ABRF 2011 Research Poster

Improvements in SOLiD™ whole transcriptome library preparation workflow to enable low input RNA amounts

Charmaine San Jose Hinahon, Jian Gu, Kristi Lea, Sheila Heater, Diana Batten, Kelli Bramlett, Penn Whitley, Jeff Schageman, Chris Mueller, and Joel Brockman,
Ambion/Life Technologies, 2130 Woodward St., Austin, TX, USA, 78744

Abstract

The SOLiD™ Total RNA-Seq (STaR-Seq) kit from Life Technologies provides a complete workflow for generating directional, random primed, whole transcriptome libraries from total RNA and fractionated RNA. Poly(A) selected RNA is commonly used for expression profiling using short read sequencing on the SOLiD™ instrument. The input requirements in the current STaR-Seq protocol is 100-500ng poly(A) selected RNA, input amounts well suited for studies using cell culture or large tissues. However, in circumstances where the available sample is limited, such as in the case of clinically derived material or the need to focus the analysis on specific cell populations, there is often too little RNA available to perform the cDNA library analysis. Improvements to the STaR-Seq workflow have been developed and validated pushing the starting input to 5ng of poly(A) selected RNA, while still maintaining high concordance with the current method. Improvements include optimizations in: 1. Reducing RNA fragmentation time to increase RNA fragments within the suitable size range; 2.Altering purification methods after RNA fragmentation to minimize sample loss; 3. Adoption of a bead purification and size selection method to reduce sample loss due to gel size selection; 4. Modifying PCR conditions to maximize cDNA library yields without biasing expression profiles. These improvements enable STaR-Seq to be a viable option for samples with small quantities of total RNA. As the field of next generation sequencing advances, it is necessary to meet the need for decreased sample requirements, while maintaining strand specificity, and accuracy.

Introduction

SOLiD™ technology coupled with the STaR-Seq library preparation workflow provides the ability to digitally detect expression levels in relatively large amounts of tissue samples (100-500 ng). This study aims to extend this capability to 5 ng input amounts. We have identified points in the STaR-Seq workflow (Figure 1.) to focus our studies. These are points in the workflow with the greatest potential for sample loss and variability. Improvements made in these areas will help to preserve sample complexity of the final library.

Methods

Poly(A) RNA was enriched from 250 µg of HeLa total RNA using the Ambion MicroPoly(A)Purist™ Kit. External RNA Controls Consortium (ERCC) Spike-in Mix 1 were added post Poly(A) selection. 5 and 25 ng of Poly(A) RNA was used in the whole transcriptome library workflow with some or all of the improvements previously described (Table 1.). All sequencing data generated was mapped using the Whole Transcriptome Analysis Pipeline and compared to standard input STaR-Seq data for HeLa Poly(A) RNA.

Results

Figure 1. Low Input Optimizations Areas of Focus

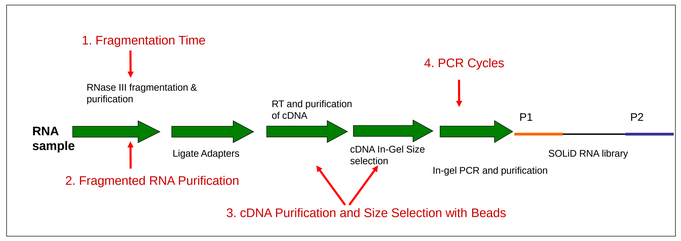

Figure 1. Diagram of the STaR-Seq library preparation workflow. Arrows indicate areas of focus for low input optimization considerations.

Table 1. Summary of Low Input Libraries and their Incorporated Improvements

Library Sample	Fragmentation	Fragmented RNA Purification	cDNA Purification with Beads	PCR Cycles
5ng HeLa Poly(A)	3 min	PureLink™ RNA Kit	None (in-gel)	18
5ng HeLa Poly(A)	3 min		2X Ampure® XP	

Figure 2. Fragmentation Time for 5ng and 25ng HeLa Poly(A) RNA

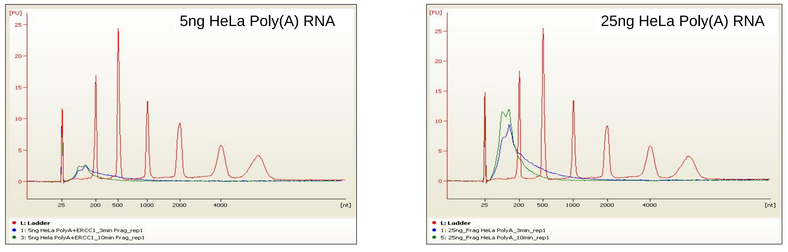

Figure 2. Traces from RNA 6000 Pico Assay on the Bioanalyzer™ Instrument comparing 3 vs.10 minute RNaseIII fragmentations of HeLa Poly(A) RNA at 37ºC incubations for 5ng and 25 ng inputs. Previously, a time point test from 1-10 minutes was performed to determine best time point for generating fragmented RNA size profiles similar to the size profile for the standard 500 ng input. Fragmentation time of 3 minutes was chosen. In addition, PureLink® RNA Micro Kit was chosen for fragmented RNA purification over RiboMinus™ Concentration Module for better recovery and reproducibility.

Figure 3. In-Gel vs. Bead Size Selection on 5ng HeLa Poly(A) RNA

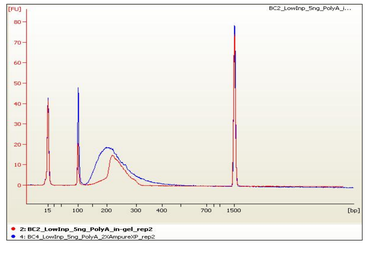

Figure 3. Profiles from Agilent® DNA 1000 Kits on the Bioanalyzer™ Instrument comparing final whole transcriptome libraries for samples with in-gel size selection at the cDNA step vs. 2X Ampure® XP bead purification at the cDNA step. Two rounds of bead capture, wash, and elution was performed to reduce excessive amount of ligation by-products. The 2X Ampure® XP library produces slightly better yield with a similar amount of by-products (peak <150 bp) as compared to in-gel library, indicating successful size selection on Ampure® XP beads.

Figure 4. Mapping Statistics for Low and Standard Input HeLa Poly(A) RNA

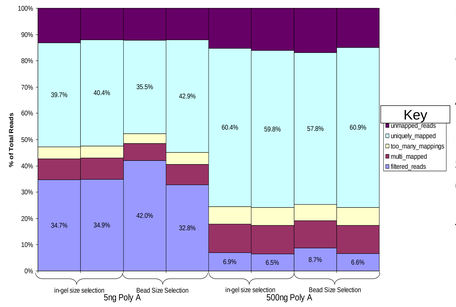

Figure 4. Mapping statistics comparing percentage of total reads mapped for 5ng and 500ng Poly(A) RNA treated with either in-gel size selection or two rounds of Ampure® XP bead purification. Uniquely mapped reads for low input 5ng Poly(A) RNA are 20% lower compared to the standard input 500ng Poly(A). However, this decrease in uniquely mapped reads does not negatively impact %detection of RefSeq transcripts (see Figure 6).

Figure 5. Replicate Correlation to Standard Method

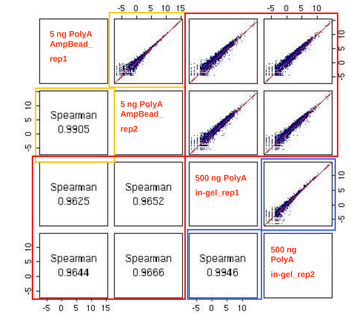

Figure 5. HeLa Poly(A) RNA Spearman correlations by RefSeq RPKM of replicate samples for low input 5ng 2X Ampure® XP (yellow box) and standard input 500ng in-gel (blue box) treated. Low input correlations to standard method (red box) are greater than 0.9625.

Figure 6. Percent Detection of RefSeq Transcripts

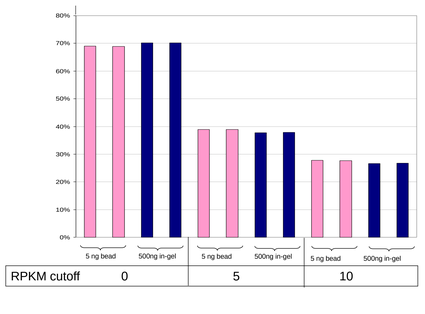

Figure 6. Low input 5ng samples show similar % detection of RefSeq transcripts compared to 500ng standard input with increasing RPKM cutoff values.

Conclusions

- This study suggests that 5ng HeLa Poly(A) with 3 minutes of RNaseIII fragmentation time followed by PureLink® Micro RNA purification is suitable for ligation.
- For cDNA purification and size selection, a double Ampure® XP bead method has been optimized to minimize by-products.
- The in-gel size selection method has been evaluated with 5ng input amounts of HeLa Poly(A) RNA.
- Sequencing data from both 5ng low input bead and in-gel methods have been analyzed and compared to the 500ng standard input method.