



KAPA Library Quantification Kits

For Roche 454 GS FLX platform

1. Product Description

Accurate quantification of the number of amplifiable molecules in a library is critical to the outcome of sequencing results on the Roche 454 FLX next-generation sequencing platform. Overestimation of library concentration results in fewer DNA-bearing beads after emulsion PCR. Underestimation of library concentration results in multiple library molecules associated with the same bead within an emulsion. Both scenarios result in suboptimal sequencing capacity. qPCR is widely regarded as the gold standard for accurate quantification of DNA libraries as it is the only technique capable of measuring the number of amplifiable molecules. The broad dynamic range of qPCR also enables accurate quantification of extremely dilute libraries (e.g. clinical, ancient, forensic and environmental samples).

KAPA Library Quantification Kits comprise DNA Standards (six 10-fold dilutions) and 10X Primer Premix, paired with KAPA SYBR® FAST qPCR Kits to accurately quantify the number of amplifiable molecules in a 454 library. The 486 bp KAPA 454 GS FLX 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.

Accurate qPCR-based library quantification ultimately depends on three factors: (i) the accuracy and reproducibility of the standards used, (ii) the ability of the DNA polymerase used in the qPCR to amplify all adaptor-flanked molecules with equal efficiency, and (iii) accurate and reproducible liquid handling. KAPA Library Quantification Kits are rigorously tested to ensure minimal lot-to-lot variation. In addition, KAPA SYBR® FAST qPCR Kits are designed for high performance, high-throughput, real-time PCR. The kit contains a novel DNA polymerase engineered via molecular evolution, resulting in a unique enzyme optimized for qPCR using SYBR® Green I dye chemistry. KAPA SYBR® FAST qPCR Kits are ideally suited to library quantification applications, as they support high-efficiency amplification of both AT- and GC-rich targets, and of fragments up to 1 kb in length.

2. Applications

KAPA 454 GS FLX Library Quantification Kits are ideally suited for the quantification of libraries:

- constructed with 454 GS FLX adaptors containing the following qPCR primer sequences:

Primer FLX A: 5'-GCC TCC CTC GCG CCA-3'
Primer FLX B: 5'-GCC TTG CCA GCC CGC-3'
- with a broad range of concentrations.
- containing fragments with a wide range of GC contents.
- containing fragment lengths ranging from 50 bp to 1000 bp.

Kit code	Components
KK4820	KK4601: KAPA SYBR® FAST Universal qPCR Kit KK4800: 1 x 1 ml 454 FLX Primer Premix (10X) 6 x 80 µl 454 FLX DNA Standards
KK4830	KK4604: KAPA SYBR® FAST ABI Prism qPCR Kit KK4800: 1 x 1 ml 454 FLX Primer Premix (10X) 6 x 80 µl 454 FLX DNA Standards
KK4840	KK4607: KAPA SYBR® FAST Bio-Rad iCycler qPCR Kit KK4800: 1 x 1 ml 454 FLX Primer Premix (10X) 6 x 80 µl 454 FLX DNA Standards
KK4850	KK4610: KAPA SYBR® FAST LightCycler 480 qPCR Kit KK4800: 1 x 1 ml 454 FLX Primer Premix (10X) 6 x 80 µl 454 FLX DNA Standards

More information on KAPA SYBR® FAST qPCR kits may be found at <http://www.kapabiosystems.com/products/name/kapa-sybr-fast-qpcr-kits>.

For reordering of DNA Standards and Primer Premix only

KK4800	1 x 1 ml 454 FLX Primer Premix (10X) 6 x 80 µl 454 FLX DNA Standards
KK4801	1 x 1 ml 454 FLX Primer Premix (10X)

Storage, handling and specifications

Store all components at -20 °C. Please refer to Section 5 for full details.

Quick Notes

- Please confirm that the qPCR primer sequences provided on this page are compatible with the adaptor sequences used to construct the libraries to be quantified. **To our knowledge, the kit provided here can be used to quantify all 454 FLX libraries, as well as 454 Titanium amplicon libraries constructed with Lib-A adaptor sequences.**
- The first time you use the kit add 1 ml 454 FLX Primer Premix to the 5 ml bottle of KAPA SYBR® FAST qPCR Master Mix (2X) and mix by vortexing for 10 seconds. Record the date of Primer Premix addition on the KAPA SYBR® FAST qPCR Master Mix bottle. 12 µl of the KAPA SYBR® FAST/Primer Premix solution is used per 20 µl qPCR reaction.
- Use 4 µl of diluted library DNA or DNA Standard regardless of reaction volume.
- Ensure that the correct version of KAPA SYBR® FAST qPCR Master Mix is selected for the qPCR instrument being used. For details on qPCR instrument and reference dye compatibility, please refer to the KAPA SYBR® FAST Technical Data Sheet.
- For accurate results ensure that all components are thawed and mixed prior to use.
- Successful library quantification is highly dependent on the accurate dilution of library DNA. Always ensure that proper pipetting techniques are employed.
- The estimated time needed to complete this protocol is 2 h 30 min, but depends on the qPCR instrument used and may be as little as 2 h.



3. Workflow

Approximate duration

5 min

Prepare qPCR/Primer mix

Add 1 ml of 454 FLX Primer Premix (10X) to 5 ml bottle of KAPA SYBR® FAST qPCR Master Mix (2X) and mix well.

Indicate date of primer addition clearly on qPCR Master Mix bottle.

10 min

Make 1:500 dilution of dsDNA library

Dilute library DNA in 10 mM Tris-HCl, pH 8.0 + 0.05% Tween 20.
Optional additional dilutions: 1:1000, 1:2000 and 1:4000

15 min

Prepare qPCR plate

KAPA SYBR® FAST qPCR Master Mix containing Primer Premix	12 µl
PCR-grade water	4 µl
Diluted library DNA or DNA Standard (1 – 6)	4 µl
TOTAL	20 µl¹

¹It is possible to use 10 µl reaction volumes with appropriate plasticware and thermocyclers. In this case add 6 µl of qPCR/Primer mix and 4 µl of template per reaction.

1 h 30 min³

Run qPCR protocol

Initial activation / denaturation	95 °C	5 min	
Denaturation	95 °C	30 sec	35 cycles
Annealing / extension / data acquisition	60 °C	45 sec ²	

Note: Dissociation (melt curve) analysis is optional, and in certain circumstances may provide a useful indication of possible primer and/or adaptor dimer contamination of libraries. Please refer to FAQs for more information.

²While KAPA SYBR® FAST qPCR reagents are generally capable of extremely fast amplification, we conservatively recommend relatively long annealing/extension times for library quantification in order to accommodate the diversity of templates in a typical library sample. If average library fragment size is >700 bp, then increase annealing/extension time to 90 sec.

20 min

Analyze data

Confirm 90 – 110% reaction efficiency for standards.

Confirm 90 – 110% reaction efficiency for library (optional).

10 min

Calculate library concentration

Use absolute quantification, against the 486 bp DNA Standard provided.

2 h 30 min³

Perform serial dilutions of undiluted DNA library for entry into emPCR

³Depends on qPCR instrument used. qPCR run may be as short as 55 min.



4. Detailed protocol

General considerations:

- The first time you use the kit, add 1 ml Primer Premix (10X) to the 5 ml bottle of KAPA SYBR® FAST qPCR Master Mix (2X) and mix by vortexing for 10 sec. **Record the date of Primer Premix addition on the KAPA SYBR® FAST qPCR Master Mix bottle.**
- This protocol is designed for 20 µl qPCR reaction volumes. It is possible to use 10 µl reaction volumes with appropriate plasticware and thermocyclers. In this case add 6 µl of qPCR/Primer mix and 4 µl of template per reaction.
- Ensure that all components are completely thawed and thoroughly mixed prior to use.
- **Optional:** 10 mM Tris-HCl, pH 8.0 + 0.05% Tween 20 is recommended for the dilution of library DNA, as it leads to improved accuracy by reducing the adherence of DNA to plastic surfaces. This solution is not supplied in the kit.

Step 1: Library sample preparation. Perform an initial 1:500 dilution of the purified library in Library Dilution Buffer (10 mM Tris-HCl, pH 8.0 + 0.05% Tween 20; not supplied in the kit). Mix thoroughly by vortexing for 10 sec. The 1:500 dilution may be prepared as follows:

Library Dilution Buffer	499 µl
Library DNA	1 µl
Total	500 µl

- **Optional:** Determine the average size distribution and quality of the purified library DNA using an Agilent Bioanalyzer assay or equivalent method.
- **Optional:** Perform three additional 2-fold serial dilutions of the 1:500-diluted library DNA. For example, add 100 µl of 1:500-diluted library DNA to 100 µl Library Dilution Buffer to obtain a 1:1000 dilution. Mix thoroughly by vortexing for 10 sec. Continue the serial dilution to obtain 1:2000 and 1:4000 dilutions. **Note:** The additional dilutions are useful for high concentration libraries, as one of the four dilutions must fall within the dynamic range of the standards supplied in the KAPA Library Quantification Kit. The dilution series also supplies information about pipetting accuracy and/or library qPCR efficiency and can be used for troubleshooting.

Step 2: Experimental design. Six DNA Standards are provided in this kit. A set of DNA Standards should be included in triplicate in each qPCR plate. In addition to the DNA Standards, each library may require a total of at least 12 reactions (triplicate reactions for the 1:500 library dilution, as well as triplicate reactions for each of the optional 1:1000, 1:2000 and 1:4000 dilutions).

Step 3: qPCR reagents. Ensure that the following reagents are completely thawed and thoroughly mixed by vortexing.

- 2X KAPA SYBR® FAST qPCR Master Mix (5 ml), to which 1 ml of 10X 454 FLX Primer Premix has been added.
- Six DNA Standards (10-fold serial dilutions, supplied in the kit).
- 1:500 dilution of library DNA and optional 1:1000, 1:2000 and 1:4000 serial dilutions of the same.

Step 4: qPCR setup. qPCR reactions for the six standards and each library dilution should be set up in triplicate. Load each well of the qPCR plate as indicated below, for a total reaction volume of 20 µl. Ensure that the qPCR plate is sealed. Collect all components in the bottom of the wells by brief centrifugation.

KAPA SYBR® FAST qPCR Master Mix containing Primer Premix	12 µl
PCR-grade water	4 µl
Diluted library DNA or DNA Standard (1 – 6)	4 µl

Note: It is possible to use 10 µl reaction volumes with appropriate plasticware and thermocyclers. In this case add 6 µl of qPCR/Primer mix and 4 µl of template per reaction.



4. Detailed protocol (continued)

Step 5: qPCR cycling. Place the reactions in the real-time thermocycler, programmed with the following qPCR protocol:

Initial activation / denaturation	95 °C	5 min	
Denaturation	95 °C	30 sec	35 cycles
Annealing / extension / data acquisition	60 °C	45 sec*	

*While KAPA SYBR FAST qPCR reagents are generally capable of extremely fast amplification, we conservatively recommend relatively long annealing/extension times for library quantification in order to accommodate the diversity of templates in a typical library sample. If average library fragment size is >700 bp, then increase annealing/extension time to 90 sec.

Note: Dissociation (melt curve) analysis is optional, and in certain circumstances may provide a useful indication of possible primer- and/or adaptor-dimer contamination of libraries. Please refer to FAQs for more information.

Step 6: Analysis

6.1 Annotate the DNA standards as follows before analyzing the data according to the qPCR instrument guidelines:

Sample name ¹	ssDNA copies/μL ^{2,3}		dsDNA copies/μL ^{2,3}
Std 1	100,000,000	or	50,000,000
Std 2	10,000,000		5,000,000
Std 3	1,000,000		500,000
Std 4	100,000		50,000
Std 5	10,000		5,000
Std 6	1,000		500

¹Each standard should be assayed in triplicate, using 4 μl of the DNA Standard supplied in the kit per reaction.

²Enter the correct number of copies/μL in the standard quantity field. If you are quantifying a dsDNA library, the dsDNA concentration of each DNA Standard must be used in calculations.

³**Note:** The concentrations provided here are for the DNA Standards as supplied in the kit, and are NOT the concentrations in the reactions. Provided that the volume of template added to each reaction is the same for Standards and for library samples (i.e. 4 μl in each case), there is no need to account for these volumes when calculating the concentrations of library samples, nor should one need to calculate the concentration of template in the reaction.

6.2 Confirm that the reaction efficiency calculated for the DNA Standard dilution series falls within the range of 90 – 110%.

6.3 **Optional:** confirm that the reaction efficiency calculated for the 2-fold library DNA dilution series (if used) falls within the range of 90 – 110%.

Successive 2-fold dilutions of a library sample should have Ct values evenly spaced approximately one cycle apart. Significant deviations from the expected spacing may indicate poor pipetting accuracy, poor amplification efficiency, or high tube-to-tube variance in your qPCR instrument and/or plasticware. Pay particular attention to the spacing between Standards 1 and 2 and between successive library dilutions in this concentration range, and examine these amplification plots to ensure that early amplification has not interfered with automatic baseline determination/subtraction on your instrument. Please refer to your instrument-specific instructions and the KAPA Library Quantification Kit FAQs for more information on this issue.

Note: We strongly recommend 2-fold library dilutions, as these data provide confirmation that the assay is functioning correctly, and can be useful for troubleshooting.



4. Detailed protocol (continued)

Step 6: Analysis (continued)

6.4 The concentration of each library is calculated as indicated in the example shown below:

- Obtain the calculated concentration of the 1:500 dilution of the library (and the calculated concentrations of the optional 1:1000, 1:2000 and 1:4000 dilutions), as determined by qPCR in relation to the concentrations of the correctly annotated DNA Standards 1– 6.
- Perform a size adjustment calculation to account for the difference in size between the average fragment length of the library and the DNA Standard (486 bp).
- Calculate the concentration of the undiluted library by taking account of the relevant dilution factor (500, 1000, 2000 or 4000).

Library dilution	Copies/μL calculated by qPCR instrument (triplicate data points)			Avg. copies/μl	Size adjusted copies/μl	Conc. of undiluted library stock (copies/μl)
	A1	A2	A3			
Library 1:500	A1	A2	A3	A	$A \times \frac{486}{\text{Avg. fragment length}} = W$	$W \times 500$
Library 1:1000	B1	B2	B3	B	$B \times \frac{486}{\text{Avg. fragment length}} = X$	$X \times 1000$
Library 1:2000	C1	C2	C3	C	$C \times \frac{486}{\text{Avg. fragment length}} = Y$	$Y \times 2000$
Library 1:4000	D1	D2	D3	D	$D \times \frac{486}{\text{Avg. fragment length}} = Z$	$Z \times 4000$

- Use the average of the triplicate data points corresponding to the most concentrated library DNA dilution that falls within the dynamic range of the DNA Standards to calculate the concentration of the undiluted library. If one of the three replicates appears to be an outlier, it may be omitted from the calculation. If more than one of the three replicates appear to be outliers, the assay should be repeated.
- Use the calculated concentration of the undiluted library to prepare an appropriate dilution of the library and proceed with emPCR.

Note: qPCR is likely to yield a lower value for the concentration of the undiluted library than would non-qPCR based methods.

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 you have previously optimized your emPCR using a different library quantification method and you are now switching over to qPCR, then you will need 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. Please refer to the FAQs for more information about these issues.



KAPA Library Quantification Kits

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5. Storage, handling and specifications

5.1 Shipping, storage and handling

KAPA Library Quantification Kits are shipped on dry ice or ice packs, depending on the country of destination. Upon receipt, store the entire kit at -20 °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.

Please refer to the KAPA SYBR® FAST qPCR Kit Technical Data Sheet for storage and handling of KAPA SYBR® FAST qPCR Master Mix (2X) and ROX dyes, if applicable.

Always ensure that all components are fully thawed and have been vortexed before use. Before first use of the kit, add 1 ml Primer Premix to the 5 ml bottle of KAPA SYBR® FAST qPCR Master Mix and mix by vortexing for 10 sec. Record the date of Primer Premix addition clearly on the KAPA SYBR® FAST qPCR Master Mix bottle.

All components of the KAPA Library Quantification Kits - as well as the combined KAPA SYBR® FAST/Primer Premix solution - are stable through more than 30 freeze/thaw cycles. We therefore recommend that all reagents are stored in the dark at -20 °C when not in use. Nevertheless, these reagents are stable in the dark at 4 °C for at least one week, and may be stored in this state 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.

5.2 Product use limitations and licenses

KAPA Library Quantification Kits are developed, designed and sold exclusively for research purposes and *in vitro* use. Neither the product, nor any individual component, has been tested for use in diagnostics or for drug development, nor is it suitable for administration to humans or animals. Please refer to the MSDS, which is available on request.

Please refer to the KAPA SYBR® FAST qPCR Kit Technical Data Sheet for further licensing information.

SYBR® is a registered trademark of Molecular Probes, Inc, Oregon. PRISM® is a registered trademark of Applied Biosystems Corporation. iCycler® is a registered trademark of Bio-Rad. LightCycler® is a registered trademark, and 454, GS Titanium, GS FLX and emPCR are trademarks of Roche.

For more detailed information and troubleshooting please refer to the Frequently Asked Questions (FAQs) supplied with the kit or visit www.kapabiosystems.com/products/name/kapa-library-quant-kits/faqs

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