KAPA Library Quantification Kits Frequently Asked Questions

Standard protocols for all three major commercial Next Generation Sequencing (NGS) platforms employ unreliable, laborious, and costly methods for quantifying library DNA molecules prior to clonal amplification of sequencing templates to form “polonies” (polymerase colonies). Accurate quantification of *bona fide* PCR-competent sequencing templates is crucial for reliable clonal amplification via bridge PCR (bPCR; “cluster amplification”) or emulsion PCR (emPCR) – underestimation results in non-clonality and/or over-clustering, while overestimation leads to poor yields of clusters or template-carrying beads.

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

In principle, quantitative PCR (qPCR) is inherently well-suited for NGS library quantification, and overcomes many of the difficulties presented by the standard approaches:

- qPCR specifically quantifies PCR-competent DNA molecules,
- is accurate across a very broad dynamic range,
- is amenable to automated liquid handling, and
- is cost-effective.

Moreover, because qPCR is extremely sensitive, it allows accurate quantification of very dilute libraries and consumes small amounts of sample. Because qPCR allows reliable cluster amplification or bead emPCR from dilute samples, less PCR amplification of libraries is required, and the biases and loss of complexity associated with library amplification can be minimized.

There are two important considerations for reliable qPCR quantification of library DNA:

i) Ideally, qPCR amplification should be efficient across the broad range of templates that constitute sequencing libraries. Traditional qPCR reagents are optimized for efficient amplification of short, “ideal” amplification targets. In contrast, factors such as target length, unbalanced GC-content, and problematic secondary structures may lead to low amplification efficiency resulting in unreliable quantification of an indeterminate proportion of library molecules.

ii) It is important to use reliable calibration standards that display minimal variability from lot-to-lot and over long periods of time.

To address these specific requirements of NGS library quantification, KAPA Library Quantification Kits comprise highly consistent sets of serially diluted DNA standards and state-of-the-art qPCR reagents, which include a DNA polymerase specifically engineered for robust, SYBR® Green I-tolerant amplification of long and difficult templates.

1. **Why is there a difference between the concentrations obtained using the qPCR-based KAPA Library Quantification Kit method, and the Agilent Bioanalyzer/Invitrogen Qubit/ spectrophotometry-based assay?**

   Many users of the qPCR-based KAPA Library Quantification Kits (LQK) notice significant differences between the concentrations determined for a given sample using qPCR versus other methods such as the Agilent Bioanalyzer. For many of the reasons stated below, we believe that qPCR quantification of libraries is the best approach for minimizing variability in cluster density or bead enrichment. In this respect, consider that the actual values obtained for the concentrations of libraries is ultimately irrelevant for the reliable generation of desirable cluster densities (or bead enrichments); rather, a reliable ***relative*** quantification of libraries with respect to one another, and in relation to their potential for producing clusters (or templated beads), is critical. While Bioanalyzer assays may remain a useful tool for assessing library quality (mainly with respect to size distribution), we do not believe that they provide the best method for quantification with the aim of predicting cluster density (or bead enrichment).

   Aside from errors in data collection and processing (i.e. qPCR instrument and software settings issues), or data analysis (i.e. calculations performed by the user), there are three obvious possible explanations for such differences:

   i) qPCR "counts" only those library molecules that are competent templates for PCR, and is therefore blind to all library molecules that cannot give rise to clusters during the bridge PCR process of cluster amplification (Illumina GA) or to beads carrying amplified sequencing template during emPCR (Roche 454 and Life Technologies SOLiD). We have therefore found that qPCR usually provides a lower estimate of library concentration than the less specific methods such as Agilent Bioanalyzer or dye-assisted spectrophotometry (e.g. Picogreen). DNA molecules in the sample may not support PCR for a number of reasons: a) they may not carry the appropriate adaptors at both ends; b) they may contain "difficult" sequences (high AT- or GC-content) and/or structures which inhibit or prevent PCR; c) they may be damaged in some way that prevents PCR amplification (e.g. nicks or UV-induced cross-linking; note - exposure to UV, especially in the presence of ethidium bromide, will damage DNA in ways that will prevent PCR amplification).

      If qPCR is consistently providing lower estimates of library concentration than your previous previous method(s), then you would need to use correspondingly "less" DNA (according to the qPCR-determined concentration) in order to generate the equivalent cluster density or bead enrichment percentage. Of course, we would expect qPCR quantification to result in ***less variability*** of cluster density or bead enrichment, because it minimizes at least one potential source of that variability -- the percentage of nucleic acid in the sample that is not amplifiable by PCR. However, there are other sources of variability for which qPCR cannot account (e.g. pipetting inaccuracies).

      Furthermore, it is worth considering that both cluster amplification and bead emPCR are expected to be less efficient than standard solution-phase PCR (as in qPCR), and one might therefore expect cluster amplification and bead emPCR to be more sensitive to "difficult templates". For this reason, even though qPCR is the most appropriate surrogate for cluster amplification titrations (or emPCR titrations), the relationship between measured library concentration and cluster density (or bead enrichment) may

be influenced by such factors as GC-content, secondary structure, template length, DNA damage, etc. You may therefore need to use different amounts of DNA to achieve equivalent cluster density (or bead enrichment) depending on the organism being sequenced, the sample type, the library construction method, etc. However, with time and experience it should be possible to gain a feel for how these factors influence the relationship between library concentration and cluster density (or bead enrichment).

ii) Pipetting accuracy is a common source of error in all library quantification methods, especially when small volumes are measured, and when serial dilutions are performed. Because qPCR is highly sensitive, requires only tiny amounts of sample, and utilizes low reaction volumes (all advantages in other respects), it is particularly important to pay close attention to the quality and condition of the equipment used, as well as to the techniques employed. We have attempted to minimize the possible effects of inaccurate pipetting in the following ways:

Strictly validated DNA Standards are supplied in a pre-prepared 10-fold dilution series, and we recommend that 4 µL of each sample be used per reaction to avoid pipetting of small volumes. Pipetting accuracy will affect the accuracy of the standard curve only in the setting up of qPCR reactions (4 µL template and 16 µL Master Mix). We recommend that new users set up reactions with the DNA Standards in triplicate, as this will help to identify outliers and to indicate whether pipetting accuracy at this point in the workflow is a potential problem. It should also be possible to use these data to distinguish between systematic pipetting inaccuracy vs. random errors in pipetting accuracy. The triplicate standard curve data may also help to identify well-to-well variability in the qPCR instrument and issues related to instrument settings, data collection, or data processing/analysis.

Apart from any initial, larger dilutions that may be required, we recommend that the sample be diluted in a 2-fold dilution series. Assuming the same pipette is used for both sample and diluent, an accurate 2-fold dilution should be produced even if pipetting is systematically inaccurate (e.g. the pipette is not accurately calibrated). Again, we recommend that dilutions be performed in triplicate, and the resulting data should allow outliers to be identified and discarded, and should help to identify any problems arising from pipetting accuracy or well-to-well variability.

iii) Bioanalyzer accuracy - the stated coefficient of variation for quantification using the relevant Bioanalyzer DNA assays is 20%. Moreover, in our experience, Bioanalyzer assays are prone to erratic behavior depending on instrument maintenance, reagent age and storage conditions, operator error, etc. Other factors that may affect quantification by Bioanalyzer relate to the size distribution of library fragments: it is possible that the size cut-offs used to define the peak to be quantified might exclude fragments, especially at the smaller end, which would give rise to clusters or to enriched beads. Similarly, a relatively small change in the position of the calculated baseline, and/or the detection limit of the instrument/assay, may under- or over-estimate a significant number of library fragments at both ends of the size distribution.

In summary, some initial work will be required to determine optimal loading concentrations for each type of library sample when customers switch from Bioanalyzer and/or dye-assisted spectrophotometry. Because qPCR quantifies a specific subset of the total DNA molecules present, and because of relatively large variability in Bioanalyzer quantification accuracy, direct comparisons between the two methods may not be particularly useful.

Based solely on the inherent variability in quantification methods, it is probably reasonable to expect up to ~ 2-fold difference in either direction between quantification by qPCR or by Bioanalyzer (or similar). Moreover, for the reasons discussed above, it is reasonable to expect qPCR would generally register significantly lower concentration than Bioanalyzer for a given library sample. Assuming no technical problems, the qPCR method for quantifying sequencing libraries should provide the most reliable predictions of cluster density or bead enrichment.

2. What is the recommended concentration of input DNA for optimal cluster density or template-to-bead ratio?

We expect that the KAPA Library Quantification Kits should allow users to dispense with cluster amplification titrations or emPCR titrations almost entirely once they are comfortable with qPCR quantification of their libraries. Indeed, many high-throughput users cannot possibly perform clustering/emPCR titrations on all of their library samples, and they rely solely on qPCR to determine optimal DNA input for cluster amplification or emPCR. Nevertheless, some facilities – especially those that process a wide variety of libraries representing a range of sample types, library construction procedures, etc. – may find that titrations are still sometimes necessary and/or useful. In such cases, qPCR is nevertheless likely to be the most informative quantification method prior to carrying out the titrations, and for inferring optimal loading concentrations for subsequent similar samples.

Inter-laboratory differences across various sample types, library construction methods, handling, etc., make it difficult to provide specific recommendations about the relationship between optimal library concentration and cluster density (bridge PCR) or template-to-bead ratio (emPCR). In the case of the Illumina sequencing platforms, the ideal cluster density varies from user to user, and has been trending upwards with upgrades to hardware and software. Furthermore, the relationship between input DNA concentration and cluster density/bead enrichment is not necessarily linear.

If your lab is following a standard sample preparation workflow, then we would expect that you should quickly be able to use your own experience to determine the best average DNA concentration that yields the required cluster density or template-to-bead ratio in your hands. While this remains a relatively inexact science, qPCR performs better than any of the alternatives (e.g. Bioanalyzer, spectrophotometry) for limiting variations in cluster density or bead enrichment. Of course, if you are working with very variable sample types (source organism, fragment sizes, etc.) and library prep techniques, then you may have to do more cluster amplification titrations, or accept more variability in cluster density or bead enrichment.

We have found that qPCR generally yields somewhat lower concentrations for a given library sample than would be obtained using less specific quantification methods such as spectrophotometry or electrophoresis. As a point of reference, one customer, performing highly automated human re-sequencing, has found that DNA input at 10 pM (determined using the KAPA Library Quantification Kit for the Illumina sequencing platform) consistently yields cluster densities around 220 000 clusters/tile. In the case of 454 emPCR, it seems that optimal input concentrations range between 0.1 - 1 copies per bead when libraries are quantified using qPCR, as opposed to inputs between 1 and 10 copies per bead when spectrophotometry and/or electrophoresis are used for library quantification.

Inconsistencies in emulsion formation may contribute to the broad range of potentially optimal input concentrations required for the 454 FLX and Titanium platforms.

3. How many libraries can I quantify with a single kit?

The number of libraries that you are able to quantify per kit depends on how many libraries you will be processing at one time, and on the qPCR format that you will be using (i.e. single tubes, 48-, 96-, or 384-well plates). By way of analogy, the situation is similar to that of a simple DNA marker/ladder for agarose gels - if you run many samples per gel, then the DNA ladder will be sufficient for many samples, but if you run only one sample per gel, then the DNA ladder will be finished quickly.

A single KAPA Library Quantification Kit is sufficient for the quantification of ~30 libraries (following our recommended protocols in a 96-well format). More libraries can be quantified

- using a 384-well plate format,
- smaller reaction volumes,
- and/or fewer replicates.

Many variables will greatly affect the ultimate number of libraries that can be quantified per kit. Below are a few guidelines based on the details of our recommended protocols:

iii) Only one (preferably triplicate) set of six DNA standards needs to be run per assay.

- If you quantify a single library sample at a time, you will be able to quantify six libraries per Kit of DNA standards. Each quantification experiment will use up 6 x 3 qPCR reactions for the standards plus 4 x 3 qPCR reactions for the library sample = 30 qPCR reactions in total. Since each Kit contains 5 mL KAPA SYBR® FAST 2X qPCR Master Mix (sufficient for 500 x 20 µL reactions), you will have 500 µL - (6 x 30 µL) = 320 qPCR reactions left over when you have finished the DNA Standards. In this case, additional DNA Standards can be purchased separately, so that the leftover qPCR reagent is not wasted.

- If you have many libraries to quantify simultaneously, and run 96-well qPCR plates, then you can load the 30 reactions described above (18 Standards and 12 samples), plus additional library samples on the same qPCR plate. Since there are 66 wells available, you can load 66/12 = 5 additional library samples on the plate, for a total of 6 libraries/plate. In this case, you will quantify 6 x 6 = 36 libraries before the DNA Standards are used up. Each plate will use 18 (triplicate standards) plus 6 x 12 = 72 (library samples), which is 90 qPCR reactions. Therefore, you will require 90 x 6 = 540 qPCR reactions in total, which means that you could expect to run out of qPCR Master Mix more or less at the same time as you run out of DNA Standards.

  As mentioned above, if you run full 96-well plates, exactly according to the recommended protocol, with 6 libraries per plate, then a single KAPA Library Quantification Kit should allow the quantification of 30 libraries.

- Similarly, it is possible to use 384-well plates or 48-well plates, and in each case the cost/library sample will be different.

iv) While the recommended qPCR volume is 20 µL, many users may choose to run 10 µL volumes (especially in 384-well formats), in which case the KAPA SYBR® FAST qPCR Master Mix and the Primer PreMix would go twice as far (the volume of DNA Standard added to each reaction remains 4 µL, regardless of the reaction volume).

v) We recommend that you perform serial dilutions of each library sample. This means that each library sample gives rise to 4 different dilutions. There are two reasons for this. First, it helps to ensure that at least one dilution will fall within the upper limit of the range of the assay. Second, it allows calculation of the qPCR efficiency for that particular library sample, which is not required for quantification, but helps with trouble-shooting. Thus, it is possible that with experience and/or with very standardized workflows, you may feel confident to stop doing these serial dilutions, in which case the number of qPCR reactions required for each quantification will be reduced.

vi) While we recommend triplicate reactions for the DNA standards and for the Library samples, some users may feel sufficiently confident (especially after some experience with the kit), to do away with some replicates of the standards and/or the samples. Of course, this would make it difficult or impossible to troubleshoot any unexpected results and the quantification accuracy may suffer. On the other hand, many more libraries could be quantified with the same amount of reagent.

4. What are the primer sequences used in the various KAPA Library Quantification Kits?

Roche 454 FLX (Primer Premix KAPA product # KP0001)

FLX Primer A: 5'-GCC TCC CTC GCG CCA-3'
FLX Primer B: 5'-GCC TTG CCA GCC CGC-3'

Roche 454 Titanium (Primer Premix KAPA product # KP0002)

Titanium Primer A: 5'-CCA TCT CAT CCC TGC GTG TC-3'
Titanium Primer B: 5'-CCT ATC CCC TGT GTG CCT TG-3'

Illumina GA II/IIx (Primer Premix KAPA product # KP0003)

Primer P1: 5' - AAT GAT ACG GCG ACC ACC GAG - 3'
Primer P2: 5' - CAA GCA GAA GAC GGC ATA CGA - 3'

Illumina GA II/IIx (Primer Premix KAPA product # KP0005)

Primer P1a: 5' - AAT GAT ACG GCG ACC ACC GA - 3'
Primer P2: 5' - CAA GCA GAA GAC GGC ATA CGA - 3'

Life Technologies SOLiD (Primer Premix KAPA product # KP0004)

Lib PCR Primer 1: 5' – CCA CTA CGC CTC CGC TTT CCT CTC TAT G – 3'
Lib PCR Primer 2: 5' – CTG CCC CGG GTT CCT CAT TCT – 3'

5. What is the primary reason for poor reproducibility across replicate data points?

Inaccurate pipetting is the most common reason for poor reproducibility. Accurate quantification requires careful pipetting. For liquid handling systems, consult the relevant user manual. Alternatively, apply the following when using a non-automated device:

- Examine the tip before dispensing to ensure that the correct volume is being added.
- Flush/rinse the tip 2-3 times after dispensing.
- Use a new pipette tip every time.
- After thawing and mixing, briefly centrifuge reagents to prevent droplets on tube walls from transferring to the outside of the pipette tip.
- Try to avoid placing the pipette tip too far under the surface when aspirating, as this may result in additional liquid adhering to the outside of the tip.
- Dispense directly into the bottom of the tube or well.
- Ensure that no residual liquid remains in the tip after dispensing.

Another common cause of poor consistency among replicates is incompletely thawed DNA template (standards or library samples); please ensure that all reagents are completely thawed and thoroughly mixed before assembling your qPCRs.

Instrument variability may also contribute to poor consistency across replicates; please ensure the qPCR instrument has been properly calibrated.

6. I am concerned that the Ct of DNA Standard 1 (and/or some of my library samples) is too low (amplification is too early).

In developing the KAPA Library Quantification Kit, we aimed to provide an assay with the broadest possible dynamic range. However, depending on the specific qPCR instrument and user-defined settings for the instrument, it is possible that amplification of the most concentrated DNA standard (Std 1) may give rise to an increasing fluorescent signal during the automatic baseline determination performed by the qPCR instrument.

Many instruments use the early cycles to calculate and set the baseline subtraction/correction, and samples that are changing significantly in fluorescence during this phase can dramatically affect this process (since there is no stable baseline). This also applies to the first DNA standard - if the "raw data" amplification plot increases visibly within the first 5-6 cycles, then this may be an issue. Unfortunately it is not possible for us to give precise guidelines on this, as it is an instrument-specific issue and in many cases depends on user-specific instrument settings. Please consult your qPCR instrument manual for more information.

It is usually possible to tell whether this is an issue by examining the amplification plots – if the corrected/processed amplification plot begins well below the baseline of samples crossing the Ct later, then it is likely that baseline determination/subtraction was not successful for that plot. Another useful way to judge whether an early Ct value has created problems during data analysis is to confirm that the expected spacing between consecutive standards is ~3.32 cycles (the standards represent a 10-fold dilution series). Similarly, consecutive two-fold dilutions of a given sample should cross the Ct ~1 cycle apart.

If you feel that the first standard and/or some sample dilutions have Ct scores that are too early, then simply exclude that standard and those sample dilutions from the analysis. This is most easily done by de-selecting them in the qPCR analysis software. If you experience this issue regularly with your instrument/settings/workflow, then you may choose to omit the first DNA standard from all future assays, and you might want to consider implementing a larger up-front dilution of your library samples in your standard work-flow. If the first standard is omitted, ensure that the library Ct score falls within the dynamic range of Standards 2 - 6.

7. What are typical Ct values for the standards?

When examining the data from the DNA standards, one should look at two things first:

i) Are the replicates very close to one another? A difference of 1 Ct is equivalent to a 2-fold difference in template concentration. In our hands, using a Corbett Rotorgene 6000 HRM qPCR instrument, we would expect to see less than 0.1 Ct difference between replicates. More variance than this may indicate a large degree of well-to-well variation in the instrument (optics and/or temperature control), or problems with pipetting accuracy.

ii) Is there consistent, even spacing between consecutive DNA standards, and is the spacing close to the expected 3.32 cycles? The DNA standards should amplify with close to 100 % efficiency, and therefore consecutive standards (10-fold dilutions) should be separated by ~3.32 cycles in Ct scores (ΔCt). If ΔCt is consistently different from 3.33, this may indicate problems either with pipetting accuracy or problems with the efficiency of the qPCR reaction.

While it is acceptable to discard the occasional "outlier", a large number of "outliers" generally indicates a problem either with the qPCR instrument or with the liquid handling equipment and/or technique.

Note: The Ct values in the table below are arbitrary and will vary significantly based on the qPCR instrument and threshold setting used.

	Given Conc	Typical Ct on Rotorgene (0.1 threshold)	dsDNA mol/µL
Roche 454 FLX DNA Standard			
Std 1	100 000 000 ssDNA molecules/µL	2.06	50 000 000 dsDNA molecules/µL
Std 2	10 000 000 ssDNA molecules/µL	5.39	5 000 000 dsDNA molecules/µL
Std 3	1 000 000 ssDNA molecules/µL	8.72	500 000 dsDNA molecules/µL
Std 4	100 000 ssDNA molecules/µL	12.06	50 000 dsDNA molecules/µL
Std 5	10 000 ssDNA molecules/µL	15.39	5 000 dsDNA molecules/µL
Std 6	1 000 ssDNA molecules/µL	18.72	500 dsDNA molecules/µL
Roche 454 Titanium DNA Standard			
Std 1	100 000 000 ssDNA molecules/µL	2.13	50 000 000 dsDNA molecules/µL
Std 2	10 000 000 ssDNA molecules/µL	5.46	5 000 000 dsDNA molecules/µL
Std 3	1 000 000 ssDNA molecules/µL	8.79	500 000 dsDNA molecules/µL
Std 4	100 000 ssDNA molecules/µL	12.13	50 000 dsDNA molecules/µL
Std 5	10 000 ssDNA molecules/µL	15.46	5 000 dsDNA molecules/µL
Std 6	1 000 ssDNA molecules/µL	18.79	500 dsDNA molecules/µL
Illumina GA DNA Standard			
Std 1	20.0000 pM	4.14	12 044 283 dsDNA molecules/µL
Std 2	2.0000 pM	7.48	1 204 428 dsDNA molecules/µL
Std 3	0.2000 pM	10.81	120 443 dsDNA molecules/µL
Std 4	0.0200 pM	14.14	12 044 dsDNA molecules/µL
Std 5	0.0020 pM	17.47	1 204 dsDNA molecules/µL
Std 6	0.0002 pM	20.81	120 dsDNA molecules/µL
Life Technologies ABI SOLiD DNA Standard			
Std 1	10.0000 pg/µL	3.74	63 424 344 dsDNA molecules/µL
Std 2	1.0000 pg/µL	7.08	6 342 434 dsDNA molecules/µL
Std 3	0.1000 pg/µL	10.41	634 243 dsDNA molecules/µL
Std 4	0.0100 pg/µL	13.74	63 424 dsDNA molecules/µL
Std 5	0.0010 pg/µL	17.07	6 342 dsDNA molecules/µL
Std 6	0.0001 pg/µL	20.41	634 dsDNA molecules/µL

	Tube code	Length	Molecular weight (dsDNA)
454 FLX DNA Standard	KQ0001	486 bp	299.66 kDa
454 Titanium DNA Standard	KQ0002	459 bp	282.98 kDa
Illumina GA Standard	KQ0003	452 bp	277.43 kDa
SOLiD DNA Standard	KQ0004	154 bp	94.95 kDa

8. Why does the recommended cycling protocol in the KAPA Library Quantification Kit protocol differ from that in the KAPA SYBR® FAST qPCR Kit protocol?

Next-generation sequencing libraries are generally complex, comprising a very wide diversity of DNA fragments. While KAPA SYBR® FAST qPCR reagents are generally capable of extremely fast thermocycling, we conservatively recommend relatively long denaturation and annealing/extension times for library quantification in order to accommodate the diversity of templates in a typical library sample. The recommended qPCR protocol for library quantification consists of an initial denaturation step at 95 ºC for 5 min followed by 35 cycles of denaturation at 95 ºC for 30 sec and combined annealing/ extension at 60 ºC for 45 sec. If the average library fragment size is >700 bp (e.g. 454 Titanium Rapid Prep Libraries), then increase annealing/extension time to 90 sec.

9. Can melt curve analysis after qPCR library quantification be used to monitor the quality of the libraries (i.e. detect adaptor dimers; average fragment sizes; library complexity; etc.)?

We do not make specific recommendations to perform melt-curve analysis after qPCR because it could be very difficult to interpret the results, for the following reasons:

- There is a very wide variety of sample types, fragment sizes, library construction techniques, qPCR instruments, etc., and each of these factors could be expected to affect the melt curve.
- Speaking very generally and superficially, the melting temperature/profile of DNA fragments above ~100 bp tend to be affected more by GC content than by length. We therefore think that it would be difficult to infer library fragment size/quality from melt curve analysis.
- Each library sample will, of course, only yield a "composite" melt curve, representing an average across the entire library sample. Since even the melt curve profile for a single DNA species can be "complex" (i.e. multiple local dsDNA to ssDNA transitions within a single fragment), even if one could discern multiple "peaks", it would be difficult to know how to interpret them. In fact, multiple, clearly definable peaks might actually indicate a low-quality library, because they would imply a low complexity (a few dominant library fragments, rather than a huge number of unique fragments).
- Although NGS library samples are expected to cross the cycle threshold (Ct) relatively early, the typical cycling profile for qPCR Library Quantification may run for 30 or 35 cycles. The melt curve analysis will only be performed after the cycling is completed, so one must keep in mind that the sample being analyzed during melting has undergone a large number of cycles. Many artifacts may be generated during these periods of extensive amplification and subsequent thermocycling, and these artifacts may be evident in the melt curve analysis and/or after electrophoresis (gel or Bioanalyzer), even though they may not be representative of the quality of the starting material used in the qPCR.

Despite the aforementioned considerations, we have received feedback from researchers who claim to detect adaptor dimers in their SOLiD libraries by melt curve analysis after qPCR. We would therefore recommend that gel electrophoresis or a Bioanalyzer assay be used to check on the quality of your libraries, and follow the melt curve analysis too. If your

library samples are consistently crossing the Ct relatively early, then you might choose to reduce the cycle numbers of your qPCR accordingly in order to reduce the possibility of artifacts as described above. With some experience and trial and error, you may be able to determine which characteristics of your melt curves might correlate with problems such as adaptor dimers.

Please note that performing melt curve analysis on the Illumina DNA Standards will reveal a multiple peak. The multiple peak is the result of differential local melting in the linear amplicon due to a heterozygous SNP and is not indicative of non-specific amplification.

10. What are the storage and stability recommendations for KAPA Library Quantification Kits and the combined KAPA SYBR® FAST/Primer Mix?

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 for at least six months from the date of receipt.

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 are not contaminated with microbes and/or nucleases.

11. How are the DNA standards in the KAPA Library Quantification Kits quality controlled to minimize batch-to-batch variance and ensure reliability?

The DNA Standards used in our Library Quantification Kits are not sequencing libraries, which are impossible to manufacture reproducibly through multiple production lots and over extended periods of time. Instead, we use a defined, pure, linear, dsDNA amplicon for each set of DNA Standards. This allows us to rigorously validate their efficiency and reproducibility for use as qPCR amplification standards.

Before accepting a newly manufactured lot into our inventory, we use a stringent qPCR assay to compare each new lot of KAPA Library Quantification DNA standards to a reference set of standards during manufacturing and quality control. We compare Ct scores for each standard in a newly manufactured set with Ct scores in a reference set of standards, and we ensure that each standard lies within 0.1 Ct of the respective reference standard, and that the resulting standard curve essentially lies on top of the reference standard curve (minimal deviations in y-intercepts and slopes).

FAQs specific to Roche 454 FLX/Titanium sequencing platforms

1. **Are the KAPA Library Quantification Kits for the Roche 454 Titanium platform compatible with the Rapid Library Preparation Kit?**

   Yes, our Library Quantification Kit for the 454 Titanium platform is compatible with the Rapid Prep libraries. Although the "adaptor moeity" used in the Rapid Prep kits is different to the adaptors used in the standard Titanium library prep, they share the same "core" sequences used for amplifying the library fragments onto the beads during emPCR, and for subsequent sequencing. Our qPCR primers bind to these "core" emPCR sequences in the library fragments.

   However, please note that the longer fragment lengths enabled by the Rapid Prep kits may require some optimization beyond our standard protocols. When using qPCR to quantify libraries of larger fragment sizes in the new Rapid Prep libraries, you may experience suboptimal qPCR amplification efficiencies. The engineered polymerase used in KAPA SYBR FAST reagents displays greater affinity for DNA and correspondingly greater processivity, so it should outperform standard qPCR reagents in this regard.

   Nevertheless, as the target length increases, amplification may become progressively more vulnerable to inhibition by other factors, such as high or low GC-content, secondary structure, and DNA damage. When using qPCR to quantify libraries with fragment sizes above ~700 bp, we suggest that you increase the annealing/extension step in the recommended thermocycling profile, as follows:

Initial denaturation:	95C	5 min
Cycling x35 cycles		
Denaturation:	95C	30 sec
Annealing/Extension:	**60C**	**90 sec**

   We also recommend that you pay particular attention to quantifying 2-fold dilution series of these samples and then use the resulting qPCR data to calculate the reaction efficiency. If you find that the large fragment libraries are not amplifying efficiently compared to the standards, then you may try the following approaches:

   i) Increase the combined annealing/extension time by a further 30 seconds, and/or
   ii) Compensate for the reduced efficiency of sample amplification via calculation.

2. The KAPA Library Quantification Kit for the Roche FLX platform is recommended for Roche 454 GS Titanium amplicon libraries.

For historical reasons, KAPA Biosystems supplies two sets of 454 Library Quantification Kits: one set is for the original "FLX" adaptor sequences, and the other set is for the newer "Titanium" adaptor sequences.

Late in 2009 Roche 454 released the Rapid Library Preparation Kit, employing a “single adaptor moiety” in place of the traditional adaptor sequences. Around the same time, the company released Titanium amplicon sequencing reagents using “FLX”-derived Fusion Primer sequences, and then subsequently released “single-read” Titanium amplicon sequencing protocols that use “Titanium”-derived Fusion Primers. These developments have created significant confusion about which KAPA Library Quantification kit should be used with each of the various possible Titanium libraries.

Please consult the following table to ensure that you obtain the appropriate KAPA Library Quantification Kit:

Type of Roche 454 library	**Compatible KAPA Library Quantification Kit**	
	for 454 FLX platform KK4820; KK4830; KK4840; KK4850	**for 454 Titanium platform** KK4821; KK4831; KK4841; KK4851
All “original” 454 **FLX** libraries	Yes	No
454 FLX **Titanium Rapid Prep** libraries	No	Yes
454 FLX **Titanium** “**Lib-L**” libraries ▪ All “standard” Titanium libraries made by ligation of adaptors to library fragments. ▪ Amplicon libraries for “unidirectional sequencing”	No	Yes
454 FLX **Titanium** “**Lib-A**” libraries ▪ Amplicon libraries for “bidirectional sequencing”	Yes	No

As noted in the table above, our Library Quantification Kit for the Titanium platform is compatible with the Titanium Rapid Prep libraries, but we suggest that customers use a longer combined annealing/extension time in the cycling protocol to accommodate the longer fragment sizes that are often used with these libraries (see FAQ 1 above).

If you know the adaptor (or Fusion Primer) sequences used to make your library, then it is best to check them against the following qPCR primer sequences to ensure compatibility with one of our two 454 Library Quantification Kits:

KAPA Library Quantification Kit for 454 FLX platform

FLX Primer A: 5'-GCC TCC CTC GCG CCA-3'
FLX Primer B: 5'-GCC TTG CCA GCC CGC-3'

KAPA Library Quantification Kit for 454 Titanium platform

Titanium Primer A: 5'-CCA TCT CAT CCC TGC GTG TC-3'
Titanium Primer B: 5'-CCT ATC CCC TGT GTG CCT TG-3'

3. What are the advantages and disadvantages of using the fluorescently-labeled adaptor supplied in the Roche FLX Titanium Rapid Library Preparation Kit for library quantification, in comparison with qPCR?

Roche 454 has released a library preparation protocol, with new reagents, which is called the "GS FLX Titanium Rapid Library Preparation Kit". This kit makes the preparation of 454 sequencing libraries quicker and easier. The new kit uses a "single adaptor moiety" in place of the two distinct adaptors used before.

Roche has addressed library quantification by incorporating a fluorescent label into the new adaptor in the Rapid Library Prep Kits. This fluorescent label increases the sensitivity of detection, so that less material is used up during library quantification, and it should help to address the problem of incorrectly "counting" library molecules that do not carry appropriate adaptors for emPCR, a major issue for Bioanalyzer and intercalating-dye assisted spectrophotometry. However, it is still possible that an unknown and variable proportion of library molecules will carry an adaptor on only one end, and may not be amplifiable during emPCR for other reasons (GC-content, secondary structures, etc).

In addition to the advantage of qPCR outlined above (i.e. it counts only *bona fide* amplifiable templates), users may find value in the following:

i) Although improved, quantification by the standard Rapid Library Prep method is still not very sensitive, and requires large volumes (20 – 50 µL) of undiluted library sample. This sample must therefore be recovered from the cuvette (or multi-well plate) after quantification, for use in sequencing. This presents additional pipetting steps and opportunities for sample confusion, contamination, and loss.

ii) The standard curve generated in the Rapid Library Prep method covers 8 x 2/3 dilutions, from 2.5 x 10^9 mol/µL down to 1.46 x 10^8 mol/µL. The KAPA Library Quantification Kits cover a dynamic range across six orders of magnitude, from 5 x 10^7 mol/µL down to 500 mol/µL.

iii) For quantification using the Roche Rapid Prep method, the user is required to produce the standard curve, making 7 serial 2/3 dilutions of the supplied standard. Aside from the extra work this entails, this also presents additional scope for variability and errors. The KAPA Library Quantification Kit contains pre-prepared and validated standards for generating the entire standard curve.

FAQs specific to Illumina GA sequencing platform

1. Compatibility of KAPA Library Quantification Kit with various types of libraries on Illumina platform.

KAPA Library Quantification Kits for the Illumina GA sequencing platform (kit codes: KK4824, KK4835, KK4844, KK4854, KK4808, KK4809 - containing Primer Premix tube code KP0005) are compatible with all Illumina GA library types.

Note: Previous versions of the kit (KK4822, KK4832, KK4842, KK4852, KK4804, KK4805 - containing Primer Premix tube code KP0003) were not compatible with small RNA or GEX (Digital Gene Expression-Taq Profiling) library adaptor sequences. We will continue to supply these original kits, containing the unmodified Primer Premix, to existing customers who specifically request to receive the original kit. Aside from the Primer Mix, all other components of the new kits remain unchanged. Existing users may request a sample of the revised Primer Mix for testing. New customers – and existing customers who do not specifically request the original kit – will receive the new kit.

References for qPCR and NGS library quantification

1. From micrograms to picograms: quantitative PCR reduces the material demands of high-throughput sequencing. Meyer *et al*. Nucleic Acids Res. 2008 Jan; 36(1):e5.

2. A large genome center's improvements to the Illumina sequencing system. Quail *et al*. Nat Methods. 2008 Dec; 5(12):1005-10.

3. Amplification-free Illumina sequencing-library preparation facilitates improved mapping and assembly of (G+C)-biased genomes. Kozarewa *et al*. Nat Methods. 2009 Apr; 6(4):291-5.

4. Titration-free massively parallel pyrosequencing using trace amounts of starting material. Zheng *et al*. Nucleic Acids Res. 2010 Apr 30.

5. Rapid quantification of DNA libraries for next-generation sequencing. Buehler *et al*. Methods. 2010 Apr; 50(4):S15-8.

6. A scalable, fully automated process for construction of sequence-ready barcoded libraries for 454. Lennon et al. Genome Biol. 2010; 11(2):R15.