



# Echo System-Enhanced SMART-Seq v4 for RNA Sequencing

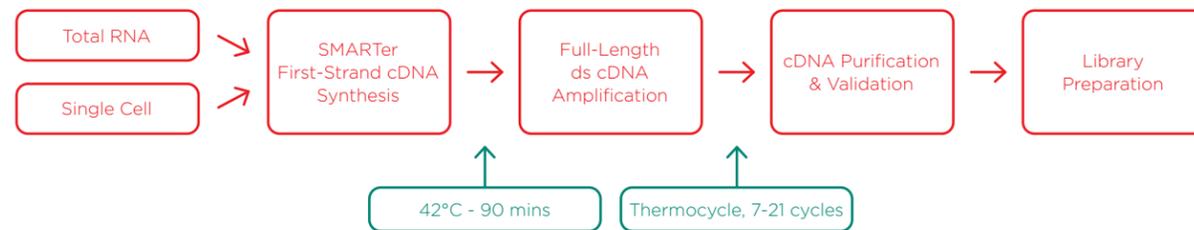
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## Introduction

As the cost of sequencing has continued to decline by orders of magnitude in the past ten years, scientists are enabled to ask deeper and more complex questions about transcriptomics. Consequently, the variety, quantity, and demands of RNA sequencing experiments have all increased. The Clontech SMART-Seq v4 Ultra Low Input RNA Kit incorporates the SMART (Switching Mechanism at 5' End of RNA Template) technology. This technology utilizes the template switching activity of reverse transcriptases to add PCR adapters directly to both the 5' and 3' ends of the first-strand cDNA before amplification using LD PCR. Here, we document information and results pertaining to the miniaturization of this process utilizing the Echo 525 Liquid Handler, effectively reducing the reagent cost and processing time for the workflow, addressing current throughput needs of RNA sequencing.

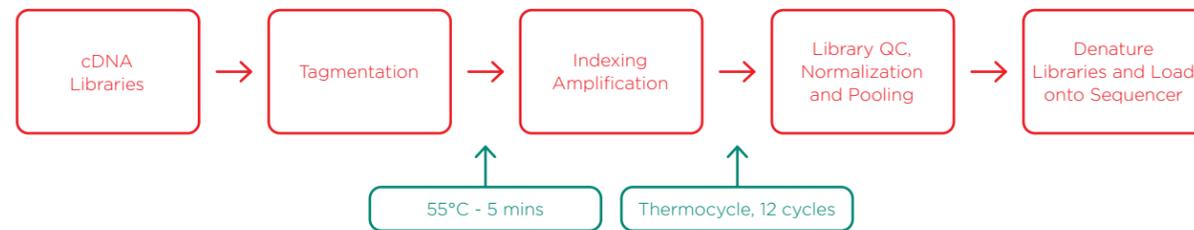
## Overview

### SMART-Seq v4 Workflow to Generate cDNA Libraries



In this experiment, we aimed to cover conditions for applications ranging from total RNA of a cell population to single-cell RNA sequencing. We tested a combination of reducing reaction volume, varying amounts of sample input, and varying the number of PCR cycles, looking for optimal parameters for quality sequencing for various situations. For this experiment, human brain total RNA was used as the input sample. We utilized the Echo 525 Liquid Handler to transfer small, accurate, and precise amounts of sample and reagents throughout the workflow to achieve miniaturized reaction volumes. Details of this method are described in the methods section.

### Nextera XT Library Preparation Workflow to Generate Libraries for Sequencing



After QC of the cDNA library generated from the SMART-Seq v4 workflow, we processed the samples through Illumina Nextera XT library preparation for sequencing. Miniaturized Nextera XT conditions have been previously optimized, as detailed in Application Note G121, as well as the publication “Low-Cost, high-throughput sequencing of DNA assemblies using a highly multiplexed Nextera process.” Shapland et al. ACS Synth. Biol., 2015. The Echo 525 Liquid Handler was used throughout this workflow to transfer small, accurate, and precise amounts of sample and reagents to achieve miniaturized reaction volumes. Details of this method are described in the methods section.

## Method

### SMART-Seq v4 Workflow

Reaction volumes tested: 1/4, 1/5, 1/8, 1/10, 1/16, 1/20, 1/32

Input RNA (human brain total RNA) amounts tested: 10pg, 30pg, 100pg, 300pg, 1000pg

Number of PCR cycles tested: 6, 9, 12, 15, 18, 21

Making 10x Reaction Buffer	Protocol (μL/rxn)	1/4	1/5	1/8	1/10	1/6	1/20	1/32
10x lysis buffer	19	4.75	3.8	2.375	1.9	1.1875	0.95	0.6
RNase inhibitor (40U/μL)	1	0.25	0.2	0.125	0.1	0.0625	0.05	0.05
<b>Total volume</b>	<b>20</b>	<b>5</b>	<b>4</b>	<b>2.5</b>	<b>2</b>	<b>1.25</b>	<b>1</b>	<b>0.65</b>

Mix and spin @ 1500g, 1 minute

Lysis	Protocol (μL/rxn)	1/4	1/5	1/8	1/10	1/6	1/20	1/32	Echo Calibration
10x reaction buffer	1	0.250	0.200	0.125	0.100	0.075	0.05	0.05	384PP_AQ_SP_High
Cells or purified RNA sample	1	0.250	0.200	0.125	0.100	0.075	0.05	0.05	384PP_AQ_BP
dH <sub>2</sub> O	8.5	2.125	1.700	1.075	0.850	0.55	0.45	0.275	384PP_AQ_BP
<b>Total volume</b>	<b>10.5</b>	<b>2.625</b>	<b>2.100</b>	<b>1.325</b>	<b>1.050</b>	<b>0.7</b>	<b>0.55</b>	<b>0.375</b>	

Spin @ 1500g, 1 minute → Incubate 5 minutes, room temperature

Add CDS Primer	Protocol (μL/rxn)	1/4	1/5	1/8	1/10	1/6	1/20	1/32	Echo Calibration
3' SMART-Seq CDS Primer II A (12 μM)	2	0.5	0.4	0.25	0.2	0.125	0.1	0.075	384PP_AQ_BP

Mix and spin @ 1500g, 1 minute → Incubate at 72°C in a preheated, hot-lid thermal cycler for 3 minutes → Snap cool on ice for 2 minutes, spin

1st Strand cDNA Synthesis	Protocol (μL/rxn)	1/4	1/5	1/8	1/10	1/6	1/20	1/32	Echo Calibration
5x ultra low first strand buffer	4	1	0.8	0.5	0.4	0.25	0.2	0.125	
SMART-Seq v4 oligonucleotide (48 μM)	1	0.25	0.2	0.125	0.1	0.075	0.05	0.025	
RNase inhibitor (40 U/μL)	0.5	0.125	0.1	0.075	0.05	0.05	0.025	0.025	
SMARTscribe reverse transcriptase (100 U/μL)	2	0.5	0.4	0.25	0.2	0.125	0.1	0.075	
<b>Total volume / reaction</b>	<b>7.5</b>	<b>1.875</b>	<b>1.5</b>	<b>0.95</b>	<b>0.75</b>	<b>0.5</b>	<b>0.375</b>	<b>0.25</b>	<b>384PP_AQ_GPSa</b>

Mix gently and spin → RT reaction → 42°C (90 min) → 70°C (10 min) → 4°C (forever) → Spin @ 1500g, 1 minute

cDNA Amplification	Manual (μL/rxn)	1/4	1/5	1/8	1/10	1/6	1/20	1/32	Echo Calibration
2X SeqAmp PCR buffer	25	6.25	5	3.125	2.5	1.5625	1.25	0.8	
PCR Primer IIA (12 uM)	1	0.25	0.2	0.125	0.1	0.0625	0.05	0.075	
SeqAmp DNA polymerase	1	0.25	0.2	0.125	0.1	0.0625	0.05	0.075	
dH <sub>2</sub> O	3	0.75	0.6	0.375	0.3	0.1875	0.15	0.1	
<b>Total volume / reaction</b>	<b>30</b>	<b>7.5</b>	<b>6</b>	<b>3.75</b>	<b>3</b>	<b>1.875</b>	<b>1.5</b>	<b>1.05</b>	<b>384PP_AQ_GPSb</b>

PCR Reaction	Time	Input amount RNA (ng)	Input number of cells	Typical number of PCR cycles
95°C	1 min	10	1000	7-8
98°C	10 sec	1	100	10-11
65°C	30 sec	0.1	10	14-15
68°C	3 min	0.01	1	17-18
72°C	10 min			
4°C	forever			

SPRI bead cleanup was performed on the cDNA product, to minimize any interference with the Nextera XT process. SPRI bead cleanup followed the Agencourt AmpureXP PCR purification protocol for 384-well format. We utilized 9μL of beads for our 5μL cDNA amplification reaction, and eluted in 20μL.

Reproducible sample preparation is dependent on the quality of the input material. Evaluation of starting total RNA quality is important. Sequence complexity and average length of the cDNA are adversely affected by quality and quantity of the starting material. When using FACS sorting single cells, avoid transferring the culture medium into the lysis buffer. Where possible wash and resuspend cells in PBS prior to lysis to avoid inhibition of the first-strand cDNA synthesis reaction.

During second strand cDNA synthesis determine the optimal number of PCR cycles for your starting material. It is not uncommon for the number to increase by 3-5 cycles above the recommended number, as optimal conditions do vary amongst cell types.

Library quantitation was performed to verify cDNA libraries and to normalize input into the Nextera XT process. The Picogreen fluorescence-based quantitation assay was used to determine the concentration of cDNA products. The Picogreen protocol was performed according to the ThermoFisher standard protocol, but volumes were miniaturized 1/100 from 2mL to 20μL in a Greiner 384-well clear-bottom plate. The Echo 525 Liquid Handler was used to dispense cDNA samples and Picogreen reagents into the Greiner 384-well plate. Results were read on the BMG Pherastar spectrophotometer.

## Nextera XT Workflow

Tagmentation	Protocol (μL/rxn)	1/10	Echo Calibration
cDNA sample	5	0.5	384PP_AQ_BP
TD buffer	10	1	384PP_AQ_GP
ATM	5	0.5	384PP_AQ_GPSB
<b>Total volume</b>	<b>20</b>	<b>2</b>	

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

Add NT	Protocol (μL/rxn)	1/10	Echo Calibration
Tagmentation reaction	20	2	From previous
NT buffer	5	0.5	384PP_AQ_SPHigh
<b>Total volume</b>	<b>25</b>	<b>2.5</b>	

Spin @ 1500g, 1 minute → Incubate 5 min RT

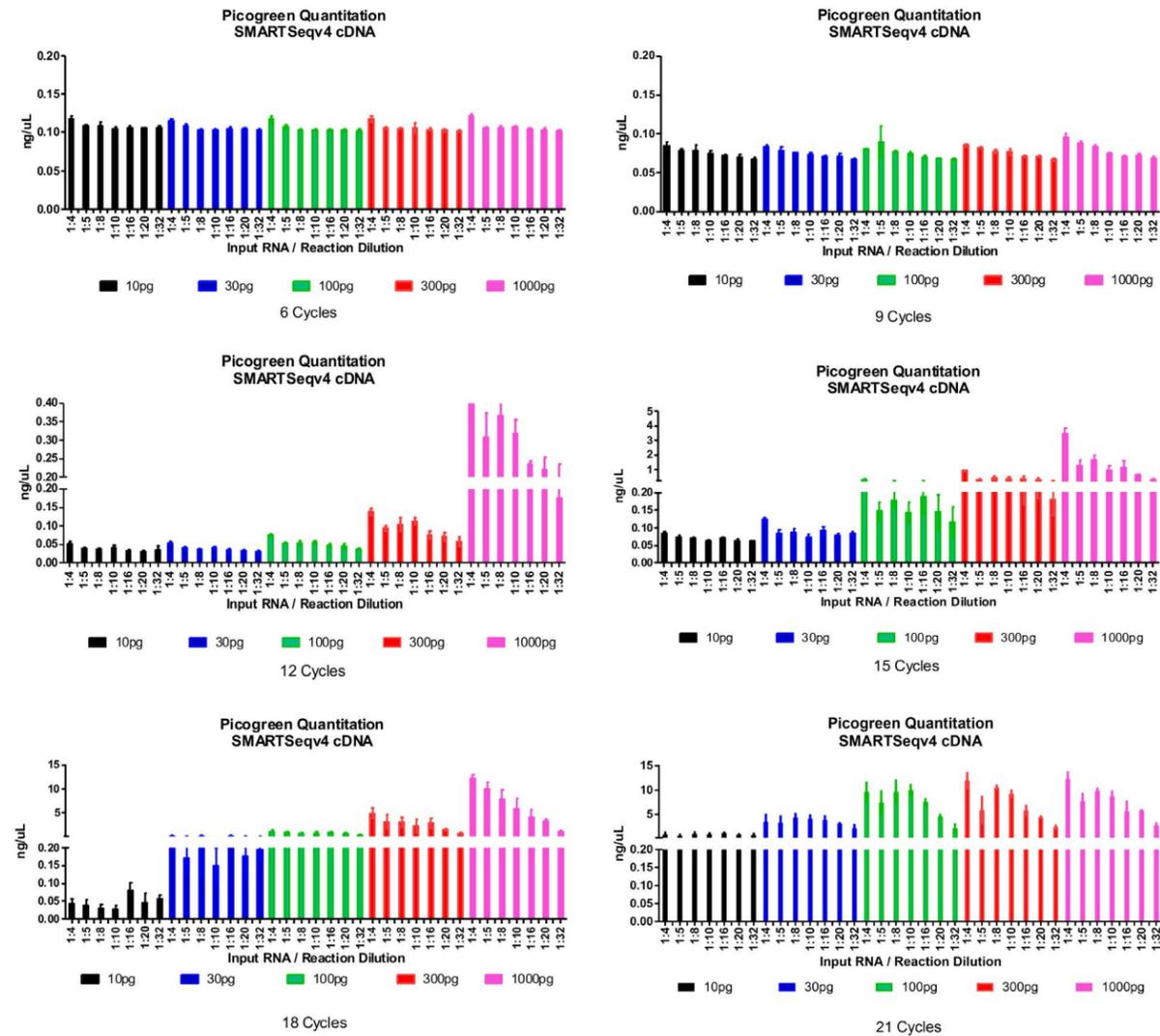
PCR Reaction	Time
72°C	3 min
95°C	30 sec
95°C	10 sec
55°C	30 sec
72°C	30 sec
72°C	5 min
4°C	forever

Library QC is necessary to verify the quantity of each sample, and fragment analysis must be considered when normalizing, pooling, and diluting for MiSeq loading. Picogreen quantitation assay was performed again as described above. Fragment size analysis was performed on the Agilent TapeStation 2200, according to the Agilent standard protocol. The Echo 525 Liquid Handler was used to transfer cDNA samples and reagents into the Agilent 96-well destination plate, then loaded and read in the TapeStation 2200.

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. Normalization can be accomplished simultaneously during pooling using the Echo 525 Liquid Handler and the worklist, in minutes. Normalization is a function of the desired amount of reads allotted to each sample. For our purposes, we seek even distribution of reads to all samples.

## Results and Discussion

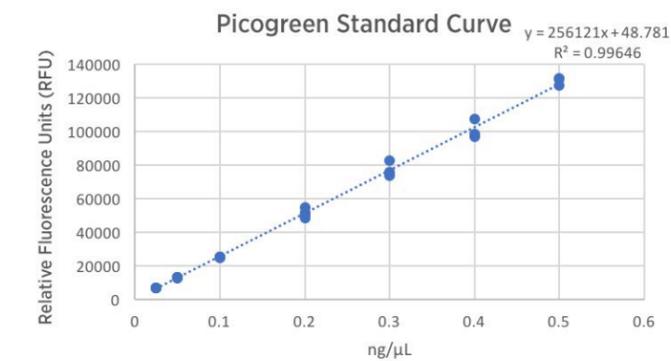
After running our matrix of sample conditions (miniaturization, reducing input, varying cycles) through the SMART-Seq v4 process, we then performed SPRI bead cleanup and quantitated all cDNA libraries. We were aiming to achieve a minimum cDNA library concentration of 0.2ng/μL, so that we have at least 0.1 ng total DNA going into a 2.5μL tagmentation reaction. In the graphs below, we have set the bottom portion of the graph to 0.2ng/μL, our QC threshold for sample minimum concentration. An additional graph stacked on top of the bottom portion indicates the quantity of those libraries that passed the 0.2ng/μL threshold for our Nextera XT library preparation workflow.



**Figure 1:** Picogreen quantitation of the cDNA libraries after SMART-Seq v4 and SPRI bead cleanup processes. Data is organized by number of thermocycles, input RNA, and reaction miniaturization amount. The bottom graph represents the quantity of each sample condition (replicated) and 0.2 ng/μL QC threshold for proceeding to Nextera XT. The top graph represents the total quantity of each sample condition (replicated) beyond the threshold mark. We found that 18x cycling is a good condition for most input RNA amounts and reaction sizes while minimizing amplification bias. Cycling does need to be increased to start from single-cell (10pg).

We picked all samples from the 18x cycle plate, and processed them through our Echo Liquid Handler-enhanced Nextera XT workflow. While cDNA libraries from 10pg input RNA did not produce the minimum amount of DNA for Nextera XT, we added max volumes (0.5μL) and ran them anyway. These samples are at risk of producing smaller fragments or not producing a Nextera XT library at all. We chose the 18x cycle plate because it was the cycling condition that was most permissive of input RNA and reaction size while minimizing amplification bias. Based on our demonstrated matrix, a few conditions can be chosen for your process depending on your input sample and desired miniaturization. After sequencing, a small portion of these libraries failed to produce enough reads (>100,000) for the RNA Express workflow on Illumina BaseSpace. To begin with 10pg input RNA, more cycling is necessary for sufficient library and read generation.

Nextera XT reagents and cDNA libraries were transferred in 1/10 volume of the recommended protocol by the Echo 525 Liquid Handler. After the Nextera XT library preparation, samples were cleaned up using SPRI beads, then QC'd using Picogreen quantitation and TapeStation 2200 fragment analysis.



**Figure 2:** Picogreen standard curve generated by using the Echo 525 Liquid Handler. The instrument is able to perform accurate and precise transfers of the lambda DNA used in this standard curve by direct dilution, avoiding propagation of errors in serial dilution.

**Picogreen Quantitation Data - RFUs converted to [DNA]**

ng/μL	1	2	3	4	5	6	7	8	9	10
A	3.547	3.48	3.574	3.719	2.76	3.031	2.563	3.311	1.201	2.669
B	2.351	3.516	1.633	5.104	2.535	3.583	2.064	3.915	1.926	1.924
C	2.132	3.728	3.764	3.263	3.51	2.6	3.23	3.082	1.873	1.628
D	4.094	3.081	3.693	4.569	2.961	2.611	2.709	1.621	1.667	1.242
E	3.678	3.564	4.182	4.47	3.321	2.942	2.92	3.063	1.986	1.282
F	3.572	3.142	3.952	3.6	3.302	2.913	2.738	3.178	2.347	1.499
G	3.168	3.122	3.275	3.163	2.46	2.919	2.609	2.783	1.552	1.418
H	1.951	1.271	2.35	2.016	2.313	2.257	1.531	1.195	1.389	1.559
I	1.084	1.207	0.89	2.024	1.489	1.738	2.122	1.239	1.933	1.228
J	1.875	1.34	1.307	1.921	1.741	1.436	0.941	0.865	1.18	1.305
K	2.268	0.873	1.034	0.67	0.947	1.113	0.757	0.998	0.96	0.941
L	1.949	1.115	0.955	0.993	1.038	0.854	1.014	0.733	1.084	0.651
M	2.092	1.021	0.936	0.576	1.161	0.52	0.585	1.023	1.213	1.091
N	1.355	1.02	0.809	0.459	0.524	0.66	0.555	0.374	0.572	0.434

**Table 1:** An overview of the sample quantities, converted from relative fluorescence units (RFUs) to concentration (ng/μL) based on the standard curve. Using the standard curve equation, we solved for concentration: [DNA] = (RFU-48.781)/256121.

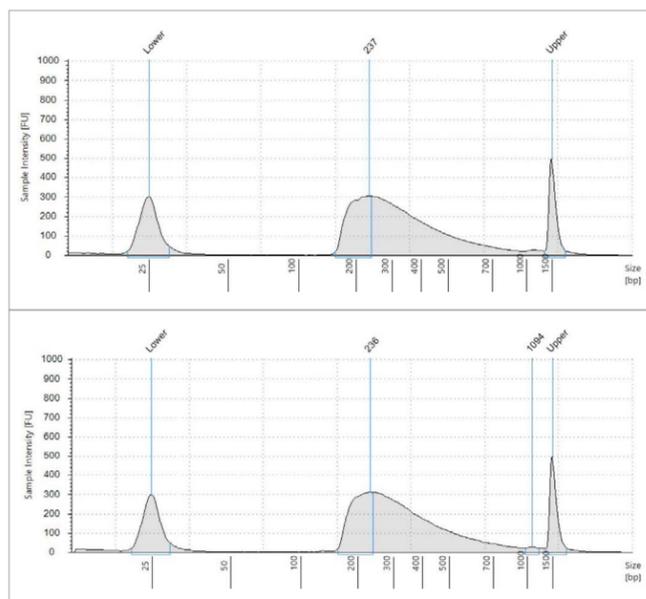


Figure 3: Fragment size analysis electropherograms of a few representative samples.

All library quantitation results from the 18-cycle cDNA library plate (carried through miniaturized Nextera XT) are printed in the table, using the given standard curve generated by the Echo Liquid Handler. These two electropherograms are representative of what the average fragment length is, approximately 237bp. Using this data, we generated a normalization worklist to achieve equimolar representation of each sample, when possible. The Echo 525 Liquid Handler was then used to simultaneously pool and normalize the libraries in two minutes. The pool was then denatured and diluted to 20pM, loaded with 1% PhiX control, and run on an Illumina MiSeq specifying 2x75 reads.

### MiSeq Run Metrics for Nextera XT Workflow

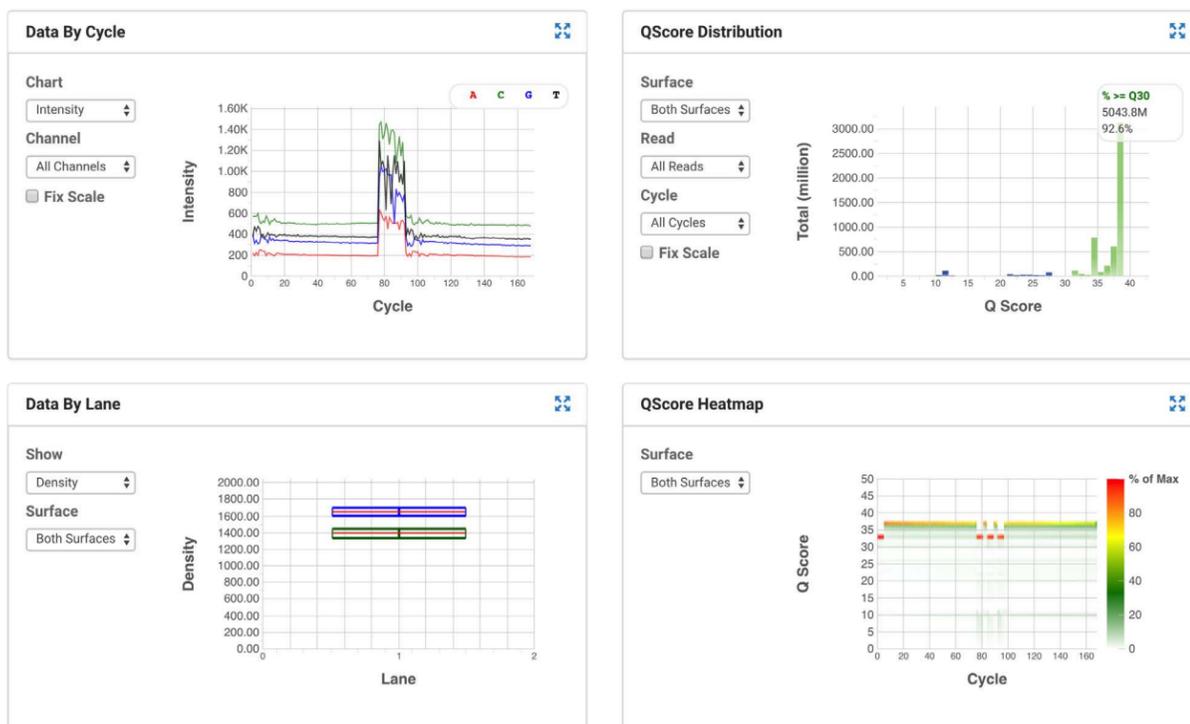


Figure 4: MiSeq run metrics of the 18-cycle cDNA samples that were processed through miniaturized Nextera XT.

MiSeq run metrics were within specifications and 92.6% of the data is between Q30 and Q40, indicating quality, reliable reads from the instrument. We then checked the read distribution by index/sample.

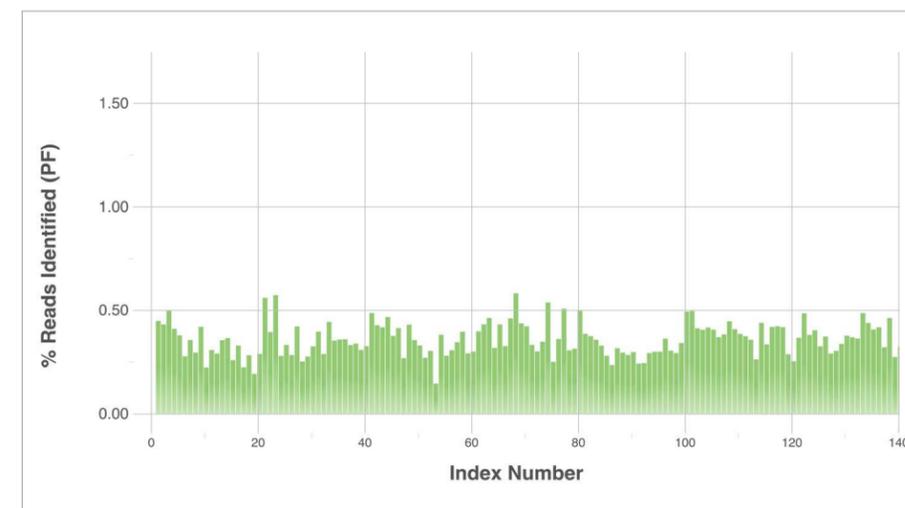


Figure 5: Index distribution of samples from the 18x cDNA library (carried through miniaturized Nextera XT) from Illumina BaseSpace.

Reads were distributed amongst the samples as shown above. There were a few samples that came from below-threshold cDNA libraries, which resulted in some underrepresentation. Furthermore, there are indexing biases that alter the efficiency of a given sample and how well it binds to the flow cell for reading in the MiSeq. Samples under 100,000 total reads were considered statistically too low to be included in the RNA Express analysis pipeline on Illumina BaseSpace. This is a cutoff determined by the software.

To assess whether SMART-Seq reaction volume and RNA input quantity of 18x cycles would reproduce data across those variables in transcript levels, we ran these samples through the Illumina BaseSpace app, RNA Express. This pipeline performs three major steps: alignment of RNA-Seq reads with the STAR aligner, assignment of aligned reads to genes, and differentiating gene expression with DESeq2. One set of replicates was chosen, at random, to be the control group, to which all other samples are referenced.

### Differential Expression

Annotation Gene Count	23,710
Assessed Gene Count	1,075
Differentially Expressed Gene Count	0
Link to Merged Gene Counts	<a href="#">Link</a>
Link to Results	<a href="#">Link</a>

Figure 6: Summary statistics from the RNA Express software pipeline in Illumina BaseSpace. It shows that in this data set, 1,075 genes were assessed, and the software found 0 of them to be differentially expressed.

## Control vs. Comparison

### Filters <sup>i</sup>

Status

OK

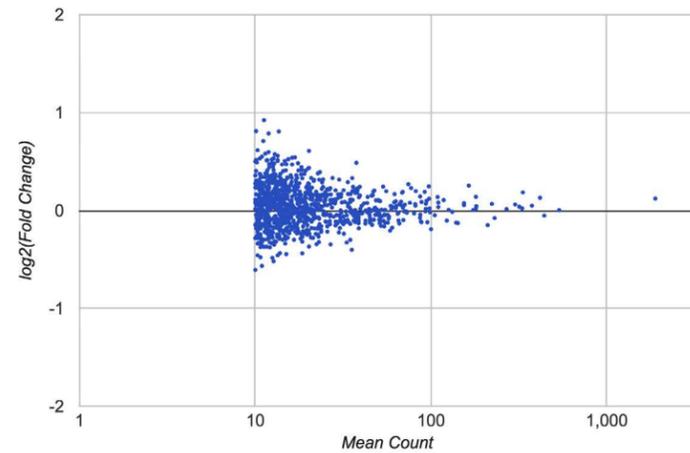
Significant

Choose a value...

$|\log_2(\text{Fold Change})|$

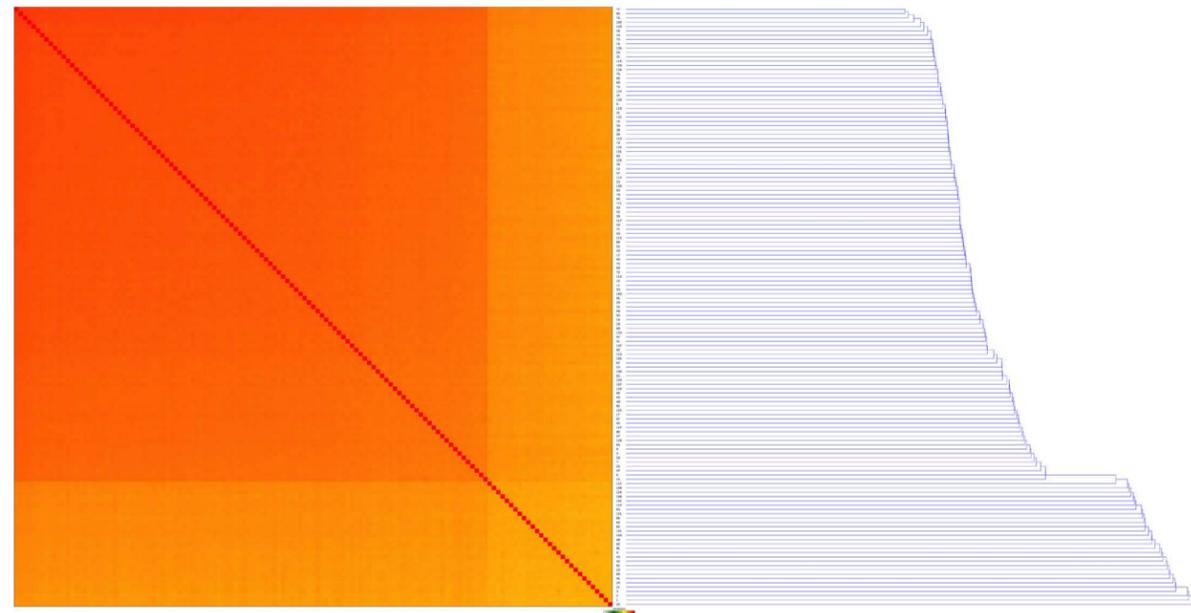
0.0 2.0

Gene



**Figure 7:** This dot plot graph shows all the assessed genes compared to the randomly chosen control replicates. We see that the expression does not differ by two-fold for any of them, which the software calls as no statistical difference in expression. We also see that as the mean count of gene is increased (moving right), the variation in expression decreases and approaches 0, which indicates there is no fold-change in expression.

## Sample Correlation Matrix



**Figure 8:** A Sample Correlation Matrix, showing a heatmap with the relative similarity between all replicates in this analysis. Each row and column represents one replicate, ordered by similarity (hierarchical clustering). The color of each field indicates the Spearman Rho correlation between these replicates. Dark orange is a correlation of 1 and lightening up to green and white indicates a correlation of -1.

Figure 6 represents summary statistics from the RNA Express analysis. It shows that we do not have any statistically significant differentially expressed gene counts, which we expect given all samples were sourced from the same RNA. The dot plot graph in Figure 7 shows that there are not any significant expression differences, all of them being within a two-fold difference. The graph further shows that with higher mean count of a certain transcript, the variation drops. In the sample correlation matrix (Figure 8), it shows a heatmap with the relative similarity between all replicates in this analysis. Each row and column represents one replicate, ordered by similarity (hierarchical clustering). The color of each field indicates the Spearman Rho correlation between these replicates. A dark orange color represents a correlation of 1 and lightening up to green and white indicates a correlation of -1. We see very strong positive correlation for the transcripts across samples.

This gives us confidence that our Echo system-enhanced miniaturized SMART-Seq v4 process can be used with a variety of sample input amounts, and with the sufficient amount of cycling, generate cDNA libraries. We further show that our miniaturized Nextera XT process can produce quality data and good sequencing metrics. Finally, the analysis shows that the entire process does not introduce significant bias to transcript levels in this RNA-seq experiment.

## Conclusion

As scientists continue to push the limits of our genomics tools and understandings, RNA sequencing experiments are growing in quantity, variety, and experimental demands. We see diversity in the questions being asked, as well as the inputs for experimental analysis, so we aimed to determine the optimal parameters for producing sufficient amounts of sequencing data at a cost-effective volumetrically-reduced process.

We found that miniaturization of SMART-Seq v4 can produce sufficient library for sequencing, across reaction sizes, with PCR cycling of 18. We showed that varying reaction miniaturization and input RNA in this 18-cycle sample set did not produce any differentially expressed genes. We also found that the variables of input RNA, amount of reaction miniaturization, and amount of PCR cycling are all interconnected. For example, low-input RNA experiments will require additional PCR cycling, especially if the reaction volume has been reduced significantly. Increasing sample RNA input or reaction volume will reduce the amount of PCR cycling necessary (and subsequent PCR bias), but the reagent cost savings will be less drastic. It is very much up to the user and the defined experiment to balance the cost-savings and throughput with the amplification bias in both SMART-Seq v4 and Nextera XT.

## Materials

Equipment	Manufacturer
Echo 525 Liquid Handler	Beckman Coulter Life Sciences
Allegra X-14 Centrifuge	Beckman Coulter Life Sciences
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

Reagents	Manufacturer	Part Number
SMART-Seq v4 Ultra Low Input RNA Kit	Clontech	#634892
Human Brain Total RNA	Thermo Fisher	#AM7962
Nextera XT DNA 96-Sample Prep Kit	Illumina	#FC-131-1096
Nextera XT 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

Consumables	Manufacturer	Part Number
384-well PP Microplate	Beckman Coulter Life Sciences	001-14555
384-well LDV Plus Microplate	Beckman Coulter Life Sciences	001-12782
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

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