

KAPA Human Genomic DNA Quantification and QC Kit

KR0454 – v5.20

This document applies to KAPA Human Genomic DNA Quantification and QC Kits (07960590001, 07960603001, 07960611001, 07960620001 and 07960689001), KAPA Human Genomic DNA Quantification and QC DNA Standards (07960638001), and KAPA Human Genomic DNA Quantification and QC Primer Premixes (07960646001, 07960654001 and 07960662001).

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KAPA/Roche Kit Codes and Components				
KK4960 07960590001 Complete kit with Universal qPCR Master Mix	KAPA SYBR® FAST Universal qPCR Master Mix (2X)	3 x 1 mL		
	KAPA ROX High (50X)	200 µL		
	KAPA ROX Low (50X)	200 µL		
	DNA Standards (1 – 5)	300 µL ea		
	41 bp Primer Premix (10X)	200 µL		
	129 bp Primer Premix (10X)	200 µL		
KK4961 07960603001 Complete kit with ABI Prism qPCR Master Mix	KAPA SYBR FAST ABI Prism qPCR Master Mix (2X)	3 x 1 mL		
	DNA Standards (1 – 5)	300 µL ea		
	41 bp Primer Premix (10X)	200 µL		
	129 bp Primer Premix (10X)	200 µL		
	305 bp Primer Premix (10X)	200 µL		
KK4962 07960611001 Complete kit with Bio-Rad iCycler qPCR Master Mix	KAPA SYBR FAST Bio-Rad iCycler qPCR Master Mix (2X)	3 x 1 mL		
	DNA Standards (1 – 5)	300 µL ea		
	41 bp Primer Premix (10X)	200 µL		
	129 bp Primer Premix (10X)	200 µL		
	305 bp Primer Premix (10X)	200 µL		
KK4963 07960620001 Complete kit with qPCR Master Mix optimized for LightCycler®	KAPA SYBR FAST qPCR Master Mix (2X) optimized for LightCycler	3 x 1 mL		
	DNA Standards (1 – 5)	300 µL ea		
	41 bp Primer Premix (10X)	200 µL		
	129 bp Primer Premix (10X)	200 µL		
	305 bp Primer Premix (10X)	200 µL		
KK4969 07960689001 Complete kit with Rox Low qPCR Master Mix	KAPA SYBR FAST qPCR Rox Low Master Mix (2X)	3 x 1 mL		
	DNA Standards (1 – 5)	300 µL ea		
	41 bp Primer Premix (10X)	200 µL		
	129 bp Primer Premix (10X)	200 µL		
KK4964 07960638001	DNA Standards (1 – 5)	300 µL ea		
	KK4965 07960646001	41 bp Primer Premix (10X)	200 µL	
		KK4966 07960654001	129 bp Primer Premix (10X)	200 µL
			KK4967 07960662001	305 bp Primer Premix (10X)

Quick Notes

- The DNA Standards provided in the kit represent a 4-fold dilution series corresponding to 2.5 ng/µL – 9.77 pg/µL hgDNA (10 ng – 0.39 ng per 20 µL reaction). This is equivalent to 3080 – 12 copies per reaction.
- Use the 41 bp Primer Premix to determine a working concentration of hgDNA samples for downstream applications.
- Use the 129 bp and/or 305 bp Primer Premixes in addition to the 41 bp Primer Premix to obtain quality information (expressed as “Q-ratios”) for hgDNA samples.

Product Description

Both the quantity and quality of input DNA have a profound impact on the outcome of library construction for next-generation sequencing (NGS). A number of innovations have reduced the amount of input DNA required for successful library construction, enabling routine sequencing of human DNA from a wide variety of sample types. Such high-throughput NGS pipelines require streamlined protocols that reduce input requirements, lower costs, improve success rates, and minimize turn-around times. A reliable method for quantifying and qualifying input DNA prior to library construction offers several benefits for any library construction workflow:

- Standardization or normalization of input DNA results in more consistent library construction outcomes (e.g., yield, library quality) with a fixed-cycle library amplification protocol, and reduces the need for sample-specific protocol adjustments.
- Quality metrics can be used to direct samples into appropriate, optimized shearing and library construction pipelines, to ensure more consistent insert sizes and data quality. Identifying the most appropriate workflow for each sample at the outset minimizes reworking of samples, and reduces average turn-around times.

Quantification methods that rely on spectrophotometry or electrophoresis (e.g., those employing a NanoDrop, Qubit or Bioanalyzer) have significant limitations for assessing input DNA, and provide poor predictions of library construction success.

Such limitations include:

- poor accuracy in the quantification of very dilute samples;
- the inability to discriminate between damaged DNA and template material suitable for PCR-based processes such as library amplification, qPCR-based library quantification, cluster amplification and sequencing; and
- sensitivity to contaminants, which can lead to significant over- or underestimation of DNA concentrations.

To address these needs, we have developed a qPCR-based kit for the reliable quantification and quality assessment of human genomic DNA (hgDNA) samples prior to NGS library construction. The kit employs the engineered KAPA SYBR® FAST DNA Polymerase, and includes three primer premixes and a set of quality-controlled, pre-diluted DNA quantification standards. The primer premixes are designed to amplify targets of 41 bp, 129 bp, and 305 bp within a conserved single-copy locus in the human genome.

Absolute quantification is achieved using the 41 bp assay, while the longer amplicons are used to assess DNA quality (Figure 1). Since DNA damage has a greater impact on the amplification of longer targets, the relative quality of a DNA sample can be inferred by normalizing the concentration obtained using the 129 bp or 305 bp assay against the concentration obtained with the 41 bp assay. This normalization generates a “Q-ratio” with a value between 0 and 1, which can be used as a relative measure of hgDNA quality prior to NGS library construction.

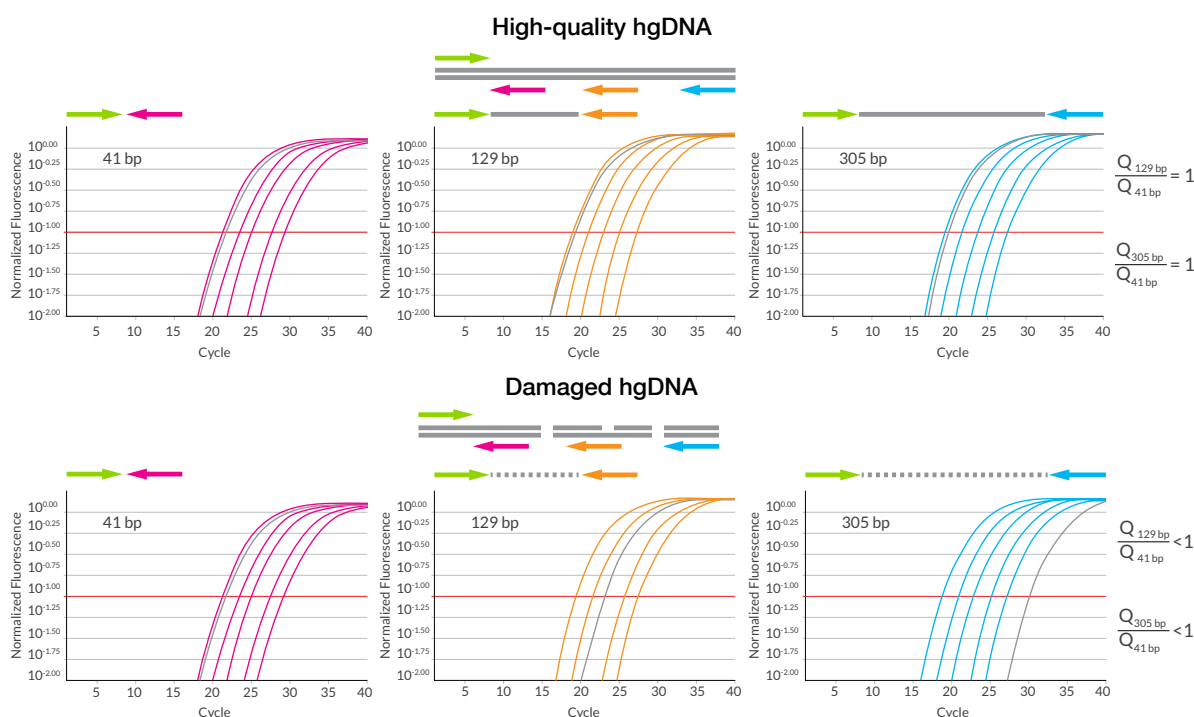


Figure 1. Principle of the hgDNA Quantification and QC assay. A single set of DNA Standards is used to generate up to three different primer pairs that amplify targets of 41 bp, 129 bp, or 305 bp within a conserved, single-copy human locus. The 41 bp assay is used for absolute quantification of DNA samples. For an assessment of DNA quality, standard curves are generated and samples assayed with the 129 bp and/or 305 bp primer premix(es). Since poor DNA quality has a greater impact on the amplification of longer targets, the relative quality of a DNA sample can be inferred by normalizing the concentration obtained using the 129 bp or 305 bp assay against the concentration obtained from the 41 bp assay. This normalization generates a “Q-ratio” (with a value between 0 and 1) that can be used as a relative measure of DNA quality.

Product Applications

The KAPA Human Genomic DNA Quantification and QC Kit is designed for the reliable quantification and quality assessment of hgDNA samples prior to NGS library construction. These include:

- formalin-fixed paraffin-embedded (FFPE) tissue samples, which are notorious for variable DNA quality as a result of degradation, cross-linking, and/or other lesions that render DNA recalcitrant to amplification
- samples obtained by laser-capture microdissection of fresh, frozen, or FFPE tissue
- DNA extracted from cells collected by flow cytometry
- free circulating DNA from plasma or serum
- any other low concentration or precious sample.

Q-ratios may be used:

- to predict the outcome of library construction from FFPE and other difficult or limited samples of variable concentration and quality. Q-ratios have been shown to correlate with post-amplification yield, insert size, and library complexity
- for library construction process control and optimization. Q-ratios can be used to assess DNA fragmentation prior to library construction; to make stop/go decisions for library construction based on sample quality; or for sample “triage”, to direct samples into the appropriate workflows
- to retrospectively troubleshoot failed samples, or samples that produce substandard sequencing results
- to detect hgDNA contamination in free circulating DNA samples.

The method is easy to automate and can be applied to any process or workflow that requires accurate quantification of dilute DNA samples, or samples that may contain a high proportion of DNA that is recalcitrant to PCR amplification.

KAPA SYBR® FAST is an antibody-mediated hotstart DNA polymerase formulation. KAPA Human Genomic DNA Quantification and QC Kits are therefore suitable for use with automated liquid handling systems for high-throughput sample quantification.

Safety Information

Precautions

- Handle all samples as if potentially infectious, using safe laboratory procedures. As the sensitivity and titer of potential pathogens in the sample material can vary, the operator must optimize pathogen inactivation and follow the appropriate measures according to local safety regulations.
- Do not eat, drink, or smoke in the laboratory area.
- Do not pipette by mouth.

- Wear protective disposable gloves, laboratory coats, and eye protection, when handling samples and kit reagents.
- Wash hands thoroughly after handling samples and reagents.

Waste handling

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

Product Specifications

Shipping and Storage

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

Handling

Always ensure that components have been fully thawed and thoroughly mixed before use. The KAPA SYBR FAST qPCR Master Mix contains isostabilizers and 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 may result in loss of fluorescent signal intensity.

All components of KAPA Human Genomic DNA Quantification and QC Kits, as well as the combined KAPA SYBR FAST/Primer Premix solution, are stable through 30 freeze/thaw cycles. It is recommended that all reagents are stored protected from light at -15°C to -25°C when not in use. Stored properly, these reagents are stable at 2°C to 8°C for long-term storage, and may be stored in this state for short-term use, provided that they do not become contaminated with microbes and/or nucleases.

Quality Control

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

Important Parameters

This kit provides a means for obtaining (i) working concentrations for dilute hgDNA samples, and (ii) quality information about dilute hgDNA samples. Since qPCR is a very sensitive analytical technique, and the dynamic range of the assay extends to low template copy numbers, the reliability of results is highly dependent on accurate liquid handling. Important considerations relating to the reliability and interpretation of data are discussed in this section.

Accurate Liquid Handling

Take care to ensure the highest degree of accuracy during the dilution of hgDNA samples and reaction setup. This can be achieved by observing the following recommendations:

- Always ensure that reagents and samples are fully thawed and thoroughly mixed before use. After thawing and mixing, briefly centrifuge the tubes to prevent droplets on tube walls from transferring to the outside of the pipette tip.
- Concentrated solutions of high molecular weight hgDNA are viscous, making it difficult to accurately dispense small volumes for analysis. Ensure that hgDNA solutions are thoroughly mixed using a vortex mixer before pipetting. Avoid making extremely large dilutions during sample preparation—see **Detailed Protocol** (step 2). If samples require very large dilutions to fall within the dynamic range of the assay, it may be preferable to perform two consecutive serial dilutions (e.g., make two consecutive 1:100 dilutions instead of a single 1:10,000 dilution). Consider preparing a diluted stock of your hgDNA sample at ~10 – 50 ng/μL, which can be used for quantification and quality assessment as well as downstream processing. When working with hgDNA at concentrations lower than ~1 ng/μL, try to avoid transferring volumes less than 4 μL.
- Use the recommended dilution buffer (10 mM Tris-HCl, pH 8.0 – 8.5 (25°C) + 0.05% Tween-20) when diluting hgDNA samples—see **Detailed Protocol** (steps 1 and 2). Tween-20 is a wetting agent, which improves pipetting accuracy, and helps to reduce DNA adsorption to plastic tubes and pipette tips. Never dilute hgDNA samples in water, as DNA is sensitive to hydrolysis at pH <7.
- Do not store diluted hgDNA samples at room temperature, or for long periods of time prior to setting up qPCRs.
- If possible, avoid the use of multi-channel pipettes when diluting hgDNA samples, or for reaction setup.
- Use a new pipette tip every time.
- 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.

- After aspirating the desired volume, examine the pipette tip before dispensing to ensure that the correct volume is being transferred.
- Dispense directly into the bottom of the tube or well.
- Flush/rinse pipette tips by pipetting up and down 2 – 3 times after dispensing.
- Ensure that no residual liquid remains in the tip after dispensing.
- Use an automated liquid handling system to dilute samples and set up reactions, especially when large numbers of samples are processed. Automated liquid handling provides the greatest consistency, both within assays, and among assays performed over extended periods of time.

Other Sources of Error and Variability

A number of factors other than inaccurate liquid handling can contribute to error and variability (low reproducibility) in the quantification and quality assessment of dilute hgDNA samples. The most important of these are detailed below:

Reaction volume: While the standard protocol for this assay specifies 20 μL reactions, the reaction volume can be scaled down to 10 μL if the assay is performed in 384-well format and/or for reasons of economy. However, please note that accurate liquid handling is particularly important when using 10 μL qPCR volumes. Manual setup of 10 μL reactions in 96-well plates using multi-channel pipettes is likely to result in unacceptable inaccuracy in the calculated concentrations and Q-ratios of samples. Manual setup of 10 μL reactions in 384-well plates is not recommended.

Sample quality: Absolute concentrations determined with this kit are expected to be accurate for diluted DNA samples falling within the range defined by Standards 1 – 3, particularly if the Q129/Q41 ratio is close to 1.0. Absolute concentrations calculated for very dilute and/or damaged DNA samples (Q129/Q41 <~0.2) may not be accurate or reproducible.

Since dilute DNA quickly degrades in an unbuffered environment:

- hgDNA samples should be diluted in a weak buffer (10 mM Tris-HCl, pH 8.0 – 8.5 at 25°C). Tween-20 should be included in the dilution buffer at a final concentration of 0.05% to improve pipetting accuracy and reduce DNA adsorption to plastic tubes and pipette tips.
- hgDNA dilutions should be made fresh for each assay and kept on ice or at 2°C to 8°C while qPCRs are set up.

Sample concentration: hgDNA samples must be pre-diluted to fall within the dynamic range of the assay (10 – 2500 pg/ μ L). Ideally, samples should be diluted to fall within a range of 0.1 – 1.0 ng/ μ L. If more than 10 ng of template DNA is added to a 20 μ L reaction, problems with data collection and/or analysis may be experienced as a result of excessively high initial background fluorescence. The initial dilution should be based on estimations from previous experience with similar samples and protocols, and/or on spectrophotometric or fluorimetric assays. Since such assays are less sensitive/accurate than qPCR, the correlation between the concentration on which the initial dilution was based, and the concentration determined by qPCR may be poor, particularly for very dilute or damaged DNA samples.

Contamination: Observe good laboratory practice at all times to avoid contamination of reagents, samples, consumables, pipettes and other equipment, and work areas with hgDNA or amplicons generated with this assay.

Incorrect product storage and/or excessive freezing and thawing: Observe all storage and handling guidelines in **Product Specifications** (p. 3).

Consumables and equipment: Use high-quality PCR tubes/plates and pipette tips. Ensure that qPCR instruments and pipettes are properly maintained and regularly calibrated according to the manufacturers' recommendations.

Replicates

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

- providing insurance against gross errors or failures with respect to individual reactions, which may be identified as outliers and excluded from the analysis;
- enabling the calculation of replicate averages, thereby discounting the impact of individual data points that are scattered around the population mean;
- providing an indication of the variability in the data, and a means to gauge the reliability of the assay; and
- sometimes assisting with troubleshooting and optimization—e.g., by providing some indication of the frequency and/or scale of liquid handling errors.

Triplicate qPCRs are recommended and are generally sufficient for standard curve data points, and for sample dilutions in the concentration range of Standards 1 – 3. However, for reliable assessment of samples in the concentration range of Standards 4 – 5, you may wish to include additional qPCR replicates, according to the level of accuracy required. Note that Standard 5 represents 12 copies per reaction (see Table 1), which will lead to unavoidable and significant sampling error among replicates.

Table 1. Concentration of DNA Standards

DNA Standard	Concentration (pg/ μ L hgDNA)	Concentration (copies/rxn)
1	2500	3080
2	625	758
3	156	189
4	39.1	47
5	9.77	12

No-template Controls

The qPCRs carried out using this protocol are capable of amplifying single-copy amounts of human genomic DNA, and the samples being assayed may be at very low concentrations. Human genomic DNA is ubiquitous in most laboratory settings, and routine use of this kit will generate extremely high numbers of amplicons. Both of these factors present considerable risks for reagent contamination. Significant contamination of qPCRs with exogenous template DNA will affect the accuracy of the estimated sample concentrations that are determined using the assay. Therefore, no-template controls (NTC) are crucial for the detection of contamination introduced during reaction set-up.

Contamination of the qPCR master mix or its components with template DNA (hgDNA or Standard) is indicated by NTC qPCR reactions having C_q values less than 3 cycles later than the average C_q value obtained for Standard 5.

Melt Curve Analysis

Each of the three amplicons generated using this kit (41 bp, 129 bp and 305 bp respectively) has a specific melting temperature (Table 2), and a characteristic melting profile (Figure 2). We recommend that a melt curve analysis be performed to confirm that specific product has been amplified in each reaction.

In the case of amplification in a NTC reaction, melt curve analysis can be used to identify the cause of the problem, by discriminating between specific amplification of contaminating template versus non-specific amplification artefacts such as primer-dimers.

Melt curve analysis may also be helpful in identifying cross-contamination, misidentification, or accidental mix-up of primer premixes.

Table 2. Amplicon melting temperatures

Amplicon	Expected T _m (±1°C)
41 bp	72.5°C
129 bp	84.5°C
305 bp	86.3°C

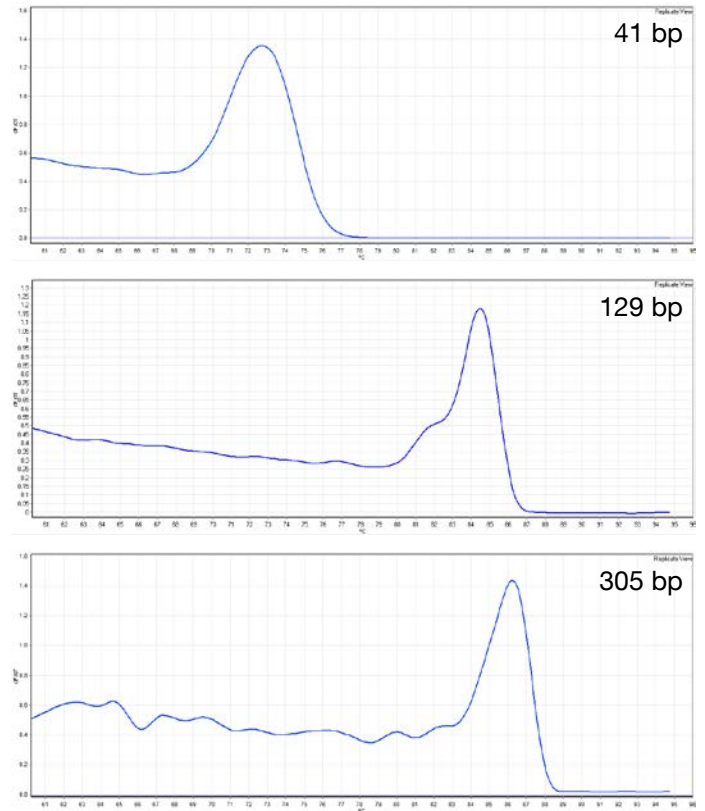
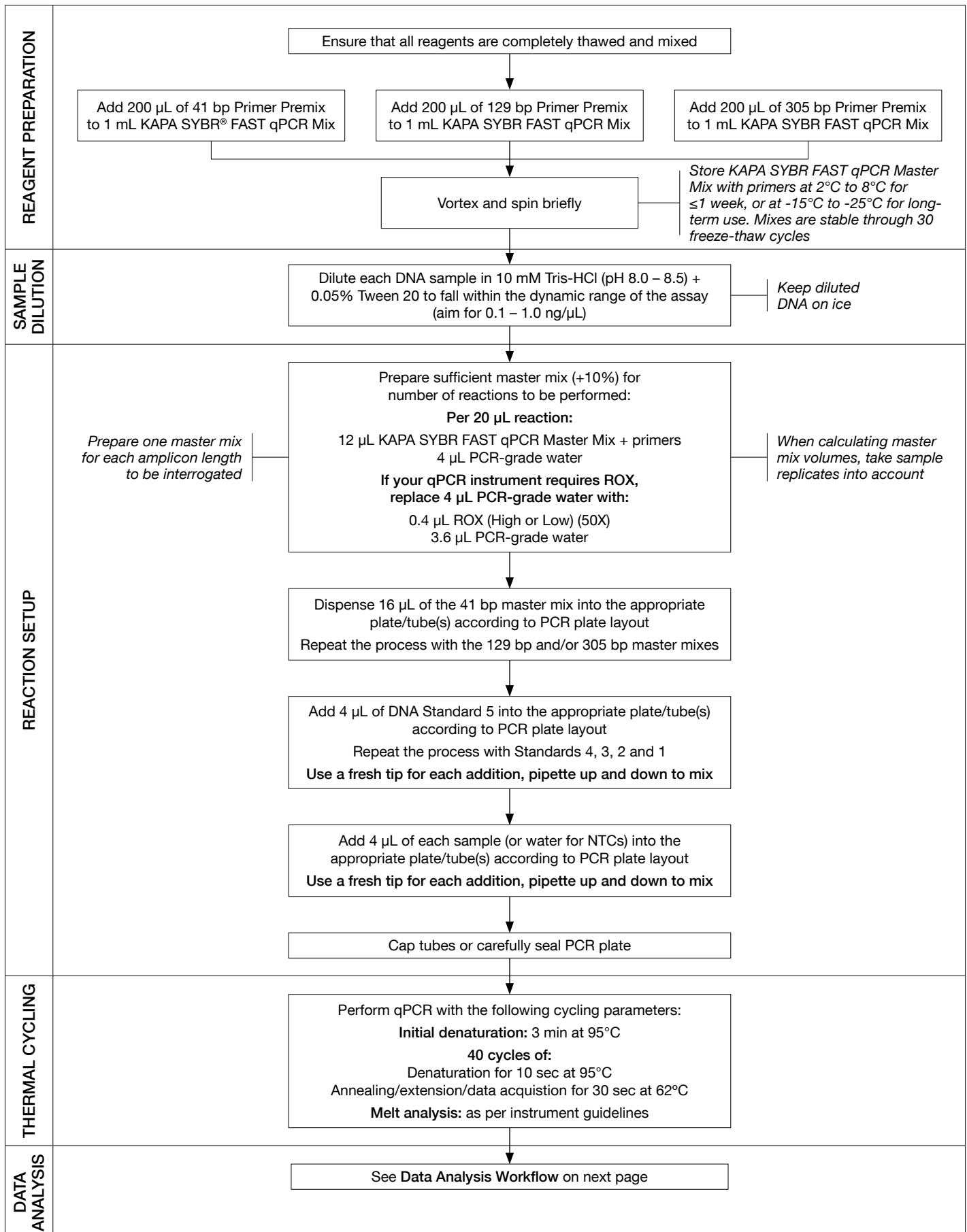
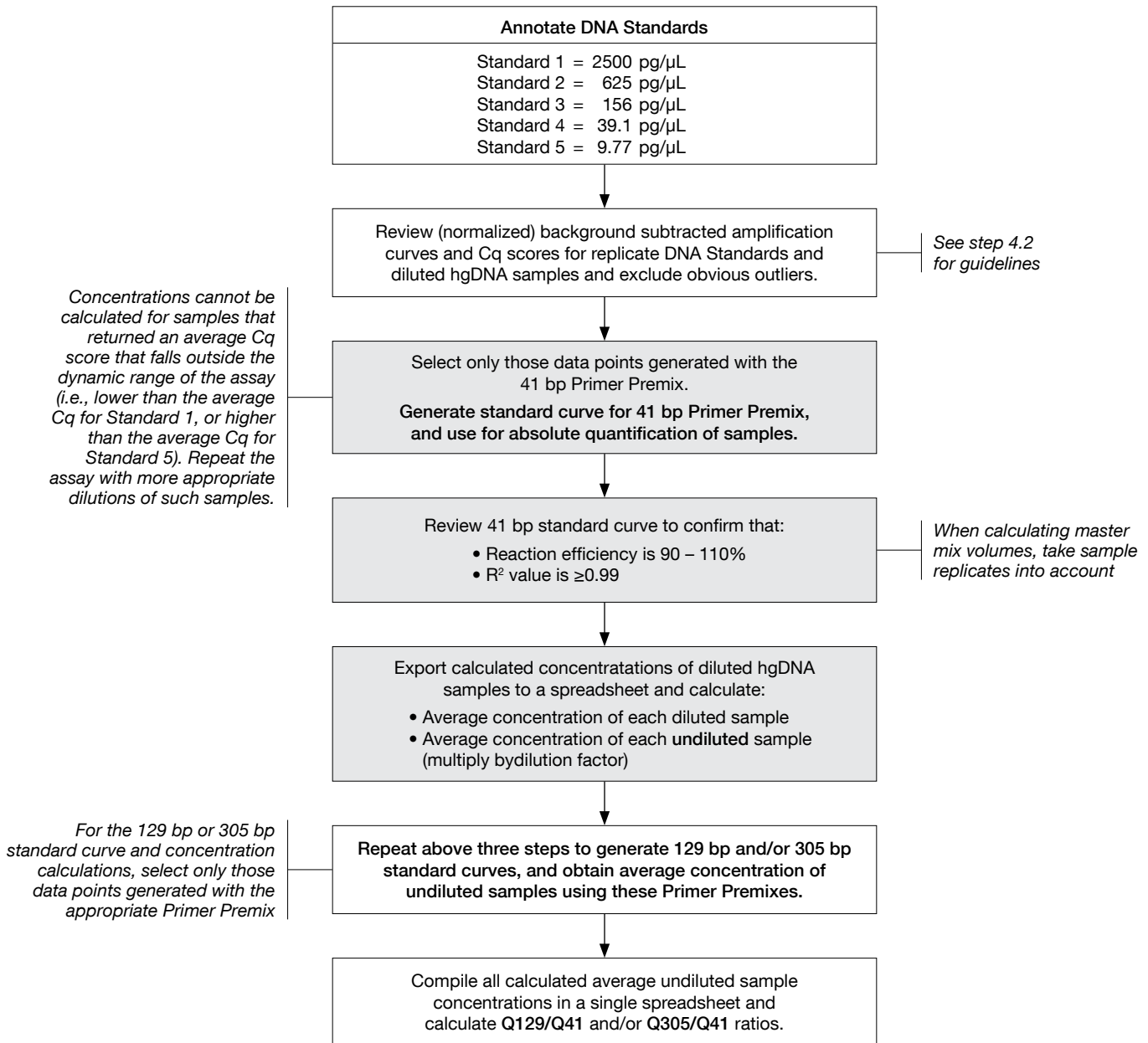


Figure 2. Typical melt curve profiles of the three amplicons (41 bp, 129 bp, and 305 bp) after qPCR

Process Workflow



Data Analysis Workflow



Detailed Protocol

1. Reagent Preparation

- 1.1 Prepare an appropriate volume of DNA dilution buffer (10 mM Tris-HCl, pH 8.0 – 8.5 (25°C) + 0.05% Tween-20).
- 1.2 Ensure that all components are completely thawed, thoroughly mixed, and then briefly centrifuged.
- 1.3 Three 1 mL aliquots of KAPA SYBR® FAST qPCR Master Mix (2X) are supplied. When the kit is first used, add 200 µL of 41 bp Primer Premix (10X) to one tube of KAPA SYBR FAST qPCR Master Mix (2X), and label the tube accordingly. Similarly, add 200 µL of 129 bp Primer Premix (10X) to the second tube, and add 200 µL of 305 bp Primer Premix (10X) to the third tube, labelling the tubes appropriately. Mix thoroughly using a vortex mixer, and then briefly centrifuge the tubes.
- 1.4 Record the date on which the primers were added. KAPA SYBR FAST qPCR Master Mixes with primers are stable through 30 freeze/thaw cycles, and should be stored in the dark at -15°C to -25°C when not in use. Alternatively, the mixes may be stored in the dark at 2°C to 8°C for up to one week, provided that they do not become contaminated with microbes and/or nucleases.

2. Sample Preparation

- 2.1 hgDNA samples must be diluted to fall within the dynamic range of the assay. Ideally, aim for a final DNA concentration of 0.1 – 1.0 ng/µL. Fresh dilutions should be made in Dilution Buffer as described in **Reagent Preparation** (step 1.1) and kept on ice during reaction setup. Diluted DNA stored at room temperature, or for prolonged periods at 2°C to 8°C, is prone to degradation and may not yield consistent results upon re-quantification. DNA diluted in water may be unstable, even when stored at room temperature for short periods of time.

3. Reaction Setup

- 3.1 Prepare a separate master mix for each primer set to be used. Based on the reaction setup outlined below, determine the volume of master mix that will be required for triplicates of each of the following reactions: five DNA Standards, one no-template control (NTC), and each of the hgDNA samples to be assayed. Remember to provide for an excess of at least 10%.

For Universal qPCR Master Mix:

Component	Volume
KAPA SYBR FAST qPCR Master Mix (2X), premixed with Primer Premix (10X)	12 µL
ROX High or Low (50X)*	0.4 µL
PCR-grade water	3.6 µL
Total volume:	16 µL

*Please refer to the KAPA SYBR FAST Technical Data Sheet included with your kit to select the appropriate ROX for your qPCR instrument.

For ABI Prism, Bio-Rad iCycler, or LightCycler® 480 qPCR Master Mix:

Component	Volume
KAPA SYBR FAST qPCR Master Mix (2X), premixed with Primer Premix (10X)	12 µL
PCR-grade water	4 µL
Total volume:	16 µL

- 3.2 Mix and briefly centrifuge each master mix.
- 3.3 Label the appropriate number of PCR tubes or decide on the appropriate plate layout.
- 3.4 Dispense 16 µL of the appropriate master mix (prepared in step 3.1) into each PCR well/tube.
- 3.5 Add 4 µL of the appropriate template (DNA Standard, diluted DNA sample from **Sample Preparation** (step 2) or PCR-grade water for NTCs) into each PCR well/tube. Use a fresh tip each time, and flush the tip by carefully pipetting up and down several times. Dispense the DNA Standards starting from the most dilute (Standard 5) and proceeding to the most concentrated (Standard 1).
- 3.6 Cap tubes or seal the PCR plate, and transfer to the qPCR instrument.
- 3.7 Select the Absolute Quantification option in the instrument software, and select/adjust parameters (e.g., reporters, reference dyes, gain settings, etc.) as required. Refer to the instrument manual for further information.
- 3.8 Refer to **Data Analysis** (step 4.1) for annotation of DNA Standards, if required at this stage.
- 3.9 Perform qPCR using the following cycling parameters:

Step	Temp	Duration	Cycles
Initial denaturation	95°C	3 min	1
Denaturation	95°C	10 sec	40
Annealing/ Extension/ Data acquisition	62°C	30 sec	
Melt analysis*	As per instrument parameters		

*See Important Parameters (p. 4) for more details.

4. Data Analysis

- 4.1 Annotate the DNA Standards as outlined in the table below. The DNA Standards comprise a 4-fold dilution series of a 610 bp dsDNA template. **Note that the specified values have already been converted to the concentration of hgDNA that is equivalent to the copy number corresponding to each DNA Standard.**

DNA Standard	Concentration (pg/μL hgDNA)
1	2500
2	625
3	156
4	39.1
5	9.77

- 4.2 Review the background-subtracted (normalized) amplification curves and the C_q scores for replicate data points, and exclude obvious outliers. Replicate data points for Standards 1 – 3, and samples in that concentration range, should differ by ≤0.2 cycles, while replicates for Standards 4 – 5, and samples in that concentration range, may vary by up to ~0.5 cycles.

If the data set contains many outliers, the results of the assay are unlikely to be reliable, and the assay should be repeated with particular focus on improving pipetting accuracy.

Exclude all samples that fall outside the range of the standard curve, i.e., that return an average C_q score lower than that of Standard 1, or higher than that of Standard 5. Where possible, such samples may be re-tested after preparing new dilutions at appropriate concentrations.

- 4.3 To generate the 41 bp standard curve and calculate sample concentrations, select only those Standards and samples that were amplified using the 41 bp Primer Premix, and deselect all data points generated with the 129 bp or 305 bp Primer Premixes.
- 4.4 Allow the software to generate the 41 bp standard curve, and to calculate the concentrations of the hgDNA samples. The standard curve and sample concentrations may also be generated manually, using a spreadsheet. For assistance in this regard, please contact sequencing.roche.com/support.
- 4.5 Review the metrics for the 41 bp standard curve. The standard curve should be used only if the following conditions are met:
- R² ≥ 0.99.
 - The calculated reaction efficiency is 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).

If the 41 bp standard curve does not meet these criteria, concentrations calculated for the hgDNA samples will not be reliable, and the assay should be repeated.

A consistent reaction efficiency (within the range of 90 – 110%) should be achieved when the assay is performed on the same qPCR cyclers, using the same plasticware. If the assay returns a reaction efficiency that is within the acceptable range, but is much higher or lower than what is routinely achieved, this may be an indication of unexpected experimental variation, and should be considered before accepting results.

- 4.6 Depending on the extent of the data analysis provided by your qPCR software, you may wish to export the qPCR data to a spreadsheet to carry out further calculations, as follows:
- Calculate the average concentration (from triplicate qPCRs) for each hgDNA sample dilution.
 - Take account of any dilution performed in **Sample Preparation** (step 2), to calculate the concentration of each undiluted sample—see **Working Example** (step 5).
- 4.7 Repeat steps 4.3 – 4.6 to generate the standard curves and calculate the sample concentrations for the 129 bp and/or 305 bp assays.
- 4.8 To perform a quality analysis of hgDNA samples, calculate Q-ratios as follows:
- **Q129/Q41 ratio:** For each sample, divide the concentration calculated using the 129 bp assay by the concentration calculated using the 41 bp assay.
 - **Q305/Q41 ratio:** For each sample, divide the concentration calculated using the 305 bp assay by the concentration calculated using the 41 bp assay.

5. Working Example

Two hgDNA samples were assessed using the KAPA hgDNA Quantification and QC Kit. One sample consisted of intact hgDNA, whereas the other contained hgDNA sheared to an average size of ~300 bp, representing damaged and/or fragmented DNA. The concentrations of both samples were determined using a NanoDrop spectrophotometer, prior to dilution and qPCR analysis (Table 3, row 1).

qPCR was performed in triplicate as described in **Reaction Setup** (step 3) using the 41 bp, 129 bp and 305 bp Primer Premixes. The following calculations were performed for the 41 bp, 129 bp, and 305 bp assays:

- Using the qPCR instrument software, the concentration of each diluted hgDNA sample was calculated from the appropriate standard curve as shown in **Data Analysis** (step 4.5). Values for each replicate qPCR are provided in Table 3, row 5).
- The average concentration of each diluted sample was calculated using a spreadsheet (see **Data Analysis** (step 4.6), and Table 3, row 6).
- Next, using the same spreadsheet, the average concentration of each undiluted sample was calculated (see **Data Analysis** (step 4.6), and Table 3, row 7).
- Finally, Q129/Q41 and Q305/Q41 ratios were calculated for each sample (see **Data Analysis** (step 4.8), and Table 3, rows 8 – 9).

The Q-ratios illustrate that high-quality, unfragmented hgDNA (represented by the intact DNA sample) yields Q129/Q41 and Q305/Q41 ratios approaching ~1.0, whereas damaged/fragmented hgDNA (represented by the sheared DNA sample) return lower Q ratio scores, with values <1. For such samples, it is expected that $Q129/Q41 \geq Q305/Q41$.

Data Interpretation

Absolute Quantification using the 41 bp Assay

The 41 bp qPCR assay offers an extremely sensitive and specific method for quantifying hgDNA. The small size of the target amplicon, combined with the robust performance of the engineered KAPA SYBR® FAST DNA Polymerase, provides reliable quantification of DNA, irrespective of quality. Nevertheless, extremely damaged DNA, or high levels of PCR inhibitors, will result in underestimated DNA concentrations when compared with mass-based methods such as spectrophotometry or electrophoresis. Even in such cases, concentrations returned by the qPCR assay are likely to be more relevant than non-qPCR-based measurements of total DNA in the context of NGS library construction, since DNA that cannot act as a PCR template is unlikely to produce viable NGS library material.

As discussed in **Important Parameters** (p. 3), the observed variance among sample replicates should be used to gauge the reliability and accuracy of the quantification data for specific samples.

Q-ratios and Quality Assessment

It is important to consider that Q-ratios are most informative (i.e., provide the highest resolution among samples of different quality) in the middle of their ranges between 0 and 1.0 (Figure 3). For this reason, one should determine which assay provides the best predictive power for the specific samples of interest and in the context of a particular NGS workflow and application. The Q129/Q41 ratio provides good discrimination among samples of relatively poor quality, and may yield values between 0.10 and 0.90; in contrast, the Q305/Q41 ratio is likely to be less informative for such low-quality samples, returning values close to zero. Similarly, Q305/Q41 ratios are likely to be more informative for relatively high-quality samples, where Q129/Q41 may approach 1.0 for all such samples.

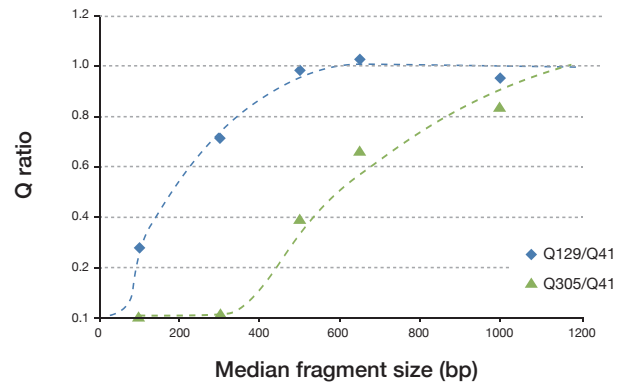


Figure 3. Q-ratios are most informative in the linear regions of their dynamic ranges

Q-ratios can be used to predict the success of library construction, and may therefore inform sample-specific processing and stop/go decisions. Unfortunately, because of the wide variety of samples and workflows, it is not possible to provide specific guidance for correlating Q-ratios with expected library construction and/or sequencing outcomes. The relationship between Q-ratios and sample processing outcomes must be determined empirically in the context of specific sample types and workflows.

Table 3. Working example of sample quantification and quality assessment

		Intact hgDNA			Sheared hgDNA		
1	Sample concentration (NanoDrop)	159 ng/μL			33 ng/μL		
2	Sample dilution—see Sample Preparation (step 2.1):	1 in 80			1 in 50		
3	Estimated concentration of diluted sample, based on NanoDrop:	1988 pg/μL			660 pg/μL		
4	Assay:	41 bp	129 bp	305 bp	41 bp	129 bp	305 bp
5	Triplicate concentration calculated by qPCR software (pg/μL):	2138 1938 2347	2034 2463 2254	2361 2173 1973	575 626 645	398 463 418	12.6 13.1 11.9
6	Average concentration of diluted sample (pg/μL):	2141	2250	2169	615	426	12.6
7	Average concentration of undiluted sample (ng/μL):	171	180	174	30.8	21.3	0.63
8	Q129/Q41:	180/171=1.05			21.3/30.8=0.69		
9	Q305/Q41:	174/171=1.01			0.63/30.8=0.02		

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