Automation and Miniaturization of Low-Input RNA-Seq Sample Prep Using Desktop Microfluidics

FORMULATRIX

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Introduction

Miniaturization and scale-down of reagent and reaction volumes for low-input RNA-Seq workflows enables researchers to work with low sample input and increased numbers of samples. As the reaction volumes are reduced, accurate delivery of reagent volumes emerges as a critical step to maintain data quality and technical reproducibility.

We present results from miniaturization of the Clontech SMART-Seq® v4 Ultra® Low Input RNA Kit for Sequencing and Illumina Nextera® XT workflows for generation of high-quality RNA-Seq samples using a compact desktop microfluidic dispensing technology.

Materials and Methods

SMART-Seq v4 Ultra Low Input RNA Kit

SMART® technology (Figure 1) is based on non-templated nucleotides that are added by an MMLV-based reverse transcriptase (RT) when it reaches the 5' end of the mRNA during cDNA synthesis. Template switching then occurs when a specially designed SMART oligo bearing a complementary sequence to these non-templated nucleotides hybridizes to the first-strand cDNA. The RT switches from using the mRNA as a template to using the SMART oligo for further cDNA synthesis. This ensures that the 5' end of the mRNA is captured and allows specific sequences to be added to each end of the cDNA for simpler amplification and enrichment of full-length cDNA.

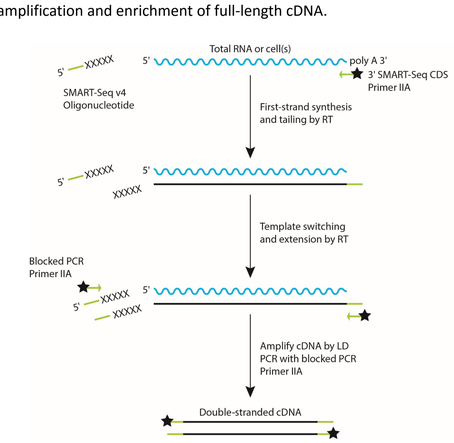

Figure 1. SMART technology for cDNA synthesis.

Mantis Low Volume Dispensing

Figure 2. Mantis microfluidic dispensing system.

The Mantis is an easy to program, low volume, low dead-volume, non-contact reagent dispenser. The Mantis can be configured with up to 24 different reagents, has a dispense range of 100 nl – 2 ml, can dispense any volume into any well, and is compatible with any plate density up to 1536.

To minimize dead volume, reagents can plug directly into microfluidic chips. Dead volumes can be further reduced to 6 µl by using pipette tips as reagent reservoirs. This feature is ideal for dispensing NGS sample prep reagents.

cDNA Synthesis and Library Prep

10 ng aliquots of mouse brain total RNA and aliquots of 1000 cells (K562 cell line) and negative control samples were processed using the standard full volume reaction conditions and compared with samples processed using reduced scale miniaturized reaction volumes using SMART-Seq v4 Ultra Low Input RNA Kit. Reagent volumes are shown in Table 1. 8 cycles of PCR were used to amplify the cDNA products. The resulting cDNA products were characterized using the Fragment Analyzer (AATI) and quantified via PicoGreen® assay.

Reagent	Standard Reaction (µl)	Miniaturized Reaction (µl)
Sample	1	1
10X Reaction Buffer	9.5	1.6
3' SMART CDS Primer	2	0.5
SMART Master Mix	7.5	1.9
PCR Master Mix	30	7.5
10X Lysis Buffer	1	0.3
AMPure® XP beads	50	12.8
Elution Buffer	17	17

Table 1. cDNA Synthesis reagent volumes for full scale and miniaturized reactions. Buffers, primers and Master Mix reagents were dispensed using the Mantis low-volume chip.

Illumina sequencing libraries were prepared from 100 pg aliquots of the amplified cDNA products using the Nextera XT fragment library kit (Illumina) using reduced scale reaction volumes as shown in Table 2. Comparison to standard full reaction volumes included for illustrative purposes. Miniaturized Nextera XT reaction conditions were used for both the full scale and miniaturized cDNA synthesis samples. 12 cycles of PCR were used to amplify the sequencing libraries.

Reagent	Standard Reaction (µl)	Miniaturized Reaction (µl)
Sample	5	1.25
Tagment DNA Buffer	10	2.5
Amplicon Tagment Mix	5	1.25
Neutralize Tagment Buffer	5	1.25
Nextera PCR Master Mix	15	3.75
Index 1	5	1.25
Index 2	5	1.25
AMPure XP Beads	30	7.5
Resuspension Buffer	50	12.5

Table 2. Nextera XT Fragment library prep reagent volumes. Reagents were dispensing using the Mantis low-volume chip.

Miniaturized cDNA synthesis and library prep conditions were used to process decreasing input of cells (K562) with samples of 1000, 100, 10, 1 cell using 8, 11, 14, 17 cycles of PCR respectively to amplify the cDNA products from the decreasing number if cells in each sample.

Sequencing and Data Analysis

Indexed samples were pooled and sequenced using MiSeq with 2x75 bp reads. Reads were trimmed by CLC Genomics Workbench (ver. 8.5.1) and mapped to rRNA and the mitochondrial genome with CLC. Unmapped reads were then mapped with CLC to the human genome (hg19) and mouse genome (mm10) with RefSeq masking, producing uniquely mapped reads and % reads that mapped to RefSeq annotations, including exons, introns and intergenic regions. The number of genes identified in each library was determined by the number of genes with a RPKM ≥0.1. Expression levels of different genes were obtained using CLC Genomics Workbench after mapping to RefSeq.

Results

cDNA Synthesis

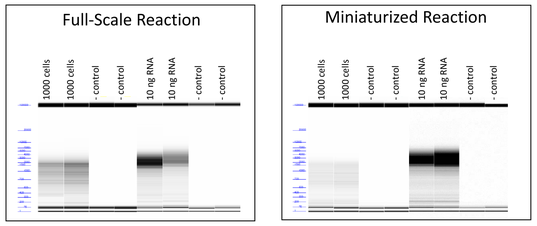

Figure 3. Electropherograms demonstrating equivalent size distribution of cDNA products for full and small scale reactions.

	Average Yield (n=6)
1000 cells – full reaction	4.63 +/- 0.88
1000 cells – miniaturized reaction	4.26 +/- 0.96
10 ng – full reaction	5.42 +/- 0.58
10 ng – miniaturized reaction	4.88 +/- 0.54

Table 3. cDNA product yield (ng/µl) measured with PicoGreen assay.

Sequencing Library Prep

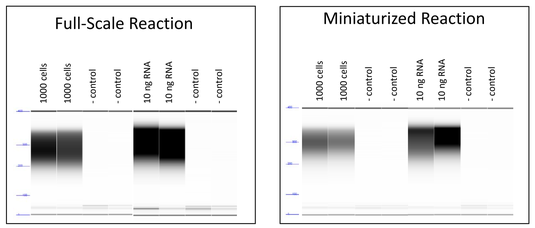

Figure 4. Electropherograms of final fragment libraries demonstrating equivalent size distribution for full and small scale cDNA synthesis reactions for both cell and RNA input samples.

Sequencing Results – Comparison of Full-Scale and Miniaturized Reaction

	Full Reaction	Mini Reaction	Full Reaction	Mini Reaction
Input	10 ng total RNA		1000 cells	
# of reads	903962	982866	484780	484740
Mapped to rRNA	3.6%	3.0%	1.7%	0.2%
Mapped to RefSeq	92%	85%	90%	90%
Mapped uniquely to RefSeq	86%	80%	80%	79%
Mapped to exons	78%	77%	83%	84%
Mapped to introns	17%	18%	13%	12%
# of transcripts with at least 0.1 RPKM	16651	16615	12550	10285
Pearson Correlation	0.98		0.94	

Table 4. RNA-Seq data analysis comparing full-scale and miniaturized reaction conditions.

Sequencing Results – Titrating Input Cells Using Miniaturized Reaction Conditions

# K562 Cells	# PCR Cycles (cDNA amplification)	Average Yield (n=6)
1000	8	5.13 +/- 0.67
100	11	3.77 +/- 0.99
10	14	5.05 +/- 1.83
1	17	3.38 +/- 0.91

Table 5. cDNA product yield (ng/µl) measured with PicoGreen assay.

	1000 cells (n=6)	100 cells (n=6)	10 cells (n=6)	1 cell (n=5)
# of reads	3.4M +/- 0.7M	2.2M +/- 0.4M	2.4M +/- 0.4M	0.8M +/- 0.2M
Mapped to rRNA	0.21% +/- 0.06%	0.21% +/- 0.09%	0.14% +/- 0.07%	0.1% +/- 0.03%
Mapped to RefSeq	87% +/- 3%	91% +/- 2%	89% +/- 2%	89% +/- 3%
Mapped uniquely to RefSeq	78% +/- 3%	83% +/- 2%	82% +/- 2%	82% +/- 3%
Mapped to exons	85% +/- 8%	89% +/- 1%	91% +/- 1%	92% +/- 1%
Mapped to introns	11% +/- 1%	8% +/- 1%	7% +/- 1%	6% +/- 1%
# of transcripts with at least 0.1 RPKM	15855 +/- 449	16092 +/- 575	15682 +/- 247	10171 +/- 1127
Pearson Correlation (to full scale reaction)	0.96 +/- 0.02	0.95 +/- 0.01	0.92 +/- 0.01	0.82 +/- 0.03

Table 6. RNA-Seq data analysis from decreasing number of cells using miniaturized sample prep conditions.

Conclusions

Reducing reagent volumes for cDNA synthesis using the Clontech SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing provides results comparable to full-scale reaction volumes when using the Formulatrix Mantis microfluidic dispensing system.

Average yield of amplified cDNA from the scaled-down reaction volume workflow is compatible with input samples of cells and total RNA.

RNA-Seq data analysis metrics are comparable between the full-scale and miniaturized reaction conditions.

The combination of high-yield cDNA synthesis chemistry and high-precision low-volume reagent dispensing provides a workflow well-suited for large-scale RNA-Seq studies.

References

Nextera XT DNA Library Prep Protocol Guide (Illumina)
SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing User Manual (Clontech)
Mantis Operators Guide (Formulatrix)