Efficient Plasmid Validation By Multiplexing and Miniaturizing Illumina Nextera XT with the Echo 525 Acoustic Liquid Handler

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Introduction

Synthetic biology is an interdisciplinary science with the potential to impact several academic and industrial applications including the creation of novel therapeutics and vaccines, plant science and biofuels, as well as bio-based chemical manufacturing capabilities that involves the application of engineering principles to biology. The ability to read and write DNA is foundational to synthetic biology. With modular design of DNA components and miniaturized reaction volumes, a dramatic increase in DNA construction throughput is possible. The ability to read these constructs with nucleotide resolution remains a crucial component of the workflow. The Illumina Nextera® XT DNA Kit (Figure 1) has a robust chemistry that is able to generate the needed sequence coverage for plasmid construct validation on an Illumina MiSeq®.

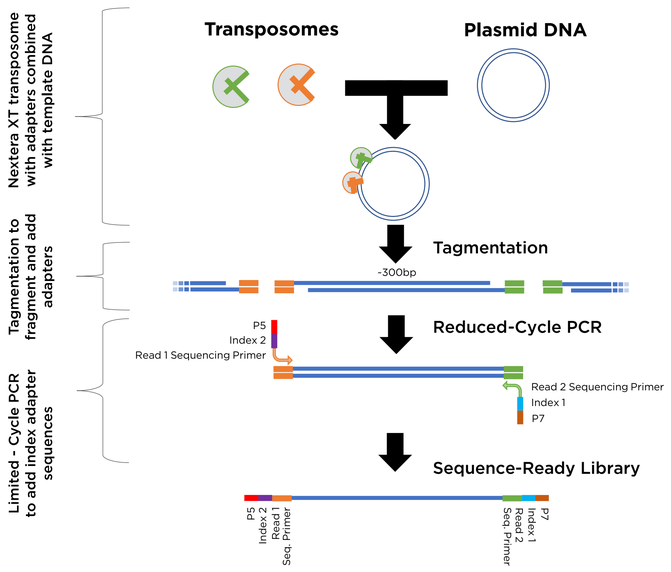


Figure 1. Illumina Nextera® XT DNA Sample Prep Kit chemistry workflow used to generate a dual-indexed linear piece of DNA capable of being sequenced on an Illumina MiSeq.

The recommended total reaction volume for each plasmid in the Nextera XT protocol is 50 µL. In this experiment, the Echo 525 Liquid Handler will be used in combination with the Illumina Nextera® XT DNA Kit and MiSeq® at ten-fold reduced volumes to obtain sequence confirmation for mutated, modular DNA constructs. The Echo 525 Liquid Handler is able to reach these minute volumes due to the accurate acoustic generation of 25 nL droplets of reagents (Figure 2). Beyond the acoustic technology, the flexible arraying capability present in the Echo 525 Liquid Handler is needed to quickly hit-pick individual wells for library pooling. The arraying speed is markedly increased from what is possible in a typical NGS pipeline with transfer volumes between 5–6 µL/sec and without tip-loading steps. By using the Illumina Nextera XT DNA Kit and MiSeq in combination with an Echo 525 Liquid Handler, plasmids are quickly sequenced at the nanoliter scale.

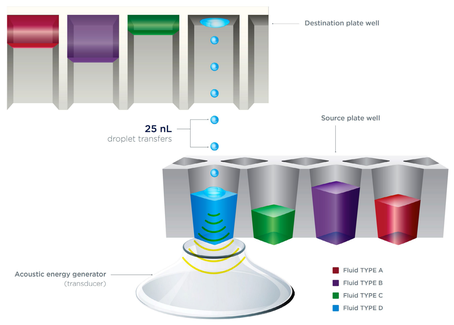


Figure 2. Represents the acoustic technology and parts used in an Echo 525 Liquid Handler to enable transfer of discrete 25 nL droplets between a source and destination plate.

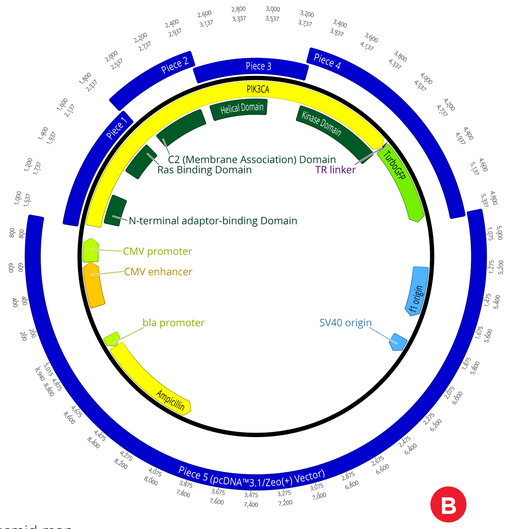
Echo® 525 Acoustic Liquid Handler

Materials and Methods

Plasmid Design

Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) is a commonly studied gene in human oncology. This gene was chosen to demonstrate a real world application of an Echo system-enabled miniaturization of the NEBuilder® HiFi kit. PIK3CA was divided into four pieces based on functional domains of interest, tagged with c-terminal GFP, and designed to assemble into a KpnI digested pcDNA™3.1/Zeo(+) Vector with 23-25 base pair DNA overlaps (Figure 3A). Three domains were designed to contain the activating mutations R38H, E542K, E545K, or H1047R (Figure 3B red). Through the interchanging of modular pieces, PIK3CA constructs with wild-type sequence along with the mutations R38H, E542K, E545K, or H1047R were generated.

A



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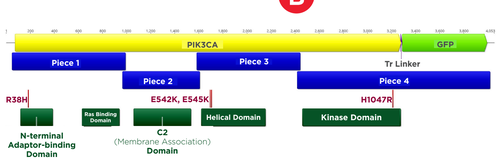

Figure 3. A: A plasmid map generated using Geneious showing the PIK3CA gene (yellow), five of the known protein domains (dark green), and the GFP tag (light green). The five assembly pieces are shown as the outermost blocks (blue).
B: Enlarged map of Pieces 1-4 showing the locations of the R38H, E542K, E545K, and H1047R mutations in red.

Construct Generation

Pieces 1-4 and their mutant analogs were synthesized by IDT as gBlocks®. These pieces were all PCR amplified using Q5® High-Fidelity DNA Polymerase from NEB on an Applied Biosystems ProFlex® for 25 cycles to generate additional material. Piece 5 was purchased as the circular vector pcDNA 3.1/Zeo(+) from Thermo Fisher. The restriction enzyme KpnI-HF was used to digest the plasmid to generate a piece with the corrrect overhangs. The linearized Piece 5 and PCR amplified pieces 1-4 were all subsequently cleaned using a NEB Monarch® PCR Cleanup Kit. All of the pieces were then visualized and quantitated. Using the Echo 525 Liquid Handler, constructs were arrayed using 1.25 fmol of DNA in a 500 nL NEBuilder HiFi DNA assembly reaction as seen in Table 1. Upon reaction completion, the entire reaction volume was transformed into chemically competent NEB 10-Beta® cells and plated onto LB+Carb100 agar plates. 192 isolated clones were cultured and their construct plasmid recovered using a standard MN NucleoSpin® 96 Plasmid Kit.

NEBuilder HiFi Modular DNA Assembly Reaction Volumes

Reagent	DNA conc. (ng/µL)	ng DNA /1.25 fmol	WT Reaction (µL)	R38H Reaction (nL)	E542K Reaction (nL)	E545K Reaction (nL)	H1047R Reaction (nL)	Echo Calibration
Piece 1 WT	23.6	16	25		25	25	25	384LDV_Plus_AQ_GP
Piece 1 R38H	25	16		25				
Piece 2 WT	14.6	11	50	50	50	50	50	
Piece 3 WT	7.69	14	75	75			75	
Piece 3 E542K	10	14			75			
Piece 3 E545K	10	14				75		
Piece 4 WT	29.6	26	50	50	50	50		
Piece 4 H1047R	30	26					50	
Piece 5	83.9	83	50	50	50	50	50	
NEBuilder Master Mix			250	250	250	250	250	

1 hr 50°C Incubation (Reaction in Thermocycler with Heated Lid)

Table 1. The different volumes of reagents by the Echo 525 Liquid Handler at a final volume of 500 nL to allow for a miniaturization of a modular DNA assembly using the NEBuilder HiFi kit.

Miniaturized Plasmid Tagmentation and Indexing

The Quant-iT™ Picogreen® dsDNA Assay Kit was used to quantify the amount of plasmid present from the plasmid recovered. The Echo 525 Liquid Handler was used to dilute the eluted plasmid DNA, standard and dye reagent for a total reaction volume of 10 µL in a 384 well plate to be read on the BMG Pherastar®. Based on these results, the DNA was normalized to provide 0.1 ng per tagmentation reaction. Illumina Nextera XT DNA tagmentation reaction and indexing reactions were then generated as shown in the 1/10 Reaction Volume Column in Table 2.

1

Tagmentation	Miniaturized (µL/rxn)	Echo Calibration
Plasmid DNA Sample	0.5	384PP_AQ_BP
TD Buffer	1	384PP_AQ_GP
ATM	0.5	384PP_AQ_GPSB
Total volume	**2**	

Spin @ 1500 x g, 1 minute -> Tagmentation reaction -> 55°C (5 min)-> 10°C (forever)

2

Add NT	Miniaturized (µL/rxn)	Echo Calibration
Tagmentation reaction	2	From previous (no transfer)
NT Buffer	0.5	384PP_AQ_SPHigh
Total volume	2.5	

Spin @ 1500 x g, 1 minute -> Incubate 5 min RT

3

Amplification	Miniaturized (µL/rxn)	Echo Calibration
Tagmentation reaction	2.5	From previous (no transfer)
Indexing Primer 1 (100 µM) (N7XX)	0.05	384PP_AQ_BP
Indexing Primer 2 (100 µM) (55XX)	0.05	384PP_AQ_BP
diH_2O	0.9	384PP_AQ_BP
NPM	1.5	384PP_AQ_GPSB
Total volume	**5**	

4

PCR Reaction		
72°C	3 min	
95°C	30 sec	
95°C	10 sec	14 x
55°C	30 sec	
72°C	30 sec	
72°C	5 min	
4°C	forever	

Table 2. Ten-fold miniaturized reaction volumes of the Nextera XT kit tagmentation and indexing reactions as compared to the standard protocol volumes. The Echo Calibration used on each transfer is listed in the rightmost column.

Library Cleanup and Pooling

After tagmentation and indexing, Agencourt AmpureXP beads were used to clean up the reactions. The SPRI bead cleanup followed the Agencourt AmpureXP PCR purification protocol for 384-well format. We utilized 9 µL of beads for our miniaturized 5 µL indexing amplification reaction, eluted in 20 µL, and removed 15 µL with care not to disturb the beads. Quantitation was performed to verify the quantity of each sample using the Picogreen quantitation assay again as previously described. Fragment size analysis was performed on the Agilent TapeStation® 2200 with a TapeStation D1000 HS Kit. Using the concentration data from the Picogreen assay, as well as fragment size information from the TapeStation 2200, we built a normalization worklist in Excel to obtain equimolar pooling of samples. The Echo 525 Liquid Handler was then used to simultaneously pool and normalize the libraries. The pool was then denatured and diluted to 20 pM, loaded with 1% PhiX control, and run on an Illumina MiSeq specifying 2x75 reads.

Results

Illumina MiSeq Sequencing

Upon completion of the Illumina MiSeq 2x75 read run, the run metrics were checked in the BaseSpace Sequence Hub. The metrics were all acceptable as seen in Figure 4 with the percent of data above Q30 at 96.2%.

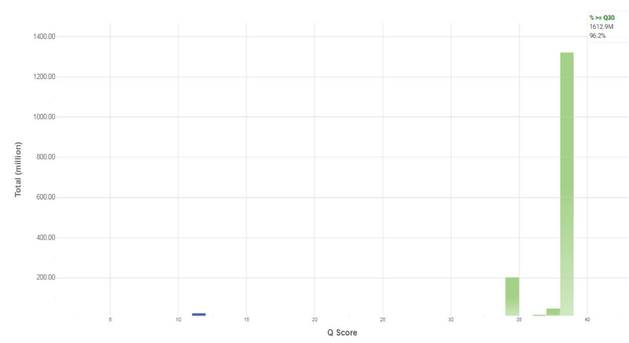

Figure 4. Q-score data generated from the Illumina MiSeq plasmid validation run.

Sequence Alignment

FASTQ files were generated on the MiSeq and aligned to an unmutated PIK3CA using Geneious® 11.0 (Figure 5). The consensus sequence between each construct and the unmutated PIK3CA is shown in gray at the top of each alignment. As expected, the wild-type mutant had no discrepancies, while each of the mutants were found in the correct locations as shown by the black bars present within the consensus map. These locations are expanded to the right of each alignment to highlight the correct amino acid change. Correct PIK3CA constructs were obtained for each of the five desired assemblies.

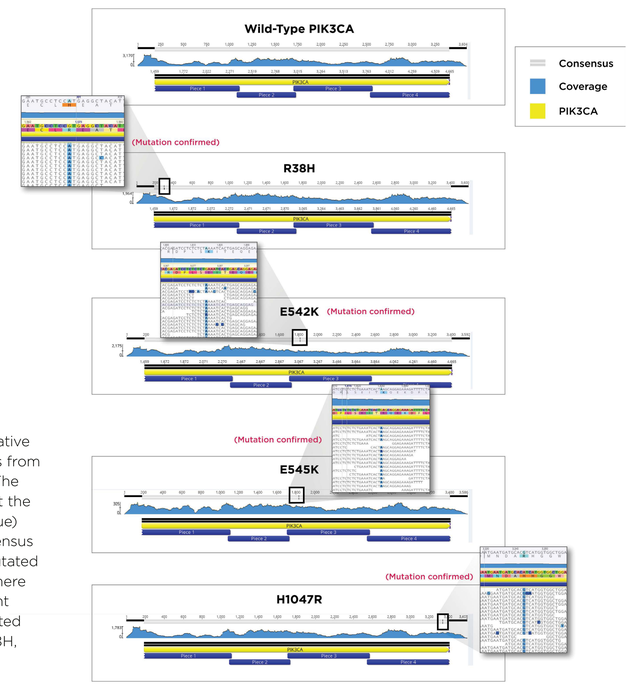

Figure 5. Representative Geneious alignments from MiSeq FASTQ files. The coverage is shown at the top of the graph (Blue) along with the consensus sequence with unmutated PIK3CA. Note that there are mutations present in each of the expected locations for the R38H, E542K, E545K, or H1047R constructs.

Summary

- Modular DNA assemblies can be generated at a 500 nL (40x reduced) scale using the NEBuilder HIFI kit in combination with the Echo 525 Liquid Handler.
- A ten-fold reduced Nextera XT plasmid verification protocol can be used with the Echo 525 Liquid Handler.

AAG-8001PST10.20