Understanding library quantification assays for next-generation sequencing applications

Introduction

Next-generation sequencing (NGS) has become an important method for applications ranging from genotyping to whole-genome sequencing. To perform an NGS experiment, users must prepare a sequencing library from a purified nucleic acid sample. Library preparation for Illumina™ NGS systems involves adaptation of the nucleic acid sample with two oligonucleotides, termed P5 and P7 adaptors, that can bind to a flow cell.

With the sequencing capacity of NGS instrumentation continuing to rise, researchers are able to pool more samples, or libraries, into a single sequencing run, greatly reducing the per-sample cost of sequencing. However, NGS library concentrations can vary widely, based on the amount and quality of nucleic acid sample inputs, as well as the target enrichment method that is used. In order to ensure that each pooled library is sequenced to the desired depth, NGS libraries must be carefully quantified and normalized so that each sample achieves the required number of reads.

Common library quantification methods include fluorometric spectroscopy and quantitative PCR (qPCR). While both methods provide relatively accurate measurement of library concentration, there are assay-specific considerations associated with these techniques. In this document, we provide a comparison of two library quantification technologies: the Invitrogen™ Qubit™ dsDNA HS Assay Kit and the Invitrogen™ Collibri™ Library Quantification Kit, which utilize qPCR or the Invitrogen™ Qubit™ Fluorometer, respectively (Table 1). We also provide a comparison of the Collibri Library Quantification Kit and the Roche KAPA™ Library Quantification Kit for Illumina platforms.

Table 1. Comparison of Qubit and Collibri library quantification assays.

	Qubit assay	Collibri assay
Specificity	Fluorophore preferentially binds dsDNA	Only library fragments containing P5 and P7 adaptors are measured
Accuracy	Within 15%	Within 10%
Sample input requirements	1–20 µL of diluted sample	4 µL of diluted sample
Hands-on time	5 min setup; 3 sec for each sample measurement	30 min setup (96-well plate)
Time to process 20 samples	8 min	90 min
Time to process 80 samples	25 min	90 min

The Qubit dsDNA HS assay is a fluorometric assay that uses dsDNA-binding dyes in order to accurately determine NGS library concentration and benefits from a simple workflow that takes just a few minutes per sample. While the Qubit dsDNA HS assay has good accuracy and sensitivity, the system is designed to read samples one at a time and thus the workflow does not scale well above 20–30 samples.

The Collibri Library Quantification Kit is a qPCR-based assay that specifically amplifies and quantifies Illumina sequencing libraries by amplifying the P5 and P7 adaptor sequences. With a 6-point standard curve, the Collibri Library Quantification Kit enables accurate and sensitive quantification across a broad range of library concentrations.

Overview of methods

Qubit dsDNA HS assay

The Qubit dsDNA HS assay utilizes a target-specific dye that emits fluorescence when bound to dsDNA. Unlike UV spectroscopy, which can overestimate sample concentration due to contaminants in a sample, the Qubit Fluorometer together with the Qubit dsDNA HS assay does not measure salts, nucleotides, or RNA that may be present. The Qubit assay is also much more sensitive than UV spectroscopy, as it is able to measure dilute DNA samples with significantly less noise.

The Qubit dsDNA HS assay utilizes two reference standards to generate a standard curve that is used to empirically determine the concentration of a sample. The assay can be used to measure samples between 10 pg/µL and 100 ng/µL of DNA. Samples with concentrations above this range have to be diluted and retested to ensure that they fall within the linear range of the reference standards. The Qubit dsDNA HS assay has good accuracy, with a coefficient of variation (CV) of <5% across a range of 0.5–200 ng/µL.

The Qubit dsDNA HS assay workflow is quick and easy. The protocol utilizes a simple mix-and-read format with incubation times of only a few minutes. Briefly, 1–20 µL of sample (or 1–10 µL of reference standard) is added to the Qubit working solution. Following a 2-minute incubation, samples are read and the DNA concentrations in the samples are calculated.

Considerations for the Qubit assay

There are a number of benefits to using the Qubit system for library quantification. The workflow is fast and efficient, making it easy to assay tens of samples in parallel. The built-in analysis software streamlines data calculation, providing library concentrations immediately after the assay is performed. Flexible sample input volumes and short incubation times allow for quick retesting of samples that might initially fall outside the range of the assay, but the amount of dsDNA in the sample can adversely affect the results.

Collibri Library Quantification Kit

The Collibri Library Quantification Kit is a qPCR-based assay that contains all of the reagents needed for the accurate quantification of Illumina sequencing libraries. The kit contains the Invitrogen™ SYBR™ Green dye–based Collibri Library Quantification Master Mix, Collibri DNA Standards, and Collibri Library Dilution Buffer.

Library quantification with the Collibri Library Quantification Kit is performed by using a set of 6 prediluted DNA reference standards to generate a standard curve, from which sample library concentrations can be empirically determined. The qPCR master mix, based on Invitrogen™ Platinum™ II *Taq* DNA Polymerase, contains primers targeting the Illumina P5 and P7 adaptor sequences. Platinum II *Taq* DNA Polymerase uniformly amplifies DNA fragments with variable GC content and fragment lengths, making it ideal for quantification of all types of sequencing libraries.

Assay setup is performed in a 96-well optical PCR plate, with master mix and 4 µL of diluted library (or standard) being added to each well, which is then run on a qPCR instrument. Due to the universal concentration of Invitrogen™ ROX™ Reference Dye, the Collibri Library Quantification Kit is compatible with all qPCR platforms. Once the run is completed, the average C_t score for each DNA standard is plotted to generate a standard curve in any data visualization software. The concentrations of diluted libraries are then calculated by absolute quantification using the standard curve.

Considerations for the Collibri assay

Compared to fluorometric approaches, qPCR assays enable greater specificity and accuracy in detecting properly adapted, amplifiable libraries that form clusters during sequencing.

The Collibri assay is very sensitive—supporting quantification of femtomolar amounts of fragment libraries. The input requirements are very low, typically only 4 µL of a diluted library sample with a concentration of >0.0002 pM. Specific amplification of library fragments with P5 and P7 adaptor sequences make this a universal assay for all types of Illumina libraries.

Experimental comparison of Qubit, Collibri, and KAPA assays

In order to assess the performance of Qubit, Collibri and KAPA assays, Illumina sequencing libraries were prepared from varying amounts of RNA input using several commercially available library preparation kits.

RNA sequencing libraries were prepared, library concentrations were measured utilizing either the Collibri or Qubit assay, and concentrations were compared across all samples. RNA input quantities between 100 ng and 1 µg were used with commercially available rRNA depletion and library preparation kits according to the manufacturer's instructions. This method resulted in 328–412 bp library fragments that were measured using Collibri and Qubit assays. In general, a high level of agreement was seen across both library quantification kits (Figure 1). Results from Collibri and KAPA qPCR-based library quantification assays were also highly correlated (Figure 2).

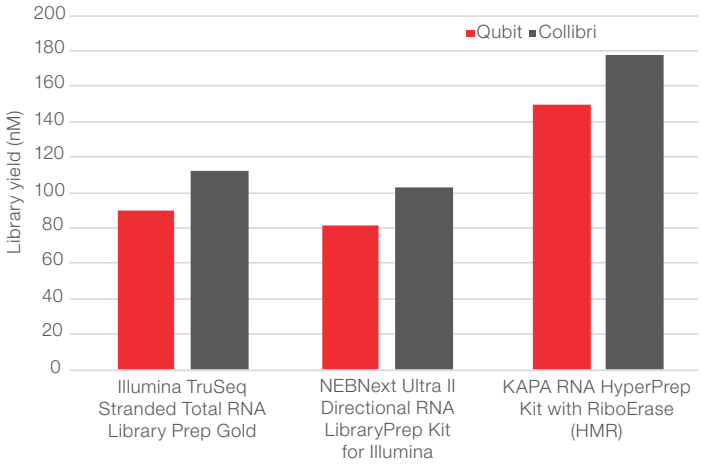

Figure 1. Measured library yields from control RNA input. RNA sequencing libraries were prepared from 100 ng of Invitrogen™ Universal Human Reference RNA (UHRR) according to the manufacturer's instructions. Library concentrations were measured utilizing either the Collibri or Qubit assays, and concentrations were compared across all samples.

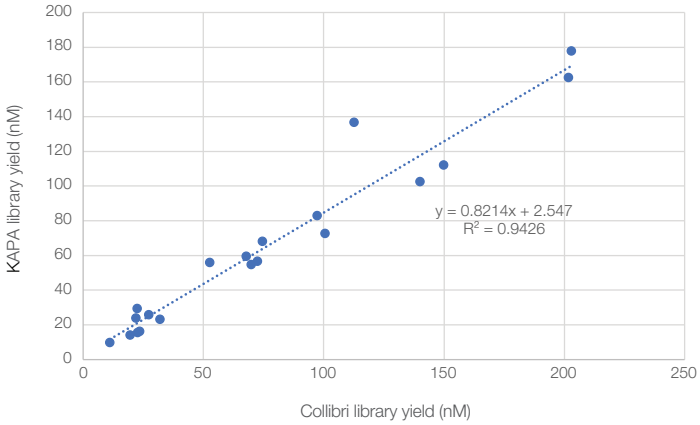

Figure 2. Collibri and KAPA quantification kits demonstrate a high level of correlation. RNA NGS libraries were generated using each manufacturer's standard recommendations using 100 ng–1 µg of input RNA from a range of sample types. All libraries were quantified using the Collibri Library Quantification Kit and KAPA Library Quantification Kit for Illumina platforms.

Conclusions

Library quantification is a crucial step in an NGS workflow, which helps ensure that each sample is sequenced to the desired depth. We have demonstrated that the Collibri Library Quantification Kit and the Qubit HS dsDNA Assay Kit can be used to precisely and accurately measure library concentrations ranging from 20 to 180 nM.

While underclustering due to overestimated library concentrations can result in diminished data output, overclustering can result in diminished quality scores and downstream analysis problems. Both the Collibri Library Quantification Kit and the Qubit dsDNA HS Assay Kit can be used to achieve optimal clustering in RNA sequencing workflows on Illumina NGS systems.

Having demonstrated equivalent results between these methods, there are key workflow considerations that users should be aware of when choosing a library quantification assay. The tighter precision of the qPCR-based Collibri assay makes it the ideal method to measure precious samples. The Qubit assay has slightly lower levels of precision, but the quick workflow and included quantitation software make it ideal for routine quantitation of libraries. Regardless of the assay that is chosen, users should utilize good laboratory technique to help ensure accurate measurement of library concentrations and high-quality Illumina sequencing data.

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Ordering information

Product	Quantity	Cat. No.
RNA-Seq kits		
Collibri Stranded RNA Library Prep Kit for Illumina*	24 preps	A38994024
	96 preps	A38994096
Collibri Stranded RNA Library Prep Kit for Illumina with H/M/R rRNA Depletion Kit*	24 preps	A39003024
	96 preps	A39003096
ERCC RNA Spike-In Mix	1 kit	4456740
ERCC ExFold RNA Spike-In Mixes	1 kit	4456739
Library quantification		
Collibri Library Quantification Kit*	100 rxns	A38524100
	500 rxns	A38524500
Qubit 4 Fluorometer	1 instrument	Q33238
Qubit 1X dsDNA HS Assay Kit	100 assays	Q33230
Qubit 4 NGS Starter Kit	1 kit	Q33240
Library amplification		
Platinum SuperFi Library Amplification Master Mix	50 rxns	A38539050
	250 rxns	A38539250
Platinum SuperFi Library Amplification Master Mix with Primer Mix*	50 rxns	A38540050
	250 rxns	A38540250
Purification		
PureLink RNA Mini Kit	25 preps	12183020
	250 preps	12183025
MagMAX *mir*Vana Total RNA Isolation Kit	96 preps	A27828
Thermo Scientific accessories		
KingFisher Flex Purification System with 96 Deep-Well Head	1 system	5400630
Applied Biosystems accessories		
Veriti 96-Well Thermal Cycler	1 instrument	4375786
ProFlex 96-Well PCR System	1 instrument	4484075
MicroAmp EnduraPlate Optical 96-Well Clear Reaction Plates with Barcode	20 plates	4483354
MicroAmp Optical 96-Well Reaction Plate	10 plates	N8010560
MicroAmp Clear Adhesive Film	100 films	4306311
MicroAmp 8-Tube Strip with Attached Domed Caps, 0.2 mL	125 strips	A30589

H = human, M = mouse, R = rat.
* Not all kits are available in all countries.

Find out more at **thermofisher.com/collibri**

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