# Automated, Accurate and Cost-Effective Next-Generation Sequencing Library Quantification using a qPCR-based method

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## Abstract

Next-generation sequencing (NGS) is an ever-growing application crucial for a variety of DNA analysis applications. As the cost of NGS has decreased, scientists are now able to take on high-throughput applications that were not possible before. However, this means the cost of library prep and quality control becomes a bigger bottleneck. Several publications have demonstrated the reduced cost of library prep with the use of low-volume Acoustic Liquid Handlers.[1-3]

Library quantification using qPCR has become a popular method for library normalization. Several vendors (e.g. Agilent, Illumina, NEB, Roche, ThermoFisher) now offer reliable and standardized kits for DNA quantification to ensure accurate library representation. Here we have shown how the Echo 525 Acoustic Liquid Handler was successfully used to prepare the standard curve for a library quantification kit (KAPA Library Quantification Kit, Roche) at volumes as low as 3 µL, with no loss in accuracy or precision. This is a roughly 7-fold or 85% reduction in reaction volume and cost, while maintaining data quality, with CVs as low as 1% for the data points in the standard curve.


## Introduction

As the sequencing cost per base pair has decreased, high-throughput applications that were not possible before, such as microbial and single-cell sequencing, are on the rise.[1] Accurate and precise quantification of prepared NGS libraries is essential to getting equimolar pooling for successful multiplexed sequencing. This equimolar pooling is crucial for enhancing the depth of coverage at the genomic size level, as well as the number of reads covered. The qPCR-based library quantification methods are the most precise, accurate and sensitive compared to other spectrophotometric methods, as they are highly specific to NGS library molecules. The commercially available kits, typically include a master mix, a sequencing platform-specific library quantification primer premix, and a pre-diluted set of DNA standards. The pre-diluted standards are used to set up a standard curve, and the concentrations of the libraries are assessed based on that curve (Figure 1). Typically, each data point is repeated three times to ensure high reproducibility.

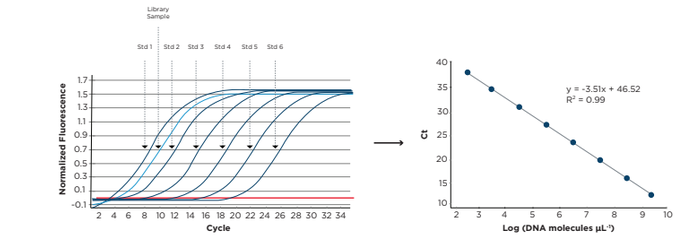


**Figure 1.** Generation of standard curve and quantification of library concentration

When performing high-throughput NGS library prep on a variety of samples, the number of qPCR reactions can easily reach a few hundred reactions per 96- or 384-sample plate. Therefore, use of automation to minimize errors and ensure high data quality enables reproducible reaction setups. While automated and traditional liquid handlers are great tools for setting up these reactions, the volumes recommended by kit manufacturers are usually around 20-25 µL to ensure high accuracy and precision of pipetting steps. However, as shown by other groups, the Echo 525 Acoustic Liquid Handler can set up qPCR reactions in a fraction of the volume with the same accuracy and precision or even better data quality, and up to a magnitude faster speed compared to tip-based liquid handlers. The Echo 525 Acoustic Liquid Handler can transfer microliters down to 25 or 2.5 nL (depending on the model), to enable fast, low-volume and low-cost sample preps for these quantification methods.[4]

## Echo Liquid Handlers

The Echo 525 Acoustic Liquid Handler uses Dynamic Fluid Analysis (DFA), to determine fluid composition, height and impedance in the source well. The required power needed to eject a droplet from the source well is then calculated within milliseconds. For the Echo 525 Acoustic Liquid Handler the 25 nL droplets are ejected from the source well to an inverted destination plate (Figure 2). By transferring a series of droplets, large volumes can be added in the destination plate at a rate of up to 6 µL/sec. The ability to rapidly transfer nanoliter-scale volumes into high-density microplates provides a fast way to automate and miniaturize library quantification, as well as normalization and pooling.

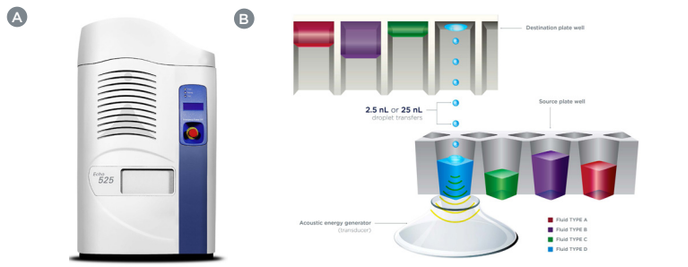


**Figure 2.** (A) Echo 525 Acoustic Liquid Handler. (B) The transducer is positioned below the source microplate well and emits focused sound energy repeatedly to the meniscus of the fluid to be transferred. A stream of 2.5 or 25 nL droplets (model dependent) is reliably ejected into a well of an inverted destination microplate, which also moves with the transducer. This will allow for a rapid transfer from any source well to any destination well for a variety of fluid types.

Here we used the Echo 525 to perform a proof of principle experiment to set up the standard curves at 20, 10, 5 and 3 µL volumes.

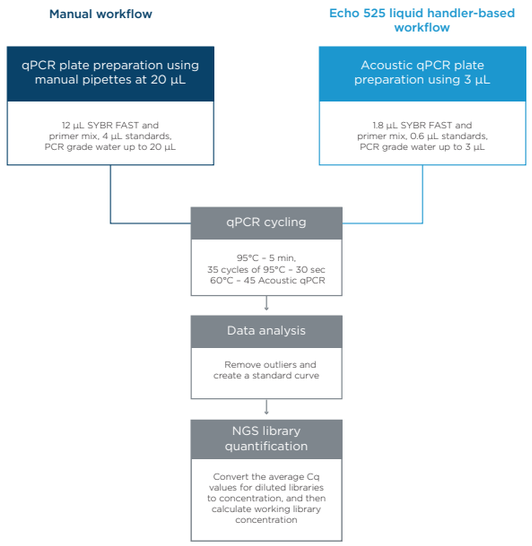


**Figure 3.** A comparison between manual protocol setup for qPCR-based quantification and an Echo 525 Acoustic Liquid Handler-enabled automated and miniaturized workflow.

## Methods

### Preparation of the reagents

Instruments, reagents and labware used in the experiment are listed in Table 1. Contents of the kit were thawed and vortexed at room temperature. The reagents were thawed and prepared quickly, while kept on ice, in order to avoid any degradation. Echo source plates were prepared by aliquoting the standards and reagents, using a manual pipette, into a 384PP 2.0 Echo source plate, and then spinning down the plates at 1500 x *g* for 5 min. Before transferring the samples and reagents on the instrument, they were brought to room temperature to ensure accurate and precise transfers on the Echo. The Echo 525 Acoustic Liquid Handler and Echo Plate Reformat Software were used to set up the qPCR reactions.

### Standard curve generation

Initial studies were conducted to identify the miniaturized reaction volume that ensured reliable standard curves and Cq values following qPCR amplification in the LightCycler 480. The the Echo 525 Acoustic Liquid Handler and Echo Plate Reformat Software were used to dispense the reagents and standards from an Echo Qualified 384-well Polypropylene Microplate using the 384PP_AQ_BP and 384PP_AQ_SP calibrations, respectively. 20, 10, 5 and 3 µL reactions were set up in 4 replicates in a Bio-Rad Hard-Shell 384-well Bio-Rad PCR plate. The 20 µL recommended qPCR protocol, as suggested by the manufacturer, and the reduced reaction volumes, were run in the LightCycler 480 with no optimization being necessary for the miniaturized reactions. Cq values obtained from this run were used to generate a standard curve using the quantification template provided by the vendor.

## Materials

| Equipment/Consumables | Manufacturer |
|---|---|
| Echo 525 Acoustic Liquid Handler | Beckman Coulter Life Sciences |
| Allegra X-14 Centrifuge | Beckman Coulter Life Sciences |
| LightCycler 480 System | Roche |
| KAPA Library Quantification Kit (KR0405 – v9.17) | Roche |
| 384-well PP Microplate (001-12380) | Beckman Coulter Life Sciences |
| Hard-Shell 384-well Bio-Rad PCR (#HSR4805K) | Bio-Rad Laboratories |
| Microseal 'C' PCR plate sealing film (#MC1001) | Bio-Rad Laboratories |

**Table 1.** Instrument, reagents and labware used

## Results

For the range of miniaturized reaction volumes tested, each displayed minimal variance, as evident by the low standard deviations and CVs, (Table 2). This kit can be miniaturized and automated with up to 85% reduction in reaction volumes, while maintaining excellent results in terms of $R^2$ value for the generated standard curve (Figure 4).

| | 20 µL | | | 10 µL | | | 5 µL | | | 3 µL | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Average Cq | Std Dev | CV (%) | Average Cq | Std Dev | CV (%) | Average Cq | Std Dev | CV (%) | Average Cq | Std Dev | CV (%) |
| Std 1 | 8.75 | 0.07 | 0.8 | 8.46 | 0.15 | 1.8 | 8.45 | 0.20 | 2.4 | 8.58 | 0.05 | 0.6 |
| Std 2 | 12.14 | 0.05 | 0.4 | 11.87 | 0.03 | 0.3 | 11.87 | 0.08 | 0.7 | 11.99 | 0.10 | 0.8 |
| Std 3 | 15.57 | 0.02 | 0.1 | 15.42 | 0.10 | 0.6 | 15.39 | 0.15 | 1.0 | 15.43 | 0.12 | 0.8 |
| Std 4 | 19.13 | 0.03 | 0.2 | 18.73 | 0.05 | 0.3 | 18.76 | 0.09 | 0.5 | 18.76 | 0.04 | 0.2 |
| Std 5 | 22.55 | 0.06 | 0.3 | 22.28 | 0.03 | 0.2 | 22.38 | 0.11 | 0.5 | 22.30 | 0.12 | 0.6 |
| Std 6 | 26.08 | 0.10 | 0.2 | 25.97 | 0.09 | 0.3 | 26.00 | 0.33 | 1.3 | 26.30 | 0.13 | 0.5 |

**Table 2.** The data reflects the tight standard deviations and low CVs for the miniaturized reaction volumes, assembled in the 384-well PCR plate via an Echo 525 Acoustic Liquid Handler.

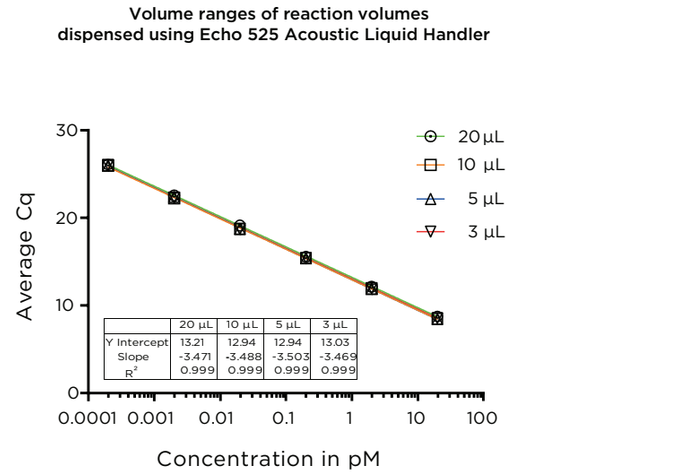


**Figure 4.** Standard curve on logarithmic scale (log 10) for four total reaction volumes tested: 20, 10, 5 and 3 µL. $R^2$ values were determined to be greater than 0.99 for each condition tested.

## Conclusions

Using an Echo 525 Acoustic Liquid Handler, this qPCR-based quantification kit was automated accurately and precisely while reducing the reaction volumes by 85%, from the standard 20 µL down to 3 µL. The data indicates high reproducibility (low standard deviations and low CVs) and good linearity ($R^2$) of the standard curves across different volume ranges. The data can be extrapolated to the quantification of the NGS libraries.

The qPCR-based methods for NGS library quantification are typically more accurate and precise compared to other methods (e.g. fluorometric or spectrophotomeric). However, due to the sensitivity of the method, accuracy and precision of pipetting to set up the qPCR reactions is critical. By using the highly accurate and precise, tip-less technology of the Echo 525 Acoustic Liquid Handler this kit can be successfully automated while reducing reaction volumes.

This helps save on costs and time required to set up reactions for quantifying libraries for Illumina and Ion Torrent platforms.

## Future work

While in this work we focused on the preparation of the standards, which are representative of the library samples, in the future we will present data on samples being analyzed using the same method for a variety of NGS libraries.

## References


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