KAPABIOSYSTEMS

Technical Data Sheet

KAPA Library Quantification Kit
Ion Torrent™ Platforms

KR0407 – v7.17

This Technical Data Sheet provides product information and a detailed protocol for the KAPA Library Quantification Kits for Ion Torrent platforms.

This document applies to KAPA Library Quantification Kits for Ion Torrent platforms (07960158001, 07960212001, 07960263001, 07960344001 and 07960301001), KAPA Library Quantification Primer and PCR Mix Kits for Ion Torrent platforms (07960450001, 07960492001, 07960549001, 07960735001 and 07960581001), KAPA Library Quantification Standards and Primer Kit for Ion Torrent platforms (07960107001), KAPA Library Quantification Standards Kit for Ion Torrent platforms (07960395001), and KAPA Library Quantification Primer Kit for Ion Torrent platforms (07960115001).

Contents

Kapa/*Roche* Kit Codes and Components	
Complete Kits with: DNA Standards 1 – 6 (80 µL each) Primer Mix (1 mL) KAPA SYBR® FAST qPCR Master Mix (5 mL)	**KK4827** – *07960158001* Universal qPCR Master Mix 50X ROX High and 50X ROX Low supplied separately
	KK4838 – *07960212001* ABI Prism™ qPCR Master Mix
	KK4847 – *07960263001* Bio-Rad iCycler™ qPCR Master Mix
	KK4874 – *07960344001* ROX Low qPCR Master Mix
	KK4857 – *07960301001* qPCR Master Mix optimized for LightCycler® 480
Kits with: Primer Mix (1 mL) KAPA SYBR FAST qPCR Master Mix (5 mL)	**KK4924** – *07960450001* Universal qPCR Master Mix 50X ROX High and 50X ROX Low supplied separately
	KK4934 – *07960492001* ABI Prism qPCR Master Mix
	KK4944 – *07960549001* Bio-Rad iCycler qPCR Master Mix
	KK4974 – *07960735001* ROX Low qPCR Master Mix
	KK4954 – *07960581001* qPCR Master Mix optimized for LightCycler 480
Kits with: DNA Standards (80 µL) Primer Mix (1 mL)	**KK4812** – *07960107001*
Kits with: DNA Standards (80 µL)	**KK4904** – *07960395001*
Kits with: Primer Mix (1 mL)	**KK4813** – *07960115001*

Quick Notes

- The DNA Standards provided in this kit represent a 10-fold dilution series (83 – 0.00083 pM/µL)
- Ensure that the libraries to be quantified are compatible with the qPCR quantification primer sequences given on the next page.
- Select the correct version of KAPA SYBR FAST qPCR Master Mix for the qPCR instrument to be used. For details on qPCR instrument and reference dye compatibility, please refer to the KAPA SYBR FAST Technical Data Sheet.
- Accurate liquid handling is critical for reliable and reproducible results.
- Unlike most TaqMan®-based assays for library quantification, this kit is compatible with all Ion Torrent libraries that contain the "A" and "trP1" adapter sequences ("trP1" forms part of the full-length "P1" adapter sequence).

Product Description

Accurate quantification of amplifiable library molecules is critical for the efficient use of the Ion Torrent next generation sequencing (NGS) platform—overestimation results in too few DNA-bearing beads, while underestimation leads to multiple template molecules per bead during emPCR. Accurate library quantification is equally important when pooling indexed libraries for multiplexed sequencing to ensure equal representation of each library.

qPCR is inherently well-suited to NGS library quantification, as it overcomes many of the difficulties posed by alternative approaches, for example it:

- quantifies only the PCR-amplifiable library molecules that are relevant to optimizing emPCR,
- is exceptionally sensitive and accurate across an extremely broad dynamic range, and
- is amenable to high sample throughput and automated liquid handling. The extreme sensitivity of qPCR enables accurate quantification of very dilute libraries. This minimizes the need for PCR amplification of libraries and the associated problematic biases.

KAPA Library Quantification Kits are comprised of DNA Standards (six 10-fold dilutions) and Primer Premix (10X), paired with KAPA SYBR® FAST qPCR Kits to accurately quantify the number of amplifiable molecules in an NGS library. The 153 bp KAPA Ion Torrent 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 vigorously tested to ensure minimal lot-to-lot variation. The kit contains the novel KAPA SYBR FAST DNA Polymerase, engineered through a process of directed evolution for high-performance SYBR Green I-based qPCR. The ability of the engineered polymerase to amplify diverse DNA fragments with similar efficiency enables the use of a universal standard for the reliable quantification of libraries with an average fragment length of up to 1kb, irrespective of library type or GC content. KAPA Ion Torrent Library Quantification Kits are suited for the quantification of libraries constructed with Ion Torrent adapters containing the following qPCR primer sequences:

Primer IT A: 5'-CCA TCT CAT CCC TGC GTG TC - 3'

Primer IT trP1: 5'-CCT CTC TAT GGG CAG TCG GTG AT-3'

Product Applications

KAPA Library Quantification Kits are suitable for any application requiring sensitive, accurate, reproducible, and/or high-throughput NGS library quantification, including:

- high-throughput library construction and quantification pipelines, especially where automation is desirable;
- pooling of indexed libraries for multiplexed sequencing, to ensure equal representation of pooled samples in sequencing data; and
- optimizing input DNA concentrations for reliable emPCR.

Product Specifications

Shipping and Storage

KAPA Library Quantification Kits are shipped on dry ice or ice packs, depending on the destination country. Upon receipt, store the entire kit at -15ºC to -25°C in a constant-temperature freezer. When stored under these conditions and handled correctly, all kit components will retain full activity until the expiry date printed on the kit label.

Handling

Always ensure that components are fully thawed and thoroughly mixed before use. The KAPA SYBR FAST qPCR Master Mix may not freeze solidly, even when stored at -15°C to -25°C.

The SYBR Green I dye (contained in the KAPA SYBR FAST qPCR Master Mix) and ROX dyes are light sensitive. Exposure to direct light for an extended period of time will result in loss of fluorescent signal intensity.

All components of the KAPA Library Quantification Kits, as well as the combined KAPA SYBR FAST/Primer Premix (10X) solution, are stable through more than 30 freeze/thaw cycles. Ensure that all reagents are stored protected from light at -15ºC to -25ºC when not in use. When protected from light, these reagents are stable in the dark at 2ºC to 8ºC for at least one week, and may be stored at this temperature for short-term use, provided that they do not become contaminated with microbes and/or nucleases. KAPA SYBR FAST is an antibody-mediated hotstart polymerase formulation, and the KAPA Library Quantification Kits are therefore suitable for use with automated liquid handling stations for high-throughput library quantification.

Quality Control

All kit components are subjected to stringent functional quality control, are free of detectable contaminating exo- and endonuclease activity, and meet strict requirements with respect to DNA contamination. Please contact Technical Support at **kapabiosystems.com/support** for more information.

Important Parameters

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

Accurate Liquid Handling

Since qPCR is a very sensitive technique and the dynamic range of this assay extends to 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, centrifuge tubes briefly 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.

Sample Concentration and Dilutions

Libraries and controls must be diluted to fall within the dynamic range of the assay. Any library dilution that amplifies before DNA Standard 1 should not be used in library concentration calculations. If only one dilution was included in the assay, it must be repeated with a more appropriate dilution of the library. If multiple dilutions were included, those that fall within the dynamic range of the assay can be used to quantify the library.

Library dilutions should be based on estimations from previous experience with libraries of the same type, or 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).

Sample Quality

Since dilute DNA degrades in an unbuffered environment, libraries and controls must be stored and diluted in a buffered solution, such as 10 mM Tris-HCl, pH 8.0 – 8.5 (25°C). Tween® 20 (0.05%) may be included in the dilution buffer to improve pipetting accuracy and reduce DNA adsorption to plastic tubes and pipette tips. Never dilute libraries or controls with water.

Prepare fresh dilutions for each assay and keep dilutions on ice during qPCR setup. 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 4ºC) prior to setting up qPCRs. If a sample has to be re-assayed, fresh dilutions should be prepared for the repeat assay.

Contamination and No-template Controls

Observe good laboratory practice at all times to avoid contamination of work areas, reagents, consumables, and equipment 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.

Always dispense the DNA Standards from the lowest to the highest concentration (i.e. from DNA Standard 6 to DNA Standard 1) and use a fresh tip for each DNA Standard. Melt curve analysis of NTC reactions may be performed to confirm whether amplification is due to contamination with DNA Standard or library DNA, or due to primer-dimer formation. Primer-dimer formation is not uncommon with this assay as the cycling times used are also much longer than those used in typical qPCR with KAPA SYBR® FAST, further increasing chances of primer-dimer formation. As long as NTC amplification is at least 3 cycles after DNA Standard 6, primer-dimer formation should have no impact on kit performance.

Reaction Volume

While this protocol specifies 20 µL reactions, volumes may be scaled down to 10 µL, if required. For improved accuracy, the volume of DNA Standards/library dilutions should be kept at 4 µL, with 6 µL of KAPA SYBR FAST qPCR master mix containing Primer Premix (10X). However, the amount of template (DNA Standard or diluted library) used per reaction may be scaled as required, provided that it can still be pipetted accurately. Always use the same volume of DNA Standard and diluted library. Ensure that plastic consumables, pipettes, and qPCR instruments are compatible with the reaction volume.

Internal Controls

The dilution of concentrated library DNA to fall within the dynamic range of this assay 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 ΔCq value for consecutive dilutions is a good indication of the reliability of calculated library concentrations (see **Data Analysis** (step 6). However, ΔCq values for serial dilutions of a library do not provide any indication of the accuracy of the initial dilution.

For this reason, it is recommended to include at least one appropriate internal process or dilution control in every assay. To be most effective, the internal control should be processed in the same way as the libraries to be assayed, i.e., the same initial dilution and serial dilutions should be prepared, and replicate reactions set up with each dilution of the internal control.

A library having previously undergone emPCR is a valuable internal control, as both qPCR-based concentration and bead clonality data will be available for such a control. The biggest risk of this control 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 libraries, which have been stored in a buffered solution at -20ºC, and have not been subjected to too many freeze-thaw cycles. Single-use aliquots of libraries can be prepared and stored at -20ºC for use as controls.

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. Triplicate qPCRs are recommended for DNA Standards, library samples, and controls.

The number of replicates may be reduced to two in order to increase throughput and reduce per-sample cost. When selecting the best strategy for workflow and throughput requirements, keep in mind that the reliability of data is inversely proportional to the number of replicates. Reducing the number of replicates increases the risk of having to re-assay libraries if 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, provided that both of these dilutions fall within the dynamic range of the assay.

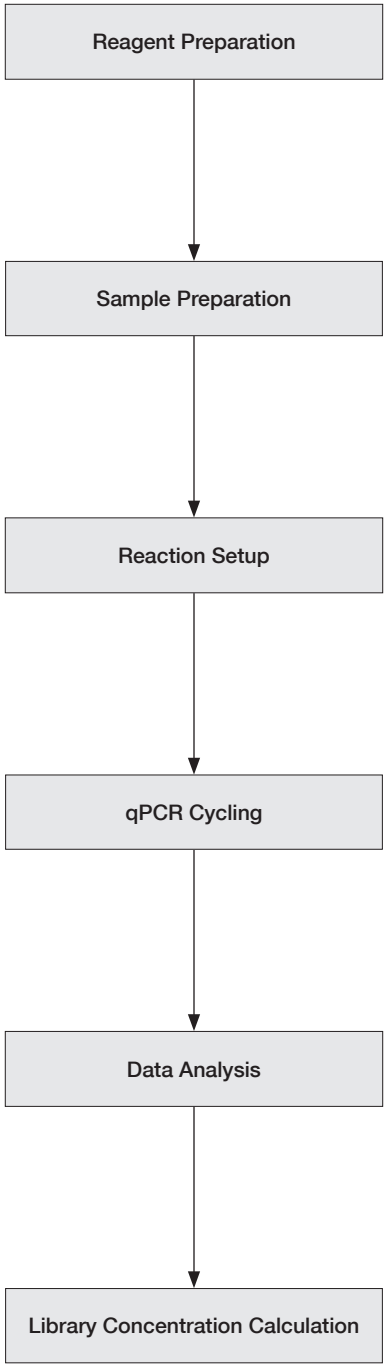
For high-throughput library construction pipelines, automated library quantification in 384-well format is highly recommended, as this offers the possibility of quantifying 96 libraries in triplicate in a single run, while reducing the per-sample cost by performing 10 µL qPCRs.

Assay Automation

Library quantification with the KAPA Library Quantification Kit is amenable to automation and the use of automated liquid handling platforms is highly recommended for high-throughput library quantification workflows.

Pre-validated KAPA Library Quantification methods are available from selected suppliers of automated liquid handling platforms. Please contact Technical Support at **kapabiosystems.com/support** for more information.

Process Workflow



Combine:

5 mL KAPA SYBR® FAST qPCR Master Mix (2X)

1 mL Primer Premix (10X)

0.2 mL ROX High or Low (50X) (optional)

Record date and lot numbers of reagents used. Store at -20°C and protect from light. Minimize freeze-thaw cycles.

Dilute library DNA and any internal controls with library dilution buffer [10 mM Tris-HCl, pH 8.0 – 8.5 (25°C) + 0.05% Tween® 20 (optional)]

Prepare fresh dilutions for each assay

Keep diluted DNA on ice to prevent degradation

Per 20 µL reaction:

*12 µL of KAPA SYBR FAST + Primer Premix (10X)**

4 µL of each DNA Standard/library dilution

PCR-grade water up to 20 µL

**If ROX is included, use 12.4 µL per 20 µL reaction. ROX can also be added separately (0.4 µL per reaction)*

Reactions may be scaled down to 10 µL

Include no-template control reactions

Work from lowest to highest DNA Standard to avoid cross-contamination

95°C – 5 min

35 cycles of 95°C – 30 sec

60°C – 45 sec [data acquisition]

Melt: 65 – 95°C (optional)

Increase combined annealing/extension to 90 sec for libraries with average fragment length >700 bp.

Remove outliers

Confirm reaction efficiency 90 – 110%

Confirm R2 >0.99

Confirm ΔCq for DNA Standards 3.1 – 3.6

Absolute quantification against standard curve)

Use the KAPA Library Quantification Data Analysis Template designed for library quantification data analysis

Confirm correct ΔCq for library dilutions

Confirm library dilutions amplify within dynamic range of assay

Perform size-adjustment calculation

Calculate undiluted library concentration

Data exported from qPCR instrument

Library concentration = qPCR conc x (153 bp/average fragment length) x dilution factor

Detailed Protocol

1. Reagent Preparation

1.1 Prepare an appropriate volume of DNA Dilution Buffer [10 mM Tris-HCl, pH 8.0 – 8.5 (25°C) + 0.05% Tween® 20 (optional)]. This buffer can be stored at room temperature or 4°C, and re-used. Always equilibrate the buffer to room temperature before use.

1.2 Ensure that all components of the KAPA Library Quantification Kit are completely thawed and thoroughly mixed.

1.3 If the kit is used for the first time, add the Primer Premix (10X) (1 mL) to the bottle of KAPA SYBR® FAST qPCR Master Mix (2X) (5 mL). Mix thoroughly using a vortex mixer.

If you are using the Universal qPCR Master Mix Kit and will only use ROX High or ROX Low, the appropriate ROX solution (50X) (0.2 mL) may be added to the qPCR Master Mix with primers when the kit is first opened. The volume of this mixture used per reaction should be adjusted accordingly (12.4 µL per 20 µL reaction or 6.2 µL per 10 µL reaction).

1.4 Record the lot numbers of all reagents, as well as the date on which the primers (and ROX) were added to the qPCR Master Mix. KAPA SYBR FAST qPCR Master Mixes with primers (and ROX) are stable through 30 freeze-thaw cycles, and should be stored protected from light at -20°C when not in use. Mixes may be stored in the dark at 4°C for up to one week, provided that they are not contaminated with microbes and/or nucleases during preparation or subsequent use in reaction setup.

Table 1. Recommended ROX concentrations for use with KAPA SYBR FAST Universal qPCR Master Mix

Instrument	ROX
Applied Biosystems® 5700, 7000, 7300, 7700, 7900HT, StepOne™, and StepOnePlus™	ROX High
Applied Biosystems® 7500, ViiA™7, QuantStudio™ 12K Flex, Agilent Mx3000P™, Mx3005P™, and Mx4000™	ROX Low
Rotor-Gene™, DNA Engine Opticon™, Opticon™ 2, Chromo 4™ Real-Time Detector, Mastercycler® ep realplex, Smart Cycler®, Roche LightCycler® 480, Roche LightCycler® Nano, Bio-Rad CFX96, and Illumina Eco™	No ROX

2. Sample Preparation

2.1 Prepare an initial 1:500 dilution of the purified library (using DNA dilution buffer).

2.2 The 1:500 dilution may be prepared as follows:

Component	Volume
Library dilution buffer	499 µL
Library DNA	1 µL
Total volume:	**500 µL**

2.3 Mix thoroughly by vortexing for 10 sec.

2.4 Prepare any additional library dilutions. Note: at least one additional 2-fold dilution of each library is recommended to ensure at least one dilution falls within the dynamic range of the standards.

2.5 Prepare the internal control dilutions (if applicable).

3. Reaction Setup and Cycling

3.1 Determine the total number of reactions that will be performed for the appropriate number of replicates of each of the following reactions:

- six DNA Standards
- each dilution of every library to be assayed
- each dilution of any internal controls
- no-template controls (NTCs)

3.2 Prepare the required volume of master mix using the reaction setup recommended below.

Reaction setup: 20 µL reactions

For Universal qPCR Master Mix	ROX	No ROX
KAPA SYBR FAST qPCR Master Mix (2X) + Primer Premix (10X)[1]	12.0 µL	12.0 µL
ROX High or Low (50X) (see Table 1)	0.4 µL	0 µL
PCR-grade water	3.6 µL	4.0 µL
Total volume:	**16.0 µL**	**16.0 µL**

[1]If ROX was added to the qPCR Master Mix and primers, use 12.4 µL of the qPCR Master Mix with Primer Premix and ROX per reaction.

For ABI Prism™, Bio-Rad iCycler™, LightCycler 480 or ROX Low qPCR Master Mix	
KAPA SYBR FAST qPCR Master Mix (2X) + Primer Premix (10X)	12.0 µL
PCR-grade water	4.0 µL
Total volume:	**16.0 µL**

Reaction setup: 10 µL reactions

For Universal qPCR Master Mix	ROX	No ROX
KAPA SYBR FAST qPCR Master Mix (2X) + Primer Premix (10X)[1]	6.0 µL	6.0 µL
50X ROX High or Low (see Table 1)	0.2 µL	0 µL
Total volume:	**6.2 µL**	**6.0 µL**

1If ROX was added to the qPCR Master Mix and primers, use 6.2 µL of the qPCR Master Mix with Primer Premix and ROX per reaction.

2The recommended reaction setup results in a total reaction volume of 10.2 µL if ROX is added during reaction setup. This does not impact performance.

For ABI Prism™, Bio-Rad iCycler™, LightCycler® 480 or ROX Low qPCR Master Mix	
KAPA SYBR FAST qPCR Master Mix (2X) + Primer Premix (10X)	6.0 µL
Total volume:	**6.0 µL**

3.3 Mix and briefly centrifuge the reagent master mix.

3.4 Dispense the appropriate volume of the master mix into each PCR tube or well.

3.5 Add 4 µL of PCR-grade water to all NTC tubes/wells.

3.6 Dispense 4 µL of each DNA Standard into the appropriate tubes/wells, working from the most dilute (Standard 6) to the most concentrated (Standard 1).

3.7 Dispense 4 µL of each dilution of libraries and internal controls to be assayed.

3.8 Cap tubes or seal the PCR plate, and transfer to the qPCR instrument.

3.9 Perform qPCR with the following cycling protocol, selecting the Absolute Quantification option in the instrument software. Adjust run parameters (e.g., reporters, reference dyes, gain settings, etc.) as required.

Step	Temp.	Duration	Cycles
Initial denaturation	95ºC	5 min	1
Denaturation	95ºC	30 sec	35
Annealing/Extension/ Data acquisition	60ºC	45 sec*	
Melt curve analysis	65 – 95°C		

*Optional; see **Important Parameters** for more details.

4. Data Analysis

In general, when standard libraries are quantified by qPCR, optimal DNA input for emPCR is 0.2 – 1 copies/bead, with the optimal input concentration varying from lab to lab according to sample type, library construction, etc. If the emPCR has been previously optimized using a different library quantification method and now qPCR is to be used, then it may be necessary to determine the optimal loading concentration (copies/bead) using qPCR-derived library concentrations. This can be accomplished empirically either via qPCR library quantification followed by emPCR titrations, or by using qPCR to retrospectively quantify a number of representative libraries that have already been used successfully in emPCR. For more information, please contact Technical Support at **kapabiosystems.com/support**.

4.1 Annotate the DNA Standards as outlined below. Note that the specified values correspond to the concentrations of the DNA Standards, and not the final DNA concentration in each reaction. It is not necessary to convert these to the actual concentrations in the reaction, as long as the same volume of template (DNA Standard, diluted library or internal control) is used in all reactions.

DNA Standard 1	83 pM
DNA Standard 2	8.3 pM
DNA Standard 3	0.83 pM
DNA Standard 4	0.083 pM
DNA Standard 5	0.0083 pM
DNA Standard 6	0.00083 pM

4.2 Review the background-subtracted (normalized) amplification curves and the Cq scores for replicate data points (DNA Standards, libraries and controls), and exclude obvious outliers. Replicate data points should differ by ≤0.2 cycles. If the data set contains many outliers, results are unlikely to be reliable. Repeat the assay with particular focus on improving pipetting accuracy.

4.3 Exclude all library dilutions that fall outside the dynamic range of the assay, i.e., that return an average Cq score lower than that of Standard 1 or higher than that of Standard 6. If all the dilutions of a library fall outside the standard curve, re-quantify a more appropriate dilution of the library.

4.4 Use the instrument software to generate the standard curve. The standard curve may also be generated manually using the **KAPA Library Quantification Data Analysis Template**.

4.5 Review the standard curve to ensure that the following criteria are met:

- The average ΔCq value between DNA Standards is in the range of 3.1 – 3.6.
- The calculated reaction efficiency is in the range of 90 – 110% (i.e., the PCR product has increased 1.8- to 2.2-fold per cycle, and the slope of the standard curve is between -3.1 and -3.6).
- R2 ≥0.99.

If the standard curve does not meet these criteria, calculated library concentrations will not be reliable, and the assay must be repeated.

4.6 Most qPCR software will calculate the concentration of the library and control dilutions using absolute quantification against the standard curve. However, we recommend exporting qPCR data to the **KAPA Library Quantification Data Analysis Template** to perform the following calculations to determine the undiluted library concentration:

- Use the standard curve to convert the average Cq score for each dilution of every library and internal control that was assayed to average concentration (in pM).
- Calculate the average size-adjusted concentration (in pM) for each dilution of every library and control that was assayed, by multiplying the calculated average concentration with the following factor:

$$\frac{\text{Size of DNA Standard in bp (153)}}{\text{Average fragment length of library in bp}}$$

- Multiply the average size-adjusted concentration calculated for each dilution of every library or control that was assayed with the appropriate dilution factor to calculate final concentration for the undiluted library or control from each of the dilutions assayed.

4.7 Review the final calculated concentrations and determine the working concentration for each sample to be used for downstream emPCR.

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