions, Cq values for all reactions are expected to be 1 cycle earlier (as compared to using the same volume of template in a 20 µL reaction).

This has no impact on the assay, unless the Cq value for DNA Standard 1 is already very low (<3 cycles) on your particular instrument.

Please refer to **DNA Standard 1 amplifies "too early"** in the **Data Analysis and Interpretation** section for a related discussion.

Accurate and reproducible setup of 10 µL reactions in 96-well plates using multi-channel pipettes is challenging. When using 10 µL reaction volumes in 96-well plates, it is important to make sure that plates are properly sealed to prevent evaporation, particularly from those wells along the edges of the plate.

Manual setup of 10 µL reactions in 384-well plates is not recommended, unless only a small portion of the plate is used for an assay. When using 20 µL reaction volumes in 384-well plates, it is important to make sure that reaction mixes do not touch the sealing film, as this will lead to inaccuracies in the detection of the fluorescent signal. It is good practice to briefly centrifuge sealed plates prior to thermocycling, regardless of whether the assay is performed in 96- or 384-well plates.

Libary concentration and dilutions

Libraries and controls must be diluted to fall within the dynamic range of the assay (20 – 0.0002 pM, which equals 5.5 – 0.000055 pg/µL, or ~12 000 000 – 120 dsDNA molecules/µL).

Although the standard curve is a straight line, data may not be extrapolated. Calculated concentrations for libraries derived from dilutions that were too concentrated or too dilute (i.e. that returned average Cq values lower than the average Cq value for DNA Standard 1, or higher than the average Cq value of DNA Standard 6) will not be accurate, as the relationship between template concentration and Cq is not linear during the very early and late stages of the assay. If only one dilution of a library was prepared and assayed in such cases, the library will have to be re-quantified using a more appropriate (larger or smaller) initial dilution. If more than one dilution of each library was prepared and assayed, the remaining dilution(s) that fell within the dynamic range of the assay may be used in concentration calculations.

Initial library dilutions should be based on estimations from previous experience with libraries of the same typeor prepared using similar workflows and/or on concentration information obtained with other methods during library construction and quality control (e.g. those employing NanoDrop™, Qubit® or Bioanalyzer instruments). Since quantification by spectrophotometric and electrophoretic methods are based on different principles than qPCR-based quantification, the correlation between the concentration on which the initial dilution was based, and the concentration determined by qPCR may be poor, particularly for very concentrated or very dilute libraries, or libraries that were over-amplified. Please refer to the **Data Analysis and Interpretation** section at the end of this document for a more detailed discussion. Guidelines for the dilution of different types of libraries are given in Table 1.

Contamination and no-template controls

The qPCRs carried out using this protocol are capable of amplifying very low amounts of DNA fragments flanked by adapters containing the Illumina® P5 and P7 sequence motifs. Suitable templates will be ubiquitous in work areas where Illumina libraries are prepared and amplified. In addition, routine use of the KAPA Library Quantification Kit will generate extremely high numbers of amplicons. Significant contamination of reactions with exogenous template DNA (libraries, DNA Standard or amplicons) will affect the accuracy of calculated library concentrations that are determined using the assay.

Good laboratory practice should be observed at all times to avoid contamination of reagents, samples, consumables, pipettes and other equipment, and work areas with libraries, DNA Standards or amplicons. It is highly recommended that no-template controls (NTCs) are included in each assay to detect contamination introduced during reaction setup. NTC qPCRs should return Cq values that are at least 3 cycles later than the average Cq value for Standard 6.

The six DNA Standards provided in the kit represent a 10-fold template dilution series. Average Cq values for consecutive Standards are expected to differ by ~3.3 cycles. A systematic decrease in the spacing of amplification curves for consecutive DNA Standards provides another indication of contamination with exogenous template, or contamination of dilute DNA Standards with more concentrated ones. The latter can be avoided by always dispensing the DNA Standards from the lowest to the highest concentration (i.e. from DNA Standard 6 to DNA Standard 1), and using a fresh tip for each Standard.

Contamination with human or bacterial DNA from a biological source (i.e. non-library DNA) is not of much concern during library quantification, as the KAPA Library Quantification Primer Premix will only amplify DNA fragments flanked by adapters with binding sites for the quantification primers.

Primer-dimer formation is not uncommon in this assay. This is a result of primer design, which is not optimal for qPCR, but dictated by the Illumina flow cell oligo sequences.

The cycling times used for library quantification are also significantly longer than those used in typical qPCR applications performed with with KAPA SYBR® FAST, thereby further increasing the probability of primer-dimer formation. As long as NTCs return average Cq values that are at least 3 cycles later then the average Cq value for DNA Standard 6, primer-dimer formation should have no impact on quantification.

Internal, process or dilution controls

Most Illumina libraries will have to be diluted to fall within the dynamic range of the KAPA Library Quantification assay (20 – 0.0002 pM). This step represents the biggest risk to accurate quantification, particularly if libraries are very concentrated and large initial dilutions are required.

If more than one dilution of each library is assayed (and falls within the dynamic range of the standard curve), the delta Cq value for consecutive dilutions is usually a good indication of the reliability of calculated library concentrations (see Table 2). Delta Cq values for serial dilutions of a library, do however, not provide any indication of the accuracy of the initial dilution.

To control for gross errors during sample dilution (or reaction setup) it is recommended that at least one appropriate internal process or dilution control be included in every assay.

One or more of the following may be used for this purpose:

- The KAPA Library Quantification Dilution Control (07960417001). This internal control or "Standard 0" is a quality-controlled 200 pM solution of the same linear, 452 bp dsDNA fragment comprising the DNA Standards.
- An Illumina library that has previously been quantified with the KAPA Library Quantification Kit, and that has been sequenced successfully.
- PhiX, a control library supplied by Illumina.

To be most effective, the internal control should be processed in exactly the same way as the libraries to be assayed, i.e. the same initial dilution and serial dilutions (if applicable) should be prepared, and replicate reactions should be set up with each dilution of the internal control.

Each of the different types of internal controls listed above has advantages and disadvantages:

- The KAPA Library Quantification Internal Control ("Standard 0") is subject to the same rigorous quality control as the set of DNA Standards supplied in the KAPA Library Quantification Kit. Absolute concentration and minimal lot-to-lot variation is guaranteed. At 200 pM, Standard 0 is, however, more dilute than most Illumina libraries. If Standard 0 is diluted to the same extent as the samples to be assayed, the Cq values for the dilutions are therefore likely to be a few cycles higher than for the libraries. This is acceptable, as long as at least one of the dilutions of Standard 0 falls within the dynamic range of the assay.
- An existing library that has been sequenced previously is a valuable internal control, as both qPCR-based concentration and cluster density data will be available for such a control. The absolute concentration of a control comprised of an existing library will, however, not have been validated, and the biggest risk is degradation of DNA quality over time – particularly if the same library is used repeatedly as an internal control. The best approach is to select one or more internal controls from a pool of recently prepared and sequenced libraries, which have been stored in a buffered solution at -20°C, and have not been subjected to too many freeze/thaw cycles. Single-use aliquots of such libraries can be prepared and stored at -20°C for use as controls.
- The use of PhiX as an internal library quantification control has similar advantages as using a previously sequenced library. However, PhiX is not recommended if only one internal control is included in a library quantification assay, due to reported batch-to-batch variation in the given concentration and average fragment length of different lots.

Kindly note that the KAPA Library Quantification Internal Control (Standard 0) is not suitable as a sequencing control, as it comprises a PCR amplicon, i.e. is a homogenous solution of a single species of dsDNA.

Replicates, data reliability, throughput and per-sample cost

qPCR is an extremely sensitive measurement technique that is vulnerable to variation arising from a number of sources. Even if the greatest attention is paid to liquid handling, inherent sources of variability such as instrument performance and sampling error lead to unavoidable scatter among replicate data points. Technical replicates are necessary and valuable because they mitigate the effects of variability in the following ways:

- They provide insurance against gross errors or failures with respect to individual reactions, which may be identified as outliers and excluded from the analysis.
- They enable the calculation of replicate averages, thereby discounting the impact of individual data points that are scattered around the population mean.
- They provide an indication of the variability in the data, and a means to gauge the reliability of the assay.
- They may assist with troubleshooting, optimization and process control, for example by providing some indication of the frequency, nature and/or scale of liquid handling errors.

Triplicate qPCRs are recommended for DNA Standards, library samples and controls. A set of triplicate qPCRs can be generated in several ways:

- By preparing a bulk reaction (volume = 3 x reaction

volume) for each DNA Standard, library or control, and splitting this across three PCR tubes or wells of a qPCR plate. This is not recommended, as it only controls for variation associated with qPCR instrumentation and plasticware.

- By preparing three individual reactions (each with volume = reaction volume) for each DNA Standard, library or control. This controls for some of the variation associated with liquid handling, as well as qPCR instrumentation and plasticware. It is the most common form of replication, and the one most compatible with high-throughput workflows.
- By performing three independent dilutions of each library to be assayed, then preparing three separate reactions, each using a replicate dilution as template. This form of replication also controls for liquid handling associated with sample dilution, but is impractical when large numbers of libraries are assayed.

The number of replicates must always be the same for DNA Standards and libraries/controls, and may be reduced to two in order to increase throughput, and reduce per-sample cost. When selecting the best strategy for your workflow and throughput requirements, it is important to keep in mind that the reliability of data is inversely proportional to the number of replicates. Reducing the number of replicates also increases the risk of having to re-assay libraries for which reliable data was not obtained.

The risk of reducing the number of replicate qPCRs can be mitigated by designing workflows in such a way that at least two serial dilutions of each library are always assayed. Because of the quantitative nature of real-time PCR, such dilutions constitute technical replicates, provided that both/all dilutions fall within the dynamic range of the assay. The relationship between replicates, data reliability, throughput and per-sample costs is summarized in Table 3. An assay configuration based on three replicates of a single dilution of each library, with at least one internal and one no template control represents the best compromise between these factors.

Assay automation

The repetitive nature of the KAPA Library Quantification assay makes it amenable to automation, and the use of automated liquid handling platforms is highly recommended for high-throughput library quantification workflows.

Automated methods for the KAPA Library Quantification assay typically consist of two parts, namely sample dilution and qPCR setup. To ensure the highest degree of accuracy and lowest consumption of precious library material, libraries are diluted in a serial fashion, starting with 1 – 2 µL of library in a total volume <200 µL. Subsequent dilutions in the series may have fixed or variable intervals. Covering a range of dilutions from 1/50 to 1/200 000 across six columns of a 96-well plate provides a versatile dilution series that should provide for any type of library to be quantified (ref. Table 1), whilst keeping dilution intervals small. For larger sample numbers (>32), the number of dilutions may be reduced, and dilution intervals increased to limit the number of dilution plates.

To ensure accurate and reliable results, sample carry-over to less dilute library solutions must be prevented, and each column of dilutions must be mixed thoroughly before the next dilution is made. Use fresh tips for each serial dilution, or wash tips between dilutions. Keep transfer volumes between dilutions as large as possible, and well within the specifications for the pipetting device used for these transfers.

qPCR setup consists of three simple steps, namely (i) broadcasting of qPCR Master Mix (including primers, passive reference dye and water, if needed) into replicate wells for each DNA Standard, library dilution or control to be assayed, (ii) transfer of template, and (iii) thorough mixing. For the standard curve, template consists of the appropriate volume (2 or 4 µL) of the appropriate, pre-diluted DNA Standard supplied in the kit. For libraries and controls, template is selected from the desired dilution(s) prepared during the first part of the assay. The capabilities of your liquid handling platform software will determine whether the same dilution(s) have to be assayed across all libraries, or whether dilutions can be "cherry-picked", i.e. whether very diverse libraries can be quantified in a single assay. A detailed protocol for qPCR setup may be found in the **KAPA Library Quantification Kit Technical Data Sheet (KR0405)**.

Pre-validated KAPA Library Quantification methods are available from selected suppliers of automated liquid handling platforms. Please contact sequencing.roche.com/support for more information. Since the assay is easy to automate, and plate layouts may already be integrated with other NGS sample preparation pipeline parameters, or determined by your LIMS system, in-house development of a tailored, automated solution may, however, be preferable.

Table 1. Factors to be considered when diluting Illumina® libraries for the KAPA Library Quantification assay.

Library type	Comments
Amplified library, prepared for direct sequencing	Examples: libraries prepared for WGS, ChIP-Seq, RNA-Seq or amplicon sequencing • Optimally amplified libraries should have a concentration in the range of 2 – 20 nM. • A **1/10 000** dilution is recommended as a first approach (diluted library should fall between Standards 2 and 3 on the standard curve). • Libraries with a higher concentration have to be diluted more to fall within the dynamic range of the assay.
Target capture library, after post-capture amplification	• Library concentration after post-capture amplification will be dependent on the specific capture protocol. • If you don't have an estimate of the library concentration (from experience or data from a different quantification method), assay a **1/100 000** dilution of libraries as a first approach. • Use the results to adjust this dilution for future quantification of libraries produced using the same capture workflow and target library.
Target capture library, after pre-capture amplification	• Single capture workflows typically require ~1 µg of library per hybridization. For multiplexed capture, the requirement for each library is lower (total amount divided by number of libraries captured together). • Library concentrations after pre-capture amplification are typically in the range of 10 – 150 µL/µg (50 – 800 µM), depending on the library volume and average fragment size. • If you don't have an estimate of the library concentration (based on experience or a different quantification method), assay a **1/100 000** dilution of libraries as a first approach (diluted libraries should fall between Standards 1 and 3 on the standard curve).
Unamplified, adapter-ligated library	Examples: libraries generated in PCR-free workflows, libraries quantified to determine optimal number of amplification cycles • For libraries prepared from 100 ng – 1 µg input DNA, try a **1/5 000** dilution as a first approach (diluted libraries should fall between Standards 1 and 3 on the standard curve). • For unamplified libraries prepared from lower inputs (10 - 100 ng), a **1/100** dilution is a good starting point (diluted libraries should fall between Standards 2 and 4 on the standard curve). • Libraries prepared from 10 ng input DNA or less should not be diluted prior to library quantification.

Table 2: Expected Delta Cq values for consecutive library dilutions of different magnitudes

Fold dilution	Expected Delta Cq	Acceptable Delta Cq*
2	1.00	0.934 – 1.08
5	2.32	2.17 – 2.51
10	3.32	3.10 – 3.59
20	4.32	4.06 – 4.67
50	5.64	5.27 – 6.10
100	6.65	6.21 – 7.18

* If the average delta Cq for consecutive dilutions does not fall within this range, an error most likely occurred during the preparation of sample dilutions. Alternatively, it may point to contamination (smaller delta Cq) or inhibition (larger delta Cq) of the assay.

Data Analysis and Interpretation

DNA Standard 1 amplifies "too early"

Due to the design specifications and broad dynamic range of the KAPA Library Quantification assay, DNA Standard 1 has a higher copy number than samples that are typically analyzed by qPCR, particularly in relative quantification assays. As a result, Standard 1 (and any samples that were not sufficiently diluted) may return very early Cq values and/or an increasing fluorescent signal during the automatic baseline determination performed by the qPCR instrument.

Most qPCR cyclers establish a signal baseline during the first 3 – 15 cycles of the qPCR, and any increase in fluorescence during those cycles is interpreted as background. This can impact the standard curve, as the Standard 1 signal may be regarded as "background". Alternatively, DNA Standard 1 may return a delayed Cq value.

It is usually possible to tell whether this is an issue by examining the amplification plots. If the baseline-corrected amplification plot for Standard 1 or any diluted library or internal control with a very low Cq value has an unusual shape, or begins well below the baseline for samples with later Cq values, it is likely that baseline determination/subtraction was not successful for that standard, library dilution or control. Another way to judge whether an early Cq value has created problems during data analysis is to confirm that the "Cq spacing" between consecutive DNA standards is ~3.32 cycles across the entire 10-fold dilution series (all six DNA Standards). Similarly, consecutive 10-fold dilutions of a library should have a delta Cq of ~3.32 cycles. The expected delta Cq for consecutive dilutions of different magnitudes are given in Table 2.

The preferred course of action is to manually decrease the number of cycles over which baseline subtraction/correction is performed (to cycles 1 – 3). Please consult your qPCR instrument manual for guidelines on how to manually adjust this setting. If this does not permanently resolve the problem, DNA Standard 1 may be omitted from future assays, i.e. the standard curve may be generated using Standards 2 – 6 only. Bear in mind though that library dilutions may have to be increased to ensure that all diluted samples fall within the dynamic range of Standards 2 – 6.

Size-adjustment calculation

Once the average Cq value for each library dilution has been converted to concentration (in pM) using the standard curve, a size-adjustment calculation has to be performed to compensate for any difference between the length of the DNA Standard (452 bp) and the average fragment length of libraries that were assayed.

The size-adjustment calculation is a simple multiplication of the concentration derived from the standard curve with the ratio between the size of the DNA Standard (452 bp) and the average fragment size for that particular library. Please refer to the **Detailed Protocol** and **Working example** in the **KAPA Library Quantification Kit Technical Data Sheet** for full details.

The size-adjustment calculation is needed because the fluorescent signal generated in SYBR® Green I-based qPCR quantification is dependent on the total mass (length) of DNA. A longer DNA fragment will carry more of the intercalating dye (reporter) molecules than a shorter fragment; hence, the relative fluorescence will be higher (and Cq value lower) for a population of long DNA fragments, compared to a population of shorter fragments with the same copy number or concentration. If the average fragment length of the Illumina® libraries that are being quantified is significantly shorter or longer than 452 bp, the size-adjustment must be done to ensure that calculated library concentrations are accurate and reliable.

Melt curve analysis

A melt curve analysis is typically included at the end of SYBR Green I-based qPCR assays to provide information about the specificity of the reaction. In library quantification melt curve analysis is less relevant, as the template being amplified is not comprised of a single DNA fragment of defined length and GC content, but is a heterogenous population of molecules, with a range of lengths and GC contents. Unlike the melt curve for a single amplicon, the melt curve for an Illumina library is a "composite" analysis, representing an average across the entire population of library fragments. It is also important to keep in mind that library samples are expected to reach their quantification cycle (Cq) relatively early, whereas the melt curve analysis is only performed after 35 cycles, i.e. at a time when substrate depletion has occurred, and amplification artifacts that are no longer representative of the original template have been generated. This complicates the interpretation of melt curves generated in library quantification assays.

Nevertheless, we have found melt curve analysis useful for the identification of carry-over adapter-dimer in Illumina libraries (Figure 2). Adapter-dimers, formed during library construction with full-length adapters, are very efficiently amplified in the KAPA Library Quantification assay, and will lead to an overestimation of library concentration if present in significant quantities. The melt curve analysis simply has diagnostic value, and does not offer a way to compensate for the contribution of adapter-dimer to the calculated library concentration.

Please note that melt curves for the DNA Standard included in KAPA Library Quantification Kits for Illumina platforms display a very characteristic double peak. This is the result of differential local melting in the 452 bp linear template (which has been confirmed by *in silico* melt curve modelling), and is not indicative of non-specific amplification.

Correlation between library concentration and cluster density

The KAPA Library Quantification Kit has become an integral part of Illumina sequencing workflows, in both low- and high-throughput settings. The assay and reagents are capable of yielding reproducible, accurate and reliable results. The guidelines and instructions provided in this document and the **KAPA Library Quantification Kit Technical Data Sheet (KR0405)** should enable both new and experienced users to determine whether each data set generated with the assay is reliable. Since the metrics used to evaluate data cannot rule out gross errors in library dilutions, calculated library concentrations can only be accepted with confidence if an internal, process or dilution control was included in the assay.

Even if data generated with the assay is determined to be reliable, there is no guarantee that libraries diluted for flow cell loading will yield optimal number of clusters. Reasons for a possible discrepancy between calculated library concentration and cluster density include the following:

- There are multiple liquid handling steps between library quantification and flow cell loading, all of which are error-prone.
- Cluster amplification is a complex process with many variables, some of which are instrument-related.
- qPCR-based quantification with the engineered KAPA SYBR® FAST DNA Polymerase ensures that all the molecules in a complex DNA population are "counted" with high and similar efficiency, irrespective of fragment length or GC content. Cluster amplification (bridge PCR) is, however, performed with a different enzyme and is more challenging. Cluster generation may therefore not be equally efficient for all library fragments.
- Anecdotal evidence suggests that not all library types cluster similarly on a specific instrument, and that libraries of the same type do not yield the same number of clusters on different instruments of the same model when loaded at a specific concentration. Reasons for this fall outside the scope of this document. The reader is encouraged to explore the *SeqAnswers* blog for insightful NGS community forum discussions.

Ultimately, the correlation between library concentration determined with the KAPA Library Quantification Kit and cluster density must be determined empirically for your libraries, instrumentation and workflow. To discover and define this correlation, it is important that the assay is performed meticulously, and that data is analyzed and interpreted correctly. If you are new to NGS and qPCR-based quantification, the following guidelines may be helpful:

- Assay different dilutions of a few selected (preferably previously sequenced) libraries on different days, to ensure that reproducible qPCR data is obtained. As indicated previously, fresh dilutions of test libraries used for this evaluation should be prepared for each assay. Variation of ≤10% in the concentration of a library calculated from different dilutions (within an assay) or in separate assays, is generally regarded as acceptable.
- Perform cluster amplification with the same set of test libraries, more than once, on the instrument(s) that will be used routinely. This is important to determine the variability of the cluster amplification process.
- Establish and maintain a database of library concentrations (determined with the KAPA Library Quantification Kit and any other quantification methods that are being used), as well as cluster density data, generated for different library types and/or instruments. This data will enable you to define the correlation between calculated library concentration and cluster density for your specific situation, and will also be invaluable for process optimization, quality control and troubleshooting.

Library concentrations determined by qPCR vs other methods

Most NGS workflows employ more than one library quantification method. Data generated with a spectrophotometer (e.g. NanoDrop™ instrument), a fluorometric assay (e.g. Qubit® fluorometer and PicoGreen® dye), or an electrophoretic device (e.g. a Bioanalyzer, TapeStation or LabChip® GX instrument) is often used to determine the most appropriate initial dilutions for accurate, qPCR-based quantification using the KAPA Library Quantification Kit. The conventional wisdom is that library concentrations determined by qPCR should be lower than those determined with spectrophotometric, fluorimetric and electrophoretic methods, as qPCR only "counts" those molecules that have the correct adapter on each end, whereas other assays "count" total DNA. However, this is often not the case, and a weak correlation between library concentrations determined with different quantification method is fairly common. The primary reasons are the following:

- Different quantification methods "count different things".
- Different quantification methods are prone to inaccuracy for different reasons.
- Library amplification can have a profound impact on library quantification.

When comparing library concentrations determined with different quantification methods, consider the following:

- Spectrophotometric methods for DNA quantification are sensitive to contaminants that absorb in the UV spectrum. These include organic solvents (e.g. ethanol) used during library purification/cleanup procedures.
- Electrophoretic assays are not primarily designed for quantification, and may have high inherent variation when it comes to quantification (as opposed to size determination). Quantification is typically dependent

on the integrity of assay components such as DNA ladders and fluorescent dyes included in the gel matrix, which are often sensitive to light exposure and/or damage induced by repeated freezing and thawing. Library concentrations determined using an electrophoretic assay may or may not correlate well with concentrations determined with other methods, and the extent of the correlation (or lack thereof) could be highly variable.

- Amplified libraries that are ready for sequencing **should** be comprised of DNA fragments that are fully double-stranded and free of inhibitors/contaminants that affect fluorimetric and qPCR assays employing dsDNA-binding dyes. Library amplification also enriches the library preparation for sequenceable molecules (e.g. fragments flanked by two adapters in the correct configuration for cluster amplification). Library concentrations determined by Qubit®/PicoGreen® assays and the KAPA Library Quantification Kit are therefore expected to correlate fairly well for optimally-amplified libraries.
- In practice, many libraries are "over-amplified", and are not comprised of individual, fully double-stranded fragments. Accurate quantification is only achievable by qPCR-based quantification, and library concentrations determined with the KAPA Library Quantification Kit are typically higher than those obtained by Qubit/PicoGreen assays, for reasons outlined below:

  During the first few library amplification cycles, each successive round of denaturation, primer annealing and extension produces more or less double the number (mass) of full-length, double-stranded library fragments. However, as thermocycling continues, primers are depleted (in NGS library amplification, this happens before dNTPs become limiting, and fairly early in the reaction if the amount of library used as template exceeds ~100 ng). At this point, denatured, single-stranded library molecules are no longer converted to double-stranded DNA. Subsequent rounds of denaturation, annealing and extension doesn't produce additional DNA (library yields are "capped"), and simply results in the separation of complementary DNA strands.

  Because libraries are extremely heterogenous, denatured fragments are statistically unlikely to ever re-anneal to their complementary strands. Primer depletion is therefore followed by imperfect annealing of non-complementary partners, driven by common adapter sequences. This results in the formation of so-called "daisy-chains" or "tangled knots", comprising large assemblies of improperly annealed, partially double-stranded, heteroduplex DNA. These species migrate slower and are observed as secondary, higher molecular weight peaks during the electrophoretic analysis of amplified libraries. However, they are typically comprised of library molecules of the desired length, which are individualized during denaturation prior to probe hybridization (in target capture workflows) or cluster amplification. Since these heteroduplexes contain significant portions of single-stranded DNA, over-amplification leads to the under-quantification with assays employing dsDNA-binding dyes.

  Over-amplification does not impact qPCR-based quantification, which involves initial denaturation of heteroduplexes, and real-time detection of perfectly complementary molecules that accumulate before substrate depletion occurs. Over-amplification should, however, be avoided in order to achieve optimal library quality. Please refer to the **KAPA Library Preparation Technical Guide** or contact sequencing.roche.com/support for assistance with the optimization of library amplification parameters.
- Libraries may be quantified after adapter-ligation, prior to library amplification. The conversion of input DNA to adapter-ligated library is an important library construction metric, as this ultimately determines library diversity. Prior to library amplification, the forked portions of Illumina® adapters are still single-stranded. Assays relying on dsDNA-binding dyes are likely to underestimate the concentrations of unamplified libraries, and the degree of under-quantification depends on the adapter design and library fragment length (i.e. the proportion of each library molecule that is single-stranded). For reasons outlined above, qPCR is recommended for the accurate quantification of unamplified, adapter-ligated libraries. The 10X Primer Premix included in the KAPA Library Quantification Kit for Illumina platforms is suitable for the quantification of unamplified libraries prepared with "full-length" adapters. For libraries prepared with truncated or custom adapters, the DNA Standards and qPCR Master Mix can be used in combination with user-supplied primers. Please contact sequencing.roche.com/support for more information.

Table 3. Relationship between replicates, reliability of data, throughput and per-sample cost.

Number of replicates	Dilutions per library[1]	Reliability of data	Through-put	Per-sample cost	Number of libraries that can be assayed per plate[3]		Number of libraries that can be assayed with a complete kit[4]	
					96-well format	384-well format	96-well format	384-well format
3	3	Very high	Lowest	Highest	8	40	36	94
3	2	High	Low	High	12 – 13	60 – 61	59	143
3	**1**	**Acceptable[2]**	**Medium**	**Medium**	**24 – 26**	**120 – 122**	**118**	**287**
2	3	High	Low	High	13 – 14	61 – 62	63	145
2	2	Acceptable[2], with increased risk of repeats	Medium	Medium	20 – 21	92 – 93	95	218
2	1	Acceptable[2], with high risk of repeats	High	Low	40 – 42	184 – 186	190	437
1	3	Acceptable[2], with increased risk of repeats	Medium	Medium	29 – 30	125 – 126	136	296
1	2	Low, with high risk of repeats	High	Low	44 – 45	188 –189	204	444
1	1	Very low. This configuration is not recommended.	Highest	Lowest	88 – 90	376 – 378	409	889

[1] Library dilutions that fall within the dynamic range of the assay, i.e. yield useable data.

[2] Especially if sample throughput is low or automated liquid handling is used for high-throughput library dilutions and reaction setup, internal controls are included, and variation related to instrumentation and consumables has been minimized.

[3] Calculations are based on the following assumptions: reaction volume is 20 µL for for 96-well format and 10 µL for 384-well format; number of replicates for DNA Standards are the same as for samples; 384-well plates are fully utilized. Where a range is given, the lower number of libraries is based on an assay configuration that includes at least one no template control (NTC) and one internal control, whereas the upper number is based on an assay configuration that excludes all types of controls to maximize the number of libraries that can be assayed. Assay configurations that include controls are always recommended.

[4] A complete kit contains 80 µL of each DNA Standard and qPCR mix and primers for 500 x 20 µL or 1 000 x 20 µL reactions. The qPCR reagents are always the limiting component, irrespective of whether 2 or 4 µL DNA Standard is used per 10 µL reaction. Calculations are based on a 20 µL reaction volume for 96-well format and a 10 µL reaction volume for 384-well format, and assumes 10% of reagent wastage. Incomplete kits (containing qPCR mix and primers only) may be purchased to utilize leftover DNA Standards from complete kits.

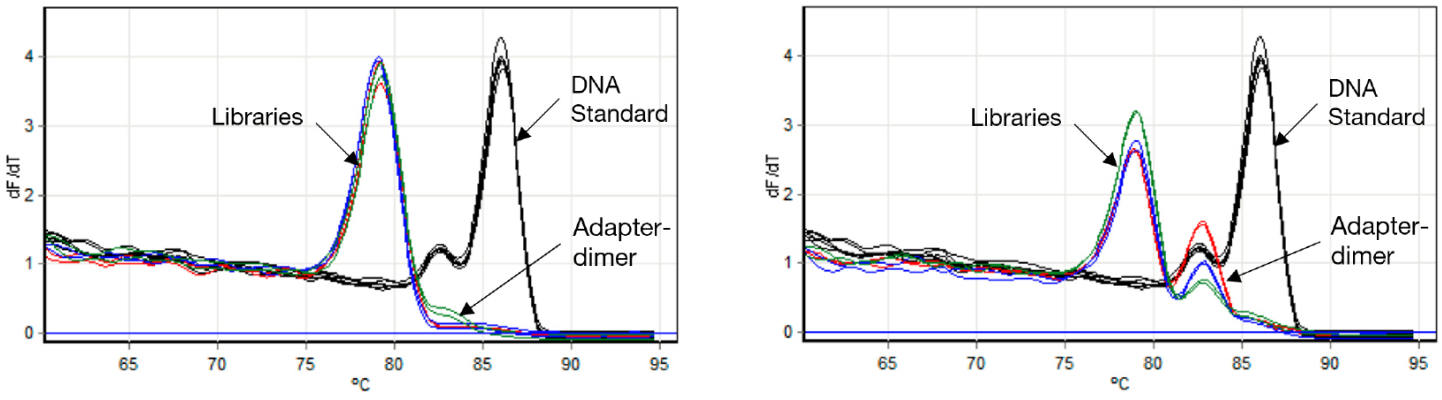

Figure 2. Melt curves for libraries and DNA Standards generated with the KAPA Library Quantification Kit for Illumina® platforms. Libraries in the top panel contain no or a negligible amount of carry-over adapter-dimer. Libraries in the bottom panel contain an unacceptable amount of adapter-dimer, which have contributed to an inflated calculated library concentration. Note the characteristic double melt peak for the DNA Standard.

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KAPABIOSYSTEMS

Manufacturing, R & D
Cape Town, South Africa
Tel: +27 21 448 8200
Fax: +27 21 448 6503

Technical Support
sequencing.roche.com/support

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