INSTRUCTIONS FOR USE OF

KAPA Library Quantification Kit

For Research Use Only. Not for use in diagnostic procedures.

June 2026, Version 14

Contents

The KAPA Library Quantification Kits contain:

Description	Kit Material Number
Complete Kits with: Primer Mix (1 mL) KAPA SYBR FAST qPCR Master Mix (5 mL)* DNA Standards 1 – 6 (80 µL each)	**KK4824** – 07960140001 Universal qPCR Master Mix 200 µL ROX High (50X) and ROX Low (50X) supplied in separate tubes
	KK4835 – 07960204001 ABI Prism™ qPCR Master Mix
	KK4844 – 07960255001 Bio-Rad iCycler™ qPCR Master Mix
	KK4873 – 07960336001 ROX Low qPCR Master Mix
	KK4854 – 07960298001 qPCR Master Mix optimized for LightCycler® 480 and PRO
Kits with: Primer Mix (1 mL) KAPA SYBR FAST qPCR Master Mix (5 mL)*	**KK4923** – 07960441001 Universal qPCR Master Mix 200 µL ROX High (50X) and ROX Low (50X) supplied in separate tubes
	KK4953 – 07960573001 qPCR Master Mix optimized for LightCycler 480 and PRO
Kits with: DNA Standards (80 µL)	**KK4903** – 07960387001 DNA Standards 1 – 6 **KK4905** – 07960409001 DNA Standards 0 – 6
Kit with: Dilution Control (80 µL)	**KK4906** – 07960417001 DNA Standard 0

*Kits with passive reference dye incorporated in the qPCR mix: ABI Prism kits (ROX High), ROX Low kits (ROX Low), Bio-Rad iCycler kits (fluorescein).

Shipping, Storage and Stability

- KAPA Library Quantification Kits are stable to be shipped at temperatures > -20°C and ≤15°C. Shipments may include the use of ice packs or dry ice, depending on the destination country. The shipment conditions might differ from the storage conditions, please check the label on the kit and store accordingly.
- Store the kit components at -15°C to -25°C in a constant-temperature freezer upon receipt.
- When stored under these conditions and handled correctly, the kit components will retain full activity until the expiry date indicated on the kit label.
- Always ensure that components have been 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 passive reference 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 KAPA Library Quantification Kits, as well as the combined KAPA SYBR FAST/Primer Premix solution, are stable through 30 freeze-thaw cycles.
- Ensure that all reagents are stored protected from light at -15°C to -25°C when not in use. When protected from light, reagents are stable in the dark at +2°C to +8°C, and may be stored at this temperature for short-term use, provided that they do not become contaminated with microbes and/or nucleases.

Application

KAPA Library Quantification Kits 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 in the Primer Premix (10X) 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.
- 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.

Warnings and Precautions

- Wear the appropriate personal protective equipment, such as gloves and safety glasses, to avoid direct contact while handling the reagents.
- Use good laboratory practices to avoid contamination when working with the reagents.
- In the event of a spill, clean up the solution with absorbent pads, allow pads to dry, and dispose of pads. Observe all national, regional, and local regulations for waste disposal and management.
- Do not eat, drink, or smoke in the laboratory area.
- Do not pipette by mouth.
- Observe all national, regional, and local regulations for waste disposal and management.
- Safety Data Sheets (SDS) are available ***online*** or upon request from the local Roche office.

Ordering Information

For a complete overview of Roche Sequencing products, including KAPA Library Quantification Kits, go to **diagnostics.roche.com**.

Trademarks

Contact and Support

If you have questions, contact your local Roche Technical Support. Go to **go.roche.com/sequencing.support** for contact information.

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Editions

Version 7.16, Oct 2016; Version 8.17, Jan 2017; Version 9.17, Sept 2017; Version 10.20, Mar 2020; Version 11.20, Dec 2020; Version 12.23, June 2023; Version 13, May 2025; Version 14, June 2026

Changes to Previous Versions

Addition of reference to LightCycler® PRO including settings and guidance using the LightCycler® PRO with KAPA Library Quantification Kit.

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Preface

Regulatory Disclaimer

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Contact Information

Technical Support

If you have questions, contact your local Roche Technical Support.

Go to *sequencing.roche.com/support* for contact information.

Manufacturing & Distribution

Manufacturer	Roche Diagnostics Cape Town, South Africa
Distribution	Roche Diagnostics GmbH Mannheim, Germany
Distribution in USA	Roche Diagnostics Corporation Indianapolis, IN USA

Conventions used in this manual

Symbols

Symbols	Description
	Important Note: Information critical to the success of the procedure or use of the product. Failure to follow these instructions could result in compromised data.
	Information Note: Designates a note that provides additional information concerning the current topic or procedure.

Text

Conventions	Description
Numbered listing	Indicates steps in a procedure that must be performed in the order listed.
Italic type, blue	Highlights a resource in a different area of this manual or on a web site.
Italic type	Identifies the external general resources or names.
Bold type	Identifies names of paragraphs, sections or emphasized words.

Chapter 1. Before You Begin

These Instructions for Use describe the product information and a detailed protocol (Figure 1) for the KAPA Library Quantification Kits.

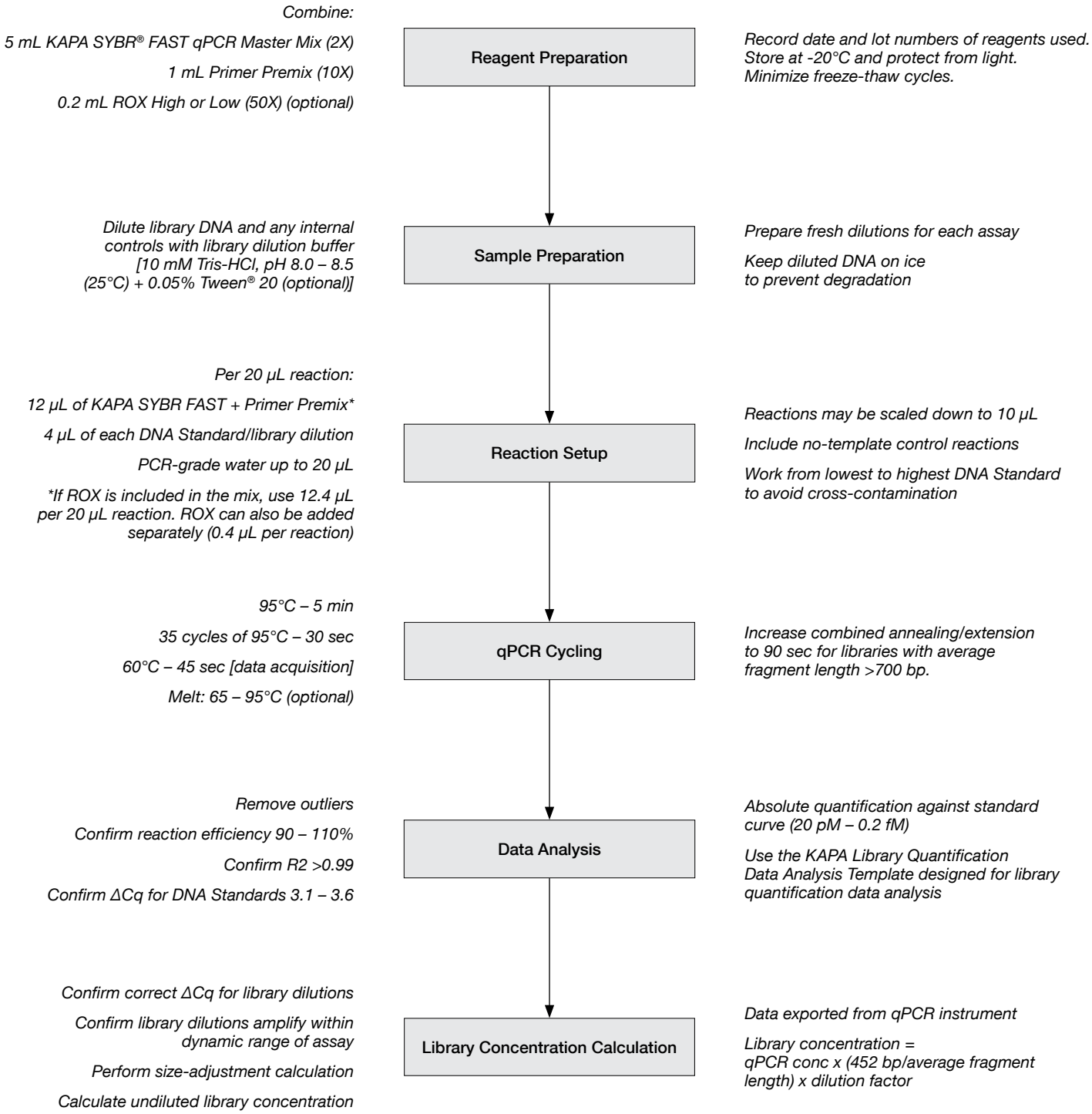
KAPA Library Quantification Kits provide:

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 (Table 1).

Figure 1 - KAPA Library Quantification Kit Quick Guide



Protocol Information & Safety

- Wear gloves and take precautions to avoid sample contamination.
- Perform all centrifugations at room temperature (+15°C to +25°C).
- Unless otherwise specified, all mixing steps are listed as ‘mix thoroughly’ and indicate that mixing should be performed by vortexing for at least 10 seconds. Mixing should be optimized for automation as applicable.
- If liquid has collected in a tube’s cap after mixing, gently tap or briefly centrifuge the sample to collect the liquid into the tube’s bottom, ensuring that the mixture remains homogeneous before progressing to the next step.

References

- Real-time thermocycler manual

Required Equipment, Labware & Consumables

The following steps should be taken before beginning the workflow:

Verify you are using the most up-to-date version of these Instructions for Use, go to **go.roche.com/sequencing.support**.

You assume full responsibility when using the equipment, labware, and consumables described below. This protocol is designed for use with the specified equipment, labware, and consumables.

Laboratory Equipment

Equipment	Supplier
Microcentrifuge (16,000 x *g* capability)	Multiple Vendors
Thermocycler	Multiple Vendors
Vortex mixer	Multiple Vendors
Plate Centrifuge (minimum 280 x *g* capability)	Multiple Vendors

Consumables Purchased from Other Vendors

Component	Supplier
10 mM Tris-HCl, pH 8.0 – 8.5	Multiple Vendors
Low binding Tubes: 0.2 mL PCR tubes 1.5 mL microcentrifuge tubes (optional)	Multiple Vendors
Nuclease-free, PCR-grade water	Multiple Vendors

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

Instrument	ROX
Applied Biosystems® 7300, 7900HT, StepOne™, and StepOnePlus™ instruments	ROX High
Applied Biosystems 7500, ViiA™7, and QuantStudio™ series of instruments, Agilent Mx3000P™, Mx3005P™, and Mx4000™	ROX Low
Qiagen Rotor-Gene™, Bio-Rad DNA Engine Opticon™, Chromo 4™ Real-Time PCR System, Mastercycler® ep *realplex*, Roche LightCycler® 480, Roche LightCycler® PRO, Bio-Rad CFX96, and Illumina Eco™ instruments	No ROX

Chapter 2.

Store and Prepare the Reagents

Step 1. Store the Reagent Kits

Reagent Kit	Storage Temperature
KAPA Library Quantification Kit	-15°C to -25°C

Step 2. Reagent Preparation

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.

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 a 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).

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 ≤1 week, provided that they are not contaminated with microbes and/or nucleases during preparation or subsequent use in reaction setup.

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

Chapter 3.

Library Quantification

In this chapter the KAPA Library Quantification Kit is used to quantify adapter-ligated, indexed libraries. The workflow requires the use of components from the following kits:

- KAPA Library Quantification Kit

Ensure that the following are available:

- Nuclease-free, PCR-grade water
- 10 mM Tris-HCl, pH 8.0 – 8.5

Sample Requirements

Sample Concentration and Dilutions

Libraries and controls must be diluted to fall within the dynamic range of the assay, i.e., 20 – 0.0002 pM or 5.5 – 0.000055 pg/µL or 12 x 10^6 – 12 x 10^1 dsDNA molecules/µL. 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™ Spectrophotometer, Qubit® assays or Agilent Bioanalyzer instrument). Refer to ***Chapter 4 - Data Analysis*** for more information.

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 or cold blocks 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 4°C) prior to setting up qPCRs. Samples diluted in a buffered solution should be stable for up to 4 h if stored at 4°C. 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. NTC qPCRs should return Cq scores that are at least 3 cycles later than the average Cq score for Standard 6.

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. This is due to primer design, which is not optimal for qPCR, but dictated by the Illumina flow cell oligo sequences. 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.

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 as in Working Example. However, ΔCq values for serial dilutions of a library do not provide any indication of the accuracy of the initial dilution.

For this reason, we recommend including at least one appropriate internal process or dilution control in every assay.

These include:

- KAPA Library Quantification Dilution Control (07960417001). This is a quality-controlled 200 pM solution of the same linear, 452 bp dsDNA fragment comprising the DNA Standards and is also referred to as DNA Standard 0.
- 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 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. Each of the internal controls listed above have advantages and disadvantages:

- The KAPA Library Quantification Dilution Control (DNA 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, however, DNA Standard 0 is more dilute than most Illumina libraries. If DNA Standard 0 is diluted to the same extent as the samples to be assayed, the Cq scores 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. Please note that the KAPA Library Quantification Dilution Control (DNA Standard 0) is not suitable as a sequencing control, as it is a homogenous solution of a single species of dsDNA and not a library.
- An existing, previously sequenced library is a valuable internal control, as both qPCR-based concentration and cluster density 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 and sequenced libraries, which have been stored in a buffered solution at -15°C to -25°C, and have not been subjected to too many freeze-thaw cycles. Single-use aliquots of libraries can be prepared and stored at -15°C to -25°C for use as controls.
- The use of PhiX as an internal library quantification control has similar advantages as that of 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.

Important Parameters

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, 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.

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 with Primer Premix. 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.

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 your 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. For more information, please contact Technical Support at go.roche.com/sequencing.support.

Library Quantification

1. Prepare the appropriate library dilutions (using DNA dilution buffer). Depending on the expected concentration of the library, 1:1,000 – 1:100,000 dilutions may be appropriate. At least one additional 2-fold dilution of each library is recommended.
2. Prepare the required internal control dilutions.
3. 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 every internal control
   - No-template controls (NTCs)
4. 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, LightCycler® PRO 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[2]:	**6.2 µL**	**6.0 µL**

[1] If 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.

[2] The 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, LightCycler® PRO or ROX Low qPCR Master Mix	
KAPA SYBR FAST qPCR Master Mix (2X) + Primer Premix (10X)	6.0 µL
Total volume:	**6.0 µL**

5. Mix and briefly centrifuge the reagent master mix.
6. Dispense the appropriate volume of the master mix into each PCR tube or well.
7. Add 4 µL of PCR-grade water to all NTC well/tube(s).
8. Dispense 4 µL of each DNA Standard into the appropriate well/tube(s), working from the most dilute (Standard 6) to the most concentrated (Standard 1).
9. Dispense 4 µL of each dilution of libraries and internal controls to be assayed.
10. Cap tubes or seal the PCR plate, and transfer to the qPCR instrument.
11. Perform qPCR with the following cycling protocol, selecting the Absolute Quantification option in the instrument software if required by the specific instrument. Adjust run parameters (e.g., reporters, reference dyes, gain settings, etc.) as required.

Step	Temperature	Duration	Cycles
Initial denaturation	95°C	5 min	1
Denaturation	95°C	30 sec	35
Annealing/Extension/ Data acquisition	60°C	45 sec[1]	
Melt curve analysis[2]	65 – 95°C		

[1]Increase to 90 sec for long-insert libraries (>700 bp).
[2]Optional; see ***Chapter 4 - Data Analysis*** for more details.

Chapter 4.

Data Analysis

Important Considerations for Data Analysis and Interpretation

Early Amplification of DNA Standard 1

The concentration of DNA Standard 1 is much higher than that of samples that are typically analyzed by qPCR. As a result, DNA Standard 1 is amplified at a very early Cq. Most qPCR instruments 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 result in significant problems with the standard curve, since Standard 1 can be interpreted purely as background or its Cq score can be somewhat delayed. To avoid problems with DNA Standard 1, the cycles used for baseline/background subtraction should be manually adjusted to cycles 1 – 3.

Size-adjustment Calculation

Size-adjustment must be performed to compensate for the difference in average fragment length between the library that was assayed and the DNA Standard. The fluorescent signal generated by SYBR® Green I is dependent on the total mass of DNA. Therefore, a longer amplicon at a lower concentration may produce the same amount of signal as a shorter amplicon at a higher concentration.

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 (see Working Example).

Melt Curve Analysis

Melt curve analysis may be useful for the identification of carry-over adapter-dimer in Illumina libraries (Figure 2) and/or to identify contamination. The melt curves for the KAPA Library Quantification DNA Standard for Illumina platforms displays a characteristic double peak. This is the result of differential local melting in the 452 bp linear template and is not indicative of non-specific amplification.

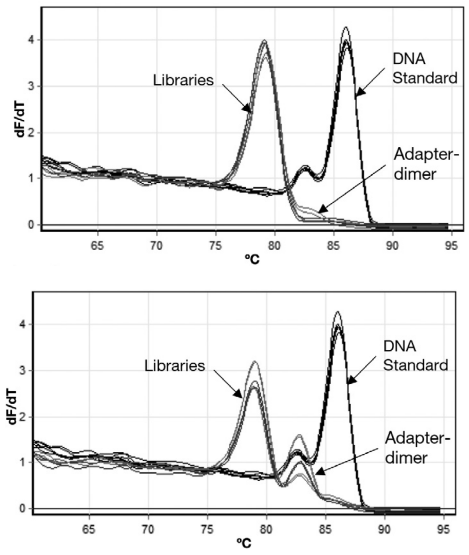


Figure 2. Melt curve analysis of libraries and DNA Standards amplified with the KAPA Library Quantification Kit. Libraries in the top panel contain no or a negligible amount of adapter-dimer. Libraries in the bottom panel contain an unacceptable amount of adapter-dimer, contributing to an inflated calculated library concentration. Note the characteristic double melt peak for the DNA Standard.

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 environments. The assay is capable of yielding reproducible, accurate, and reliable results. The guidelines and instructions provided in this document, particularly in **Data Analysis** and **Working Example**, 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 the result generated with the assay is determined to be reliable, there is no guarantee that libraries diluted for flow cell loading will yield an optimal number of clusters. Reasons for possible discrepancies between calculated library concentration and cluster density include:

- There are multiple liquid handling steps between library quantification and flow cell loading, all of which are prone to error.
- 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 quantified with high efficiency, irrespective of fragment length or GC content. Cluster amplification 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 libraries of the same type do not yield the same number of clusters on different instruments of the same type when loaded at a specific concentration. Reasons for this fall outside the scope of this document. The SeqAnswers blog is an excellent resource 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 workflows. To discover and define this correlation, it is important that the assay is performed meticulously and that data analysis and interpretation is performed correctly. If you are new to NGS and qPCR-based quantification, the following may be helpful:

- Assay different dilutions of a few libraries (preferably previously sequenced) on different days, to measure the reproducibility of qPCR data 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. 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.

Comparison with Other Quantification Methods

Most NGS workflows employ more than one library quantification method. Data generated with a NanoDrop™ spectrophotometer, a fluorometric (e.g., Qubit® or PicoGreen®) assay, or an electrophoretic device (e.g., a Agilent Bioanalyzer, TapeStation or Revvity LabChip® GX instrument) may be used to determine the most appropriate initial dilutions for accurate, qPCR-based quantification using the KAPA Library Quantification Kit. Theoretically, library concentrations determined with the Library Quantification Kit should always be similar to or lower than concentrations determined with spectrophotometric methods, which quantify total DNA, since qPCR measures only those molecules that can be amplified in PCR. However, when quantifying very concentrated or overamplified libraries, this may not be the case. In over-amplified libraries, a large proportion of the DNA may be partially single-stranded due to substrate depletion and subsequent formation of heteroduplex molecules, which leads to severe underestimation of library concentrations when dsDNA-binding dyes are used. Conversely, library quantification by qPCR, involves denaturation denaturation of the entire library to single-stranded form, allowing accurate quantification of all PCR-competent molecules.

Data Analysis

For analysis guidance on the Roche LightCycler® PRO, see *Appendix A* for details.

Step 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	Concentration
DNA Standard 1	20 pM
DNA Standard 2	2 pM
DNA Standard 3	0.2 pM
DNA Standard 4	0.02 pM
DNA Standard 5	0.002 pM
DNA Standard 6	0.0002 pM

Step 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.

Step 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.

Step 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** available from your local Roche Technical Support. Go to go.roche.com/sequencing.support for contact information.

Step 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).
- R^2 ≥0.99.

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

Step 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:
  - Size of DNA Standard in bp (452)
  - 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.

Review the final calculated concentrations and determine the working concentration for each sample to be used for downstream processing (e.g., pooling for target enrichment or cluster amplification).

Working Example

Three indexed DNA libraries were prepared from 250 ng of Covaris-sheared human genomic DNA using the KAPA EvoPrep Boost Kit, for 2 x 150 bp paired-end whole-genome sequencing on the Illumina NovaSeq 6000 system. Adapter-ligated libraries were size-selected (250 – 450 bp) and amplified (6 cycles). Amplified libraries were analyzed using an Agilent Bioanalyzer High Sensitivity DNA Assay to determine the average fragment size and approximate concentration of each library (Table 2, rows 1 and 2).

An initial 1:10,000 dilution and one additional 2-fold (i.e., 1:20,000) dilution of each library was prepared. As a process control, 1:10,000 and 1:20,000 dilutions of the KAPA Library Quantification Internal Control (Illumina DNA Standard 0, 200 pM) were also made. Samples were assayed using the KAPA Library Quantification Kit. All DNA Standards and library dilutions were assayed in triplicate, and triplicate NTCs were included.

The triplicate and average Cq scores for the six DNA Standards and NTCs are given in the following table.

DNA Standard	Concentration (pM)	Cq score	Average Cq	ΔCq*
1	20	7.15	7.20	–
		7.22		
		7.23		
2	2	10.63	10.66	3.46
		~~10.90~~		
		10.69		
3	0.2	14.13	14.13	3.47
		14.12		
		14.13		
4	0.02	17.72	17.68	3.55
		17.65		
		17.66		
5	0.002	21.01	21.08	3.40
		21.14		
		21.08		
6	0.0002	~~25.00~~	24.44	3.36
		24.45		
		24.42		
NTC	N/A	34.5	>34	N/A
		ND		
		33.9		

*Should be 3.1 – 3.6 for a 10-fold template dilution series.

After excluding outliers (>0.2 Cq difference) for DNA Standards 2 and 6, the standard curve was generated (Figure 3). The quality control metrics for the standard curve were as follows:

- ΔCq for all pairs of DNA Standards was within the specified range of 3.1 – 3.6.
- The reaction efficiency of 95% was within the specified range of 90 – 110%.
- The R^2 value of 0.9999 met the specification of ≥0.99.

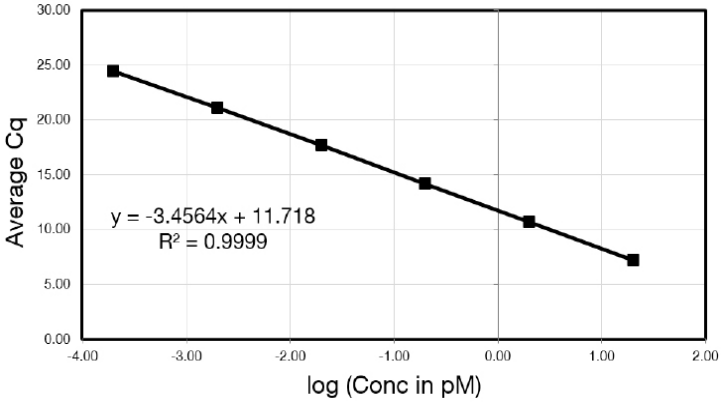


Figure 3. Standard curve generated with the KAPA Library Quantification Kit in this working example

Triplicate Cq scores for each dilution of the three libraries and internal control are given in Table 2, row 4. Outliers (>0.2 Cq difference) for the 1:20,000 dilution of Library 1 and the 1:10,000 dilution of Library 3 were excluded before the average Cq score (row 5) was calculated for each sample.

For an indication of the reliability of concentrations to be calculated, the ΔCq for the two dilutions of each library and the internal control was calculated. For consecutive 2-fold dilutions of a sample, a ΔCq of 1.0 is expected, and values between 0.9 and 1.1 are acceptable. This indicates that the concentration of a library calculated from two or more dilutions is likely to differ by <10%. If the ΔCq values for consecutive dilutions of a sample fall outside the acceptable range, indications are that quantification may not be reliable, as a result of poor liquid handling and/or sample contamination.

Next, the average Cq score for each library/control dilution was converted to concentration (in pM) using the standard curve (Table 2, row 7). The average size-adjusted concentration of each library dilution was subsequently calculated (as outlined in Detailed Protocol (step 4.6); Table 2, row 8). Finally, the average final concentration (in nM) of each undiluted library and the control was calculated from each of the dilutions that were assayed (Table 2, row 9). The calculated concentration of the internal control (205.4 pM and 207.8 pM, from the 1:10,000 and 1:20,000 dilutions, respectively) was within 10% of the given value, indicating that no gross errors occurred during sample dilution, or setup and execution of the assay.

To calculate the working concentration of each library, the variation between the two different dilutions was determined. Since calculated concentrations varied by <10% for all libraries, the average value of the two dilutions was selected as the working concentration. If the values differ by >10%, working concentrations should be based on one of the following:

- The average value from dilutions that returned final calculated concentration values that varied <10%.
- The final calculated concentration for the lowest dilution of each library that appears to be reliable.

Table 2. Working example of qPCR-based quantification of libraries prepared for Illumina sequencing during sample dilution, or setup and execution of the assay.

Row	Parameter	Library 1		Library 2		Library 3		Std 0 control	
1	Average fragment length (electrophoretic)	340 bp		335 bp		351 bp		452 bp	
2	Estimated concentration (other method)	17 ng/µL = 80.9 nM		18 ng/µL = 87.0 nM		27 ng/µL = 124.5 nM		200 pM (as supplied)	
3	Dilutions for qPCR	1:10K	1:20K	1:10K	1:20K	1:10K	1:20K	1:10K	1:20K
4	Triplicate Cq scores	9.24 9.25 9.21	10.21 10.12 ~~10.53~~	8.79 8.84 8.88	9.79 9.81 9.94	8.30 ~~8.65~~ 8.34	9.40 9.22 9.26	17.50 17.58 17.57	18.53 18.62 18.57
5	Average Cq score	9.23	10.17	8.84	9.85	8.32	9.29	17.55	18.57
6	ΔCq	0.93		1.01		0.97		1.02	
7	Average concentration for sample dilution calculated using standard curve (pM)	5.23	2.81	6.82	3.48	9.62	5.03	0.021	0.010
8	Average size-adjusted concentration for library dilution (pM)	6.96	3.74	9.20	4.69	12.39	6.48	0.021	0.010
9	Average final calculated concentration of undiluted library dilution (nM)	69.6	74.8	92.0	93.9	123.9	129.5	0.205	0.208
10	Deviation between final concentrations calculated from different dilutions	7.5%		2.1%		4.6%		1.2%	
11	Working concentration	72.2 nM = 15.2 ng/µL		92.9 nM = 19.2 ng/µL		126.7 nM = 27.5 ng/µL		(206.6 pM)	

Appendices

Appendix A. Data and melt curve analysis settings for LightCycler® PRO development software

This guidance is based on LightCycler® PRO development software v1.3.0. Certain features are not available on previous versions of the software.

Define the appropriate analysis settings for absolute quantification by doing the following:

To create a new single plate analysis, continue to Step 1. Or, to create a new sample group, for partial plate analysis or multi-plate analysis, continue to Step 2. Or, if a sample group already exists, continue to Step 3

Step 1: Create a new analysis.

a. Choose the **Projects** app.

b. At the end of the project row, choose the > button of the project for which you want to create a sample group.

c. Select the plate to perform the analysis on by choosing the tick box.

d. On the **Perform single-plate analysis** panel, choose the **Create** button.

e. In the **Add analysis** panel, choose the **New analysis** option.

f. From the **Define analysis type** group box, choose **Absolute quantification**.

g. Activate the SYBR™ Green 1 channel.

h. Choose the **Add analysis** button and proceed to *Step 4*.

Step 2 : Create a new sample group and a new analysis.

a. Choose the **Projects** app.

b. At the end of the project row, choose the > button of the project for which you want to create a sample group.

c. On the **Manage sample groups and existing analyses** panel, choose the **Create** button.

d. In the **Create sample group** dialog box, enter the sample group name.

e. Choose the **Create** button.

f. On the **Choose run or plate** panel, choose the appropriate runs or plates you want to use.

g. Choose the **Proceed** button.

h. Choose the wells on the plate setup you want to use for the analysis.

i. Choose the **Assign** button.

j. If you selected more than 1 plate, navigate between these plates by choosing **Run ID** or **Plate ID** from the drop-down list above the plate setup.

k. Repeat steps (h) to (i) until all required wells are assigned to the sample group.

l. Choose the **Create** button.

m. The **Add Analysis** dialog box is displayed. You can either proceed to perform the analysis, or choose the **Cancel** button to add the analysis later.

n. If a current analysis has been created for the sample group, choose the ⊕ button left of the current analysis tab to create a new analysis.

o. In the **Add analysis** panel, choose the **New analysis** option.

p. From the **Define analysis type** group box, and choose **Absolute quantification**.

q. Activate the **SYBR™ Green 1** channel.

r. Choose the **Add analysis** button and proceed to *Step 4*.

Step 3: Create a new analysis for an existing sample group.

a. In the **Projects** app, choose the > button of the project which includes the runs to which you want to add the analysis.

b. On the **Manage sample groups and existing analyses** panel, choose the **View sample group and analyses** button.

c. Choose the > button at the end of the sample group row.

d. If there is no current analysis created for the sample group, choose either **Add analysis** or the ⊕ button, top left, next to the sample group name.

e. If a current analysis has been created for the sample group, choose the ⊕ button left of the current analysis tab to create a new analysis.

f. In the Add analysis panel, choose the **New analysis** option.

g. From the **Define analysis type** group box, and choose **Absolute quantification**.

h. Activate the **SYBR™ Green 1** channel.

i. Choose the **Add analysis** button and proceed to *Step 4*.

The software will proceed to analyze the data using the default settings. This may take some time. You must edit the parameters and recalculate your results (Step 4).

Step 4: Define parameters.

a. Choose the **Define parameters** button.

b. Set the following parameters.

Target definition subtab	Channel selection: SYBR™ Green I; Activate **Color compensation** and deactivate the **Internal control** toggle button.

Targets Subtab	SYBR™ Green I		
Define the following:	**Cq threshold**	Lower Cq limit: **5**	Upper Cq limit: **30**
		Advanced settings: **On**	
	Limit of validity	Limit of validity: **0**	
	Positive/negative calling	Lower EPF limit: **0.2**	Minimum slope: **0.02**
		Lower titer limit: **0.0002**	Upper titer limit: **20**
	Cq calling	Choose **2nd derivative PRO** (**only** available in software versions 1.3.0 and above)	

c. **Save** the chosen parameters before proceeding to define the parameters for the **Controls**.

Step 5: Controls

a. Set the following parameters for the controls.

Controls subtab	Keep the default SYBR™ Green I. If SYBR™ Green I is not chosen, edit and choose SYBR™ Green I.

Standards subtab	Select **cp/ml** (copies per mL). Select the target **SYBR Green I** to edit the settings.		
	In-run standard		
	SYBR™ Green I (should already be chosen).		
	Cq limits		
	Lower Cq limit: **5**	Upper Cq limit: **30**	
	Curve fitting method		
	Select Linear		
	Acceptance criteria		
	Efficiency higher than **90**	Efficiency lower than **110**	Standard error lower than **1**
	Keep off the toggle for **Turn off internal control check for positive targets**		
	Update the **List of standards**. In the **List of standards**, choose the icon to modify standard concentration, as necessary: • Assign the appropriate titer to each of the KAPA DNA Standards 1 - 6 (20 pM to 0.0002 pM). • Ensure SYBR™ Green I is chosen as a target from the dropdown menu. • Choose the 💾 icon to save the changes.		

b. **Save** the chosen parameters at the bottom of the page before proceeding back to the main Analysis page.

Step 6: Calculate results

a. Return to the main screen.

b. Choose the **Calculate results** button. The results are calculated according to the defined settings.

Step 7: Melt curve analysis

In LightCycler PRO Development Software v1.3.0, the melt curves for the KAPA DNA Standards, if assigned as quantitation standards, are not visible. To visualize the melt curves for the KAPA DNA Standards, the supported workaround is to relabel these samples as unknown.

a. Choose the ⊕ icon left of the current analysis tab.

b. In the **Add analysis** panel, choose the **New analysis** option.

c. Enter the **Analysis name**.

d. **Define the analysis type**. Ensure **TM calling** is chosen.

e. Define the **SYBR™ Green 1 channel**.

f. **Add analysis** and proceed to the next step.

g. Choose the **Define parameters** button.

h. On the **Channel parameters** subtab, in the **Define curve parameters for a target** panel, modify the following parameters:

- **Lower temperature limit (°C)** : 65 °C
- **Upper temperature limit (°C)** : 95 °C
- **Max. No. of peaks** - 2
- **Shoulders** - toggled off
- **Advanced settings** - toggled off

i. Choose **Add target** and define the following two targets:

- Target name 1: NGS Library
  - ☐ Lower Tm limit (°C) : 75 °C
  - ☐ Upper Tm limit (°C) : 80 °C

- Target name 2: DNA Standard
  - ☐ Lower Tm limit (°C) : 85 °C
  - ☐ Upper Tm limit (°C) : 90 °C

j. When all required changes have been made, choose **Save.**

k. On the Controls subtab, choose the ⚙ icon to open the **Add control** dialog box. Choose both targets, NGS Library and DNA Standard.

l. Choose the **Update** button.

m. Choose the **Calculate results** button.

Appendix B. Troubleshooting

This appendix provides guidance for interpreting unexpected results and recommendations for implementing corrective action if problems occur. For technical questions, contact your local Roche Technical Support.
Go to **go.roche.com/sequencing.support** for contact information.

Symptom	Possible causes
Efficiency not in specified range (90 – 110%)	• Efficiency above 100% may be indicative of contamination, where Standard 6 is <3.1 cycles from Standard 5, and NTC reactions amplify less than 3 cycles from Standard 6. Examine melt curves to determine the source of contamination (Standard DNA or library DNA). • Baseline setting may delay Cq score for Standard 1, affecting efficiency. Adjust baseline manually. • Poor liquid handling.
Poor R^2 value (<0.99)	• Poor liquid handling. Ensure that all reagents are thoroughly mixed before use. • Instrument-related issues. Ensure that the correct reference dye was used, at the correct concentration.
Standards are not spaced correctly (3.1 – 3.6 cycles apart)	• ΔCq of <3.1 between DNA Standards 5 and 6 is indicative of contamination. Examine melt curves to determine whether contaminant is Standard or library DNA. • ΔCq of <3.1 between DNA Standards 1 and 2 may be indicative of problems with background subtraction. Adjust the baseline manually. • ΔCq of >3.6 points to poor reaction efficiency. Ensure that all reagents are thoroughly mixed before use. Confirm that all reaction components were added at the correct concentration, and that the correct cycling protocol was used. • Severe light exposure of KAPA SYBR® FAST qPCR Master Mix will reduce total fluorescence and may result in a delay of Cq scores, resulting in ΔCq values of >3.6.
Poor reproducibility between replicates	• Poor liquid handling. Ensure that all reagents are thoroughly mixed before use. • Instrument-related issues. Ensure that the correct reference dye was used, at the correct concentration.
ΔCq of library dilutions is not within expected range (0.9 – 1.1 for 2-fold dilutions)	• Poor liquid handling in preparation of library dilutions. • Library is challenging to amplify, i.e., extremely GC- or AT-rich, or has an average fragment length >1 kb. • Library DNA has degraded. Prepare fresh dilutions and keep on ice during reaction setup.
Concentrations calculated from different library dilutions differ by more than 10%	• Poor liquid handling in preparation of library dilutions. • Library is challenging to amplify, i.e., extremely GC- or AT-rich, or has an average fragment length >1 kb. • Library DNA has degraded. Prepare fresh dilutions and keep on ice during reaction setup.
Library dilutions do not fall within dynamic range of standard curve	• Dilutions that amplify before DNA Standard 1 should not be used. Use only those dilutions that fall within the range of the standard curve. If no such dilutions were assayed, repeat the assay with a more appropriate dilution factor. • Libraries that amplify after Standard 6 are unlikely to contain a significant amount of library DNA. Repeat the quantification to confirm the result.
Standard 1 amplification plot appears abnormal	• Instrument is subtracting early amplification as background. Adjust the baseline settings to use cycles before any signs of amplification (1 – 3).
Standards amplify, but libraries do not, or very late amplification of libraries	• Library does not contain the appropriate adapter sequences for quantification primers to bind to. • Gross error with initial dilution, or library DNA has degraded. Re-assay fresh dilutions.

Appendix C. Limited Warranty

1. Limited Warranty

A. Products: Roche Sequencing Solutions, Inc. (“Roche”) warrants that its Products conform to its published specifications and are free from defects in material or workmanship. Customer’s sole and exclusive remedy (and Roche’s sole and exclusive liability) under this limited warranty shall be to either (a) replace the defective Products, or (b) provide Customer with a refund, as solely determined by Roche.

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Evidence of original purchase is required. It is important to save your sales receipt or packaging slip to verify purchase.

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