A COMPETITIVE qPCR ASSAY ELIMINATES THE NEED FOR A CALIBRATION CURVE AND ENABLES STREAMLINED QUANTIFICATION AND NORMALIZATION OF TARGETED NEXT-GENERATION SEQUENCING LIBRARIES

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SUMMARY

- Complex workflows in next-generation sequencing (NGS) clinical applications have spotlighted the need for simple and integrated assay solutions.
- Accurate library quantification is a critical component of wet-bench NGS that balances sample-to-sample read coverage and optimizes available sequencing bandwidth.
- Current library quantification assays are limited by either non-specific quantification or require a standard curve, which increases both the costs and the hands-on time for the assay.
- Here we describe the development and performance of a streamlined, calibration-curve-free, competitive qPCR assay for the quantification and normalization of targeted NGS libraries.

INTRODUCTION

Library quantification guides sample loading onto the sequencing instrument, ensuring optimal utilization of NGS resources. Quantitative PCR (qPCR) is the method of choice for functional library assessments since it directly measures on-target amplifiable DNA, requires very low sample input, and provides high specificity and sensitivity. To facilitate streamlined library quantification within our comprehensive NGS workflows we developed a calibration-curve-free, library quantification assay that balances workflow efficiency and simplicity with reliable library quantification.

METHODS

An exogenous DNA standard was designed for use as an internal single-point reference[1] for the quantification of targeted NGS libraries using qPCR, hereafter referred to as library quant PCR (LQ PCR). Differences in cycle threshold (ΔCt) between the exogenous standard and the input library were used to determine the concentration for each sample library and subsequently guide the normalization of sample pooling. LQ PCR assay performance was compared to a direct quantification method (Quant-iT™ DNA Assay Kit (high sensitivity), Life Technologies) and to a common standard curve-based qPCR assay that relies on absolute quantification (KAPA Library Quantification Kit, KAPA Biosystems). The assay's capability to guide NGS loading was evaluated using sample uniformity within NGS runs. Source gDNA was derived from FFPE, fresh frozen, and plasma residual clinical samples as well as cell-line extracts. NGS studies were performed using the QuantideX® NGS Pan Cancer Kit[2,3] (Asuragen) and the MiSeq® system (Illumina).

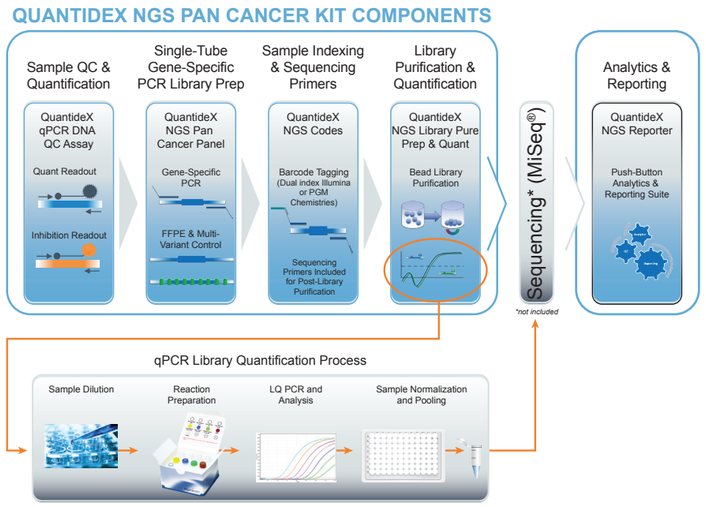

Figure 1. QuantideX Library Quant as part of Asuragen's integrated NGS workflow. The QuantideX NGS Pan Cancer Kit provides an all-in-one NGS solution. The LQ PCR assay is used after library purification and serves to guide sample pooling prior to sequencing.

RESULTS

Results comparing the correlation between LQ PCR and two common alternative library quantification methods on a set of 81 residual clinical samples are shown in Figure 3A. LQ PCR normalization is illustrated in Figure 3B. Repeatability results for LQ PCR are shown in Figure 4. Table 1 gives a side by side comparison of features offered by LQ PCR and 2 alternative methods.

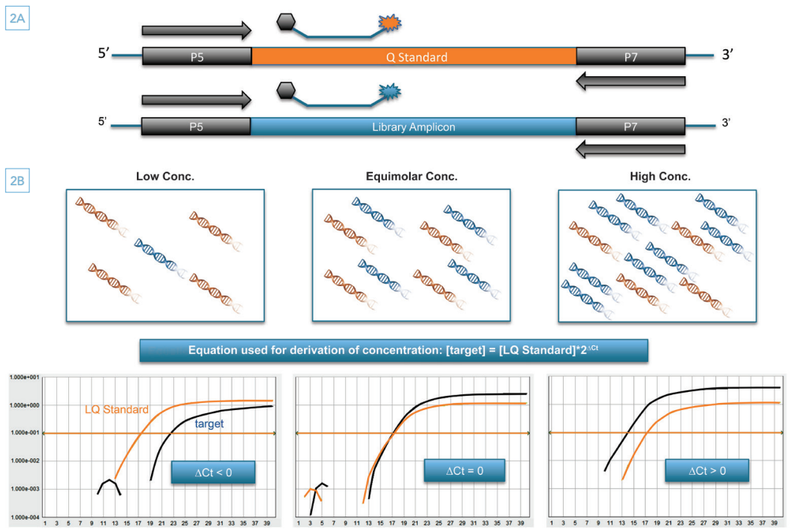

Figure 2. LQ PCR approach for library quantification. An exogenous DNA standard (orange) was designed for use as an internal single-point reference for the quantification of targeted NGS libraries (blue) within a two-color multiplex qPCR assay. A) The DNA standard is co-amplified with tagged library amplicons by priming off universal adapter sequences in a multiplex TaqMan® qPCR reaction. B) Differences in cycle threshold (illustrated in above qPCR amplification plots) between the exogenous DNA standard (orange) and the input library (black) were used to determine the concentration for each sample and subsequently guide normalization of sample pooling.

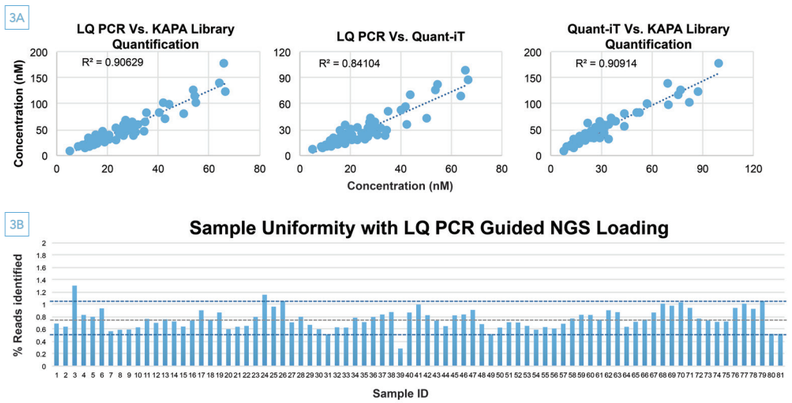

Figure 3. Correlation analysis between LQ PCR, KAPA Library Quant, and Quant-iT assays. A) Correlation analysis comparing LQ PCR, KAPA, and Quant-iT revealed high correlation with R^2 >0.84 (n=81). **B)** LQ PCR guided sample normalization resulted in sample-to-sample sequencing uniformity to within 1.45-fold from the median (gray-dotted line) for 95% of samples (blue-dotted lines).

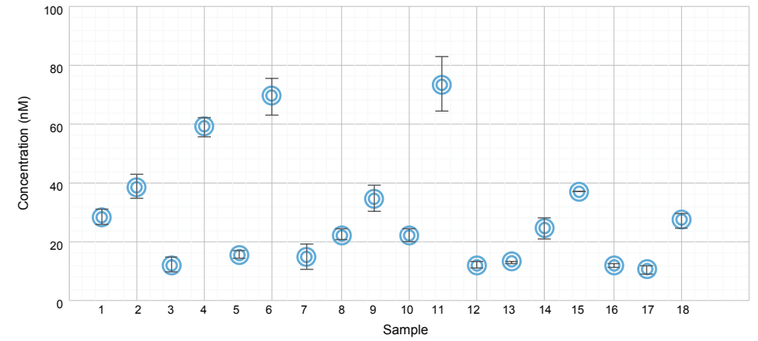

Figure 4. Repeatability analysis. A set of 18 samples was used for repeatability studies. Samples were run in 3 independent LQ PCR experiments by 2 different operators. Repeatability studies demonstrate assay precision with an average of 12% CV.

	LQ PCR	KAPA	Quanti-iT
Kit components	5	10	10
Number of pipetting steps*	54	127	98
Calibration standards/batch	1	18	24
Minimal sample input LOQ (pg/rxn)	0.65	0.01	5000
Calibration-curve-free	Yes	No	No
Quantification independent of template size	Yes	No	No
Specific to amplifiable molecules	Yes	Yes	No
Part of an integrated and optimized NGS workflow	Yes	No	No

*Pipetting steps for a batch of 8 samples

Table 1. Side-by-side comparison of 3 library quantification assays.

CONCLUSIONS

- LQ PCR guided normalization resulted in NGS read uniformity to within ± 1.45 fold from the median for 95% of samples, despite a 12.9-fold distribution in sample concentration.
- Comparisons of LQ PCR library quant with a non-specific fluorescent dye binding assay and a calibration-curve-based qPCR assay revealed significant correlation between approaches, with R^2 >0.84.
- The LQ PCR assay was performed in less than half the pipetting steps routinely required for alternative methods and eliminated the need to use an external calibration curve.
- The LQ PCR offers quantitative accuracy and consistency, simplifies library analysis, and provides a new tool for integration into comprehensive workflows for clinical NGS.

References and Acknowledgments

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