Scaling Workflows for Growing Microbiome Applications

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Introduction

Our understanding of the role of the human microbiome in health and disease has been growing rapidly in recent years and is being increasingly elucidated every day. Amplicon sequencing of highly conserved 16S ribosomal RNA (rRNA) regions has long been the standard technique used to assess microbiome diversity in a patient, however there are limitations to this method. 16S rRNA amplicon sequencing only captures bacterial diversity, and misses fungal and viral components of the microbiome. While a separate amplicon sequencing of the ITS1 region captures fungal diversity, there is no known parallel technique for viruses. Furthermore, these rRNA amplicon methods are generally only specific to the genus, and to obtain accurate species information, different variable regions of the rRNA need to be assessed in repeated experiments. Even with that, important strain information is never ascertained, in which strain-to-strain variation is responsible for pathogenicity, toxins, virulence factors, epitopes, and antibiotic resistance characteristics. Here we show that the Echo® 525 Liquid Handler can be used to miniaturize both 16S rRNA amplicon sequencing, as well as metagenomic whole-genome sequencing (WGS). This miniaturization makes WGS cost-competitive with 16S rRNA amplicon sequencing, while providing much greater amounts of information. In addition, running smaller volume tagmentation reactions reduces the amount of sample gDNA required. As researchers continue to deepen our understanding of human microbiome implications on health and disease, our tools and analyses need to scale accordingly. We demonstrate that the Echo 525 Liquid Handler can miniaturize whole-genome sequencing of the microbiome, and can cost-effectively replace 16S rRNA amplicon sequencing to meet the demands of this new era of microbiome research.

Echo® Liquid Handler

Echo® 525 LIQUID HANDLER

The Labcyte Echo 500 series liquid handlers revolutionize liquid transfer by using acoustic energy to eject fluids. Transfer with the Echo Liquid Handler 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.

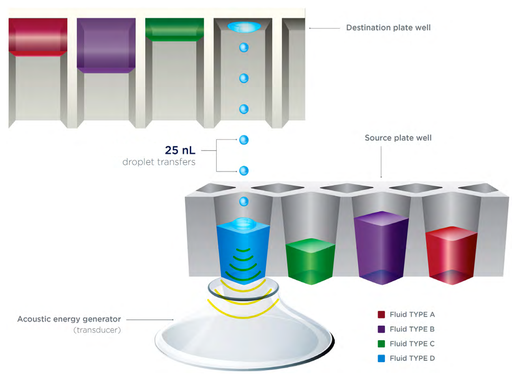

FIGURE 1: The Echo 525 Liquid Handler has a transducer that emits low energy sound waves to eject 25 nL droplets from a source plate to an inverted destination plate above.

Materials

Equipment	Manufacturer
Echo® 525 Liquid Handler	Labcyte Inc.
Allegra® X-14 Centrifuge	Beckman Coulter
MixMate®	Eppendorf
Qubit®	Thermo Fisher
TapeStation 2200	Agilent
BMG PHERAstar	BMG Labtech
ProFlex™ PCR System	Thermo Fisher
384-well Post Magnet Plate	Alpaqua
MiSeq®	Illumina

Consumables	Manufacturer	Part Number
384-well PP Microplate	Labcyte Inc.	#P-05525
384-well LDV Plus Microplate	Labcyte Inc.	#LPL-0200
TapeStation Plate	Agilent	#5067-5150
Qubit Microtube	Thermo Fisher	#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 A	Illumina	#FC-131-2001
PhiX Control v3	Illumina	#FC-110-3001
KAPA HiFi HotStart ReadyMix (2X)	KAPA Biosystems	#KK2602
16S rRNA V4 Region Primers	Integrated DNA Technologies	Custom Oligos
TapeStation D1000 HS Kit	Agilent	#5067-5584, #5067-5585
Qubit® dsDNA HS Assay Kit	Thermo Fisher	#Q32851
Quant-iT™ Picogreen® dsDNA Assay Kit	Thermo Fisher	#P11496
Agencourt® AMPure® Beads	Beckman Coulter	#A63881
200 Proof Ethanol	Sigma Aldrich	#E7023
MiSeq Reagent Kit v3 (600-cycle)	Illumina	#MS-102-3003

Methods

16S rRNA Amplicon Sequencing

A 10-organism microbial community standard from Zymo Research (D6305), containing 8 bacterial and 2 fungal strains, was selected for this study. In addition, a negative control (Saccharomyces cerevisiae, ATCC 204508D-5) and a positive control (Staphylococcus aureus, ATCC 25923D-5) were run in parallel. All samples were run in technical replicates. The Echo 525 Liquid Handler was also used to transfer gDNA, KAPA HiFi 2x Master Mix, water, and the V4 region primers (5' – TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGYCAGCMGCCGCGGTAA) (3' – GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACNVGGGTWTCTAAT) into a PCR plate for a total reaction volume of 5 µL. First stage PCR was performed following the Illumina 16S Metagenomic Sequencing protocol, then SPRI bead cleanup was performed manually. Then, product from this PCR was transferred via the Echo 525 Liquid Handler into another PCR plate, along with KAPA HiFi 2x Master Mix, water, and the Nextera XT Index primers, totaling 5 µL reaction volume. Second stage PCR was performed to append the Illumina sequencing primers on the amplicons, then SPRI bead cleanup was performed manually. The Echo 525 Liquid Handler was then used to transfer samples and reagents for Picogreen quantitation and TapeStation fragment analysis. The Echo 525 Liquid Handler was also utilized to create a standard curve, and results were read on the BMG PHERAstar. The Echo 525 Liquid Handler was then able to simultaneously normalize and pool the libraries together by transferring accurate, precise, and variable volumes of libraries into one well. This pooled library was then quantified via Qubit, normalized to 10pM, a concentration of 10% PhiX was spiked in for a low-diversity library, then loaded onto an Illumina MiSeq instrument for a 2x300 run. Data was then analyzed in the Illumina BaseSpace App, "16S Metagenomics."

Whole Genome Sequencing

The same Zymo microbial community standard, positive and negative controls were run through a 10x-miniaturized Illumina Nextera XT protocol. All samples were run in technical replicates. The Echo 525 Liquid Handler was used to normalize and transfer the sample gDNA into a 2.5 µL tagmentation reaction, then was used to precisely dispense the appropriate TD + ATM buffer volumes. 55ºC tagmentation incubation was performed in the thermocycler. To neutralize tagmentation, NT buffer was dispensed using the Echo 525 Liquid Handler. PCR amplification of tagmented libraries was performed at a miniaturized volume of 5 µL. Indexing primers were dispensed utilizing a hit-pick worklist loaded into the Echo 525 Liquid Handler, and Nextera PCR Mix (NPM) was accurately dispensed as well. Amplification was run according to the Nextera XT protocol. Libraries were then cleaned up manually utilizing SPRI bead cleanup at a ratio of 0.6x. Each library was then quantitated via the Picogreen assay. Using a combination of quantitation 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 accurate, precise, and variable volumes of libraries into one well. This pooled library was then quantified via Qubit, normalized to 20pM, then loaded onto an Illumina MiSeq instrument for a 2x300 run with 1% PhiX. Data was then analyzed in the Biomatters Geneious software, aligned to their respective reference sequences from NCBI.

Results

Organism*	Reported GC	Measured GC	gDNA Abun. (%)	Measured Abun. (%)	Reads
Pseudomonas aeruginosa	66	67	12	14	209,673
Escherichia coli	57	53	12	16	242,562
Salmonella enterica	52	53	12	20	297,457
Lactobacillus fermentum	53	53	12	11	162,806
Enterococcus faecalis	38	41	12	8	113,919
Staphylococcus aureus	33	38	12	8	126,185
Listeria monocytogenes	38	41	12	8	124,416
Bacillus subtilis	44	46	12	12	179,820
Saccharomyces cerevisiae	38	39	2	2	27,137
Cryptococcus neoformans	48	48	2	3	46,946
Source: ZymoBiomics D6305				**Total Reads**	**1,487,546**

TABLE 1: Metagenomic whole-genome sequencing data. Reads were successfully aligned to reference genomes. The given GC and organism abundance was compared to the measured. Process bias is low, despite it being an enzymatic process.

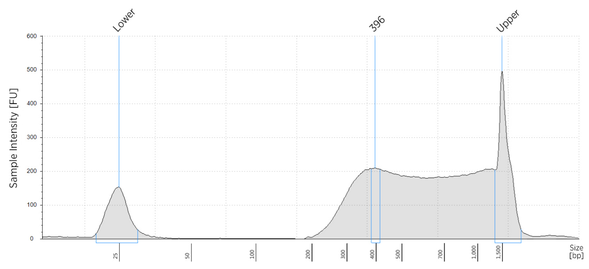

FIGURE 2: TapeStation electorpherogram of metagenomic whole-genome sequencing after library prep and magnetic bead cleanup. Size selection cutoff was performed using 0.6x SPRI beads. Average fragment length is about 396bp with a right tail, appropriate for 2x300 reads.

Results

16S rRNA Amplicon Sequencing

		Reference		Sample gDNA Abundance (%)		
Source	Organism	gDNA Abun. (%)		2.5 ng input	1 ng input	0.5 ng input
ZymoBiomics D6305	*Pseudomonas aeruginosa*	12		7.50	7.28	6.95
	Escherichia coli	12		2.10	2.05	2.01
	Salmonella enterica	12		1.60	1.56	1.52
	Lactobacillus fermentum	12		18.70	18.41	17.94
	Enterococcus faecalis	12		7.00	7.20	7.60
	Staphylococcus aureus	12		15.50	15.19	15.50
	Listeria monocytogenes	12		11.60	11.78	12.32
	Bacillus subtilis	12		18.20	18.64	19.25
	Saccharomyces cerevisiae	2		0	0	0
	Cryptococcus neoformans	2		0	0	0
	Total Reads			**382,386**	**360,318**	**341,417**
Positive Control	*Staphylococcus aureus*	100		94.55	95.36	94.71
	Total Reads			**305,673**	**313,864**	**258,883**
Negative Control	*Saccharomyces cerevisiae*	100		0	0	0
	Bacterial	0		3.08	3.10	2.99
	Total Reads			**9,066**	**6,988**	**7,251**

TABLE 2: 16S rRNA amplicon sequencing data post-analysis from Illumina BaseSpace App "16S Metagenomics." Identification was based on genus-level filtering. Species data was highly inaccurate, and would require variable region optimization in repeated runs. Furthermore, no fungal data was detected using this method.

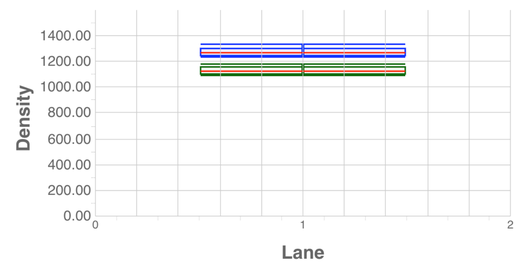

FIGURE 3: MiSeq sequencing statistics showing cluster density for 16S rRNA amplicon sequencing.

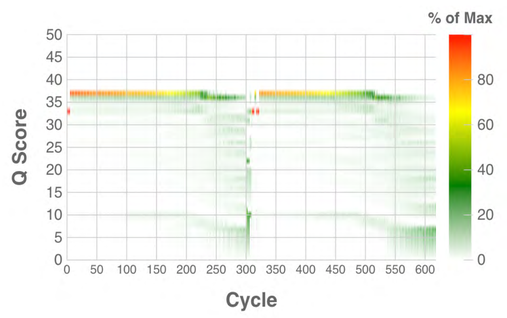

FIGURE 4: MiSeq sequencing statistics for 16S rRNA amplicon sequencing. Shown above is the Q-Score per cycle. The 16S rRNA amplicon sequencing is extremely low diversity, and 10% PhiX was spiked in to achieve these metrics.

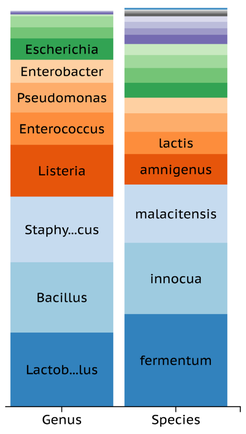

FIGURE 5: Genus and species-level classification from 16S rRNA sample data. Analysis used was Illumina BaseSpace app "16S Metagenomics." Variable region surveyed was V4. Organism abundance should be even. Strain classification is highly incorrect when compared to the known reference. Obtaining correct strain identification would require surveying and analyzing multiple variable regions in the 16S rRNA gene.

Summary

- **The Echo 525 Liquid Handler enables miniaturization of 16S rRNA amplicon sequencing as well as Nextera XT library prep, saving reagent cost, time, and valuable sample input.**
- **Samples prepared through whole-genome sequencing are accurate down to the species and strain level while 16S rRNA amplicon sequencing provided reliable data only to the genus level.**

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