Effective Miniaturization of Small Whole Genome Next-Gen Sequencing by Utilizing Illumina® Nextera XT Library Preparation with Echo 525® Liquid Handler

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Introduction

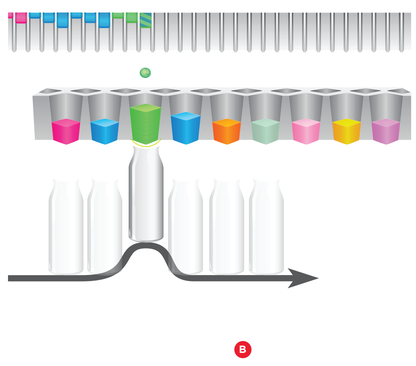
The cost of sequencing per nucleotide has been declining by orders of magnitude in the past ten years, due to continuous improvement in next-generation sequencing technologies. However, library preparation of samples remains a bottleneck for reducing sequencing costs. In synthetic biology, whole genome sequencing is becoming a routine process to validate genome editing, deconvolute mutagenesis campaigns, confirm high-throughput DNA construction, and more. Current efforts to sequence small genomes (bacterial, fungal) using an Illumina Nextera XT based workflow are constrained by minimum working volumes needed for manual processing and once processed, only a small fraction of the library generated is used for the sequencing run. By utilizing the Echo 525 liquid handler with the Nextera XT kit, we perform the reactions at a miniaturized scale that reduces excessive library generation, thus saving costs on expensive reagents. In addition, running smaller volume tagmentation reactions reduces not only reagent volumes but also the amount of sample gDNA required. We demonstrate that the tagmentation reaction can be scaled from 25 µL to 1.25 µL, and the amplification reaction can be scaled from 50 µL to 2.5 µL. These libraries were multiplexed on an Illumina MiSeq paired-end sequencing run and shown to have equivalent quality metrics. The nanoliter-precision and speed of the Echo 525 liquid handler enables accurate miniaturization of Nextera XT library preparation, thus reducing cost and increasing throughput while providing high quality sequencing data. Furthermore, we show that the Echo 525 liquid handler can scale the Quant-iT Picogreen DNA quantification assay from 200 µL to 20 µL, as well as enable library fragment analysis on the Agilent Tapestation 2200 for accurate and high-throughput sample transfer. Lastly, the Echo 525 liquid handler enabled us to normalize and pool our libraries for sequencing at a speed unrivaled by any tip-based liquid handler.

The Echo Liquid Handler

The Echo 500 series liquid handlers revolutionize liquid transfer by using acoustic energy to eject fluids. Transfer with Echo Liquid Handlers is completely touchless—no tips or nozzles, and no material contacts the sample as it moves from source to destination. This protects the integrity of samples and precious reagents while providing additional cost savings and eliminating waste, carry-over effects and cross contamination. The Echo 525 Liquid Handler can transfer in 25 nL increments to allow miniaturization with accuracy and precision.

A

Figure 1A. The Echo 525 Liquid Handler



B

Figure 1B. Echo 525 Liquid Handlers have a transducer that emits low energy sound waves to eject 25 nL droplets from a source plate to an inverted destination plate above.

Materials

Equipment	Manufacturer
Echo® 525	Beckman Coulter Life Sciences
Allegra® X-14 Centrifuge	Beckman Coulter Life Sciences
MixMate®	Eppendorf
Qubit®	ThermoFisher
TapeStation 2200	Agilent
BMG PHERAstar	BMG Labtech
ProFlex™ PCR System	ThermoFisher
384-well Post Magnet Plate	Alpaqua
MiSeq®	Illumina

Consumables	Manufacturer	Part Number
384-well PP Microplate	Beckman Coulter Life Sciences	#P-05525
384-well LDV Plus Microplate	Beckman Coulter Life Sciences	#LPL-0200
TapeStation Plate	Agilent	#5067-5150
Qubit Microtube	ThermoFisher	#Q32856
384-well PCR Plate	Bio-Rad	#HSP3805
384-well Black Flat Clear-Bottom Microplate	Greiner	#781096
1.5 mL DNA LoBind Tubes	Eppendorf	#022431021

Reagents	Manufacturer	Part Number
NexteraXT™ DNA 96-Sample Prep Kit	Illumina	FC-131-1096
NexteraXT™ Index Kit v2 Set	Illumina	FC-131-2001
PhiX Control v3	Illumina	FC-110-3001
TapeStation D1000 HS Ki	Agilent	#5067-5584, #5067-5585
Qubit® dsDNA HS Assay Kit	ThermoFisher	#Q32851
Quant-iT™ Picogreen® dsDNA Assay Kit	ThermoFisher	#P11496
Agencourt® AMPure® Beads	Beckman Coulter	#A63881
200 Proof Ethanol	Sigma Aldrich	#E7023
MiSeq Reagent Kit v3 (600-cycle)	Illumina	MS-102-3003

Methods

NexteraXT Library Preparation

The *E.coli* K-12 genome was selected for this study, because of its widespread use in academic and industrial settings. Its representative "small genome" size allows for a high degree of multiplexing conditions of library prep on one MiSeq run. All samples were run in technical replicates. The Echo 525 liquid handler was used to transfer differing amounts of the same sample gDNA into a variety of tagmentation reaction volumes (see **Table 1**), then was also used to precisely dispense the appropriate TD + ATM buffer volumes, except for the 25 µL manual control. 55°C tagmentation incubation was performed in the thermocycler, once for each volume size. To neutralize tagmentation, NT buffer was dispensed using the Echo 525 liquid handler. PCR amplification of tagmented libraries were performed at a panel of volumes (see **Table 1**). Indexing primers were then dispensed utilizing a hit-pick worklist loaded into the Echo 525 liquid handler. Nextera PCR Mix (NPM) was accurately dispensed at varying amounts using the Echo 525 liquid handler. Amplification was run on a reaction volume basis following the NexteraXT PCR conditions. Libraries were then cleaned up manually utilizing SPRI bead cleanup at a ratio of 0.6x. Each library was then quantified via the Picogreen assay. The Echo 525 liquid handler was utilized in this assay to dispense the samples, as well as Picogreen reagents to a miniaturized 20 µL reaction in a Greiner 384-well plate, and results were read on the BMG PHERAstar. Each library also underwent fragment size analysis via the TapeStation 2200. The Echo 525 liquid handler was used to dispense sample and reagent into the assay plate. Using a combination of quantification data and average fragment size data, we generated a normalization worklist. The Echo 525 liquid handler was then able to simultaneously normalize and pool the libraries together by transferring small, accurate, and precise amounts of libraries into one well. This pooled library was then quantified via Qubit, normalized to 4 nM, then loaded onto an Illumina MiSeq instrument for a 2x300 run. Data was then analyzed in the Biomatters Geneious software, aligned to *E.coli* K-12 MG1655 reference genome from NCBI.

Results

Input DNA	Tagmentation Volume	PCR Volume	Fragment Size	Coverage		Mean Q-Score	> Q30
				Average	StDev		
1 ng	25	25	419	9	4.5	33.7	82.70%
1 ng	25	2.5	410	8.5	4.6	33.5	81.80%
0.5 ng	12.5	6.25	372	8.4	4.6	34.4	85.50%
0.25 ng	6.25	6.25	378	9.4	5	33.9	83.70%
0.05 ng	1.25	2.5	335	9.7	5.5	34.1	84.40%

Table 1. Individual library conditions and resulting coverage and quality

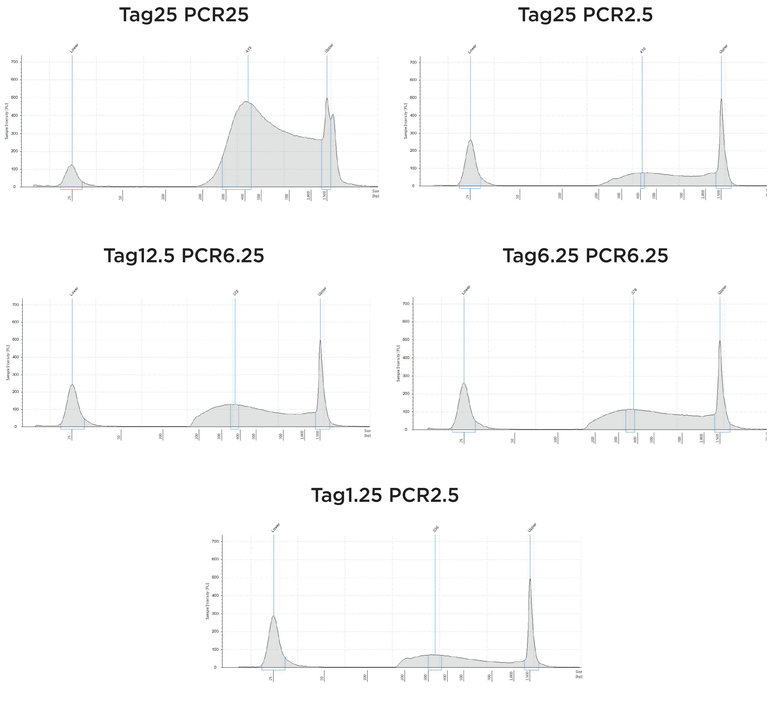


Figure 2. Electropherograms of library conditions tested. Averaged fragment length is 383 with a right tail, which is appropriate for 2x300 reads. Size selection cutoff was performed using 0.6x SPRI beads.

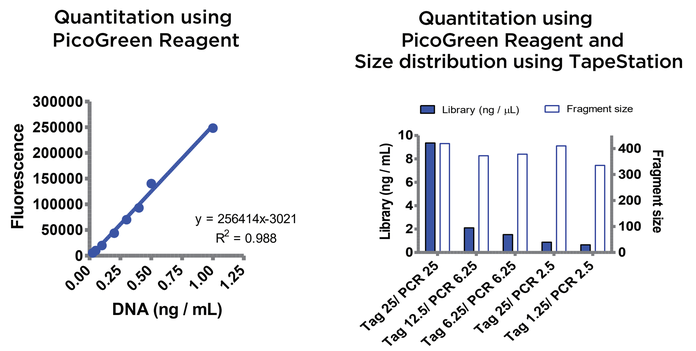


Figure 3. Picogreen DNA quantification results. Standard curve was generated using lambda DNA from 25pg/µL to 1 µg/µL. Results agree with TapeStation fragment analysis. Yield dropoff is expected due to reduced PCR volume, and is shown to be reproducible.

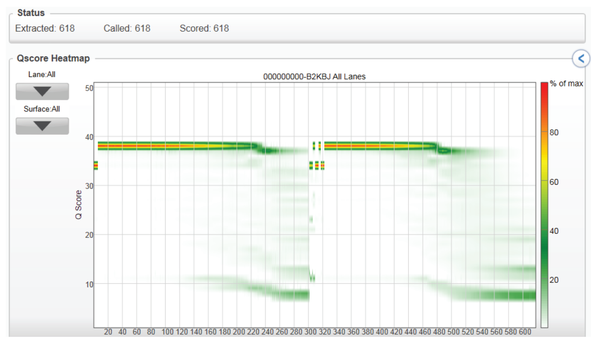


Figure 4. Quality read scores per cycle of the entire multiplexed MiSeq run, demonstrating that various reaction volumes can be run together and produce quality scores similar to conventional 2x300 genomic runs.

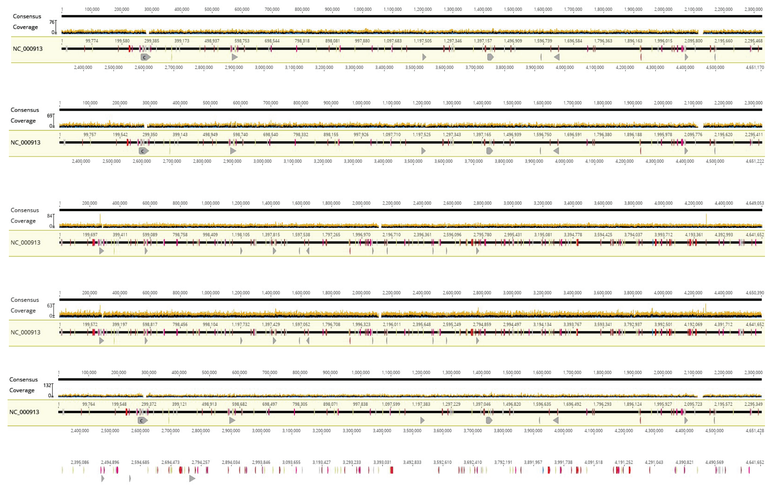


Figure 5. Coverage graphs from Geneious. From top to bottom: Tag25/PCR25, Tag25/PCR2.5, Tag12.5/PCR6.25, Tag6.25/PCR6.25, and Tag1.25/PCR2.5. Coverage is highly equivalent regardless of reaction volumes used. *E.coli* K-12 gDNA from ATCC was aligned to E.coli K-12 MG1655 reference genome from NCBI.

Summary

- The Echo 525 Liquid Handler enables miniaturization of reaction volumes to prepare libraries using the Illumina Nextera XT DNA library prep kit. We show that the tagmentation can be reduced 20-fold from 25 µL to 1.25 µL, and that the PCR can be reduced 20-fold from 50 µL to 2.5 µL.
- Sample input, tagmentation volume, PCR reaction volume, and Picogreen assay can be miniaturized using the Echo liquid handler to increase throughput and reduce costs.
- TapeStation QC analysis can be streamlined for time efficiency by utilizing the Echo 525.
- Sequencing of the libraries prepared by these methods demonstrated equivalent quality metrics when compared to the manual reaction.

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