**Introduction**

Deep sequencing of mRNA (mRNA-Seq) is rapidly gaining momentum for transcript profiling, discovery of novel transcripts, and identification of alternative splicing events. Current methods for making RNA-Seq libraries generally consist of (1) ribosomal RNA (rRNA)-depleted RNA; and (2) platform-specific cDNA libraries for sequencing. However, these methods are time-consuming with significant hands-on time for mRNA fragmentation, end-polishing, adapter ligation, size selection, multiple clean-up steps and require the use of high quality, intact total RNA samples. As importantly, rRNA contamination poses a significant barrier to performing transcriptome analysis.

We describe the development of a novel rRNA removal procedure (Ribo-Zero™ Technology) and a novel ligation-free cDNA library synthesis procedure (ScriptSeq™ Technology) for preparing di-tagged cDNA fragments and a novel ligation-free cDNA library synthesis process (ScriptSeq™ Technology) for preparing directional, uniformly mappable reads in about 6 hours from both intact and degraded RNA samples, and with low rRNA background.

**Methods Overview**

**Ribo-Zero™ rRNA Removal**

T-Ribo-Zero rRNA removal process uses a proprietary method that is optimized for removal of all sizes of RNA. Intact or degraded total RNA samples (50 ng to 5 µg) are mixed with the Ribo-Zero Reagents in solution (25 minutes). T-Ribo-Zero is then added to Ribo-Zero Micspheres and incubated for 20 minutes followed by the removal of Micspheres with a spin-filter column (2 minutes). The recovered rRNA-depleted RNA is recovered either by ethanol precipitation or a column-purification method of choice.

**ScriptSeq™ Library Preparation**

T-ScriptSeq library preparation method employs random-primer, first-strand cDNA synthesis from rRNA-depleted mRNA (10 µg) that incorporates a platform-specific 3'-sequencing tag (30 minutes; Fig. 1). T-RNA and excess oligonucleotides are then enzymatically hydrolyzed (13 minutes) and a mixture comprising a terminal-tagging oligonucleotide (TTO) and a DNA synthesis reagent is added. T TTO contains a known 5'-sequencing tag (A), a 3'-random sequence and a terminally blocked 3' end to prevent priming of DNA synthesis. T-RNA ends of the cDNA molecules are extended, incorporating a complement to the sequencing tag (18 minutes), forming cDNA molecules with known sequence tags at their 5' (A) and 3' (B) ends for directionality. Excess TTO is enzymatically degraded (13 minutes) and the di-tagged cDNA molecules are purified (10 minutes). T-RNA complete di-tagged cDNA synthesis process is performed in a single reaction tube. Next, platform-specific capture sequences, which can include a barcode, are added to the di-tagged cDNA molecules using limited-cycle PCR, and the products are purified (70 minutes). T-RNA adaptor-tagged library is then ready for cluster generation in preparation for deep sequencing.

**RNA-Seq workflow comparison**

Table 1 compares the EPICENTRE ScriptSeq library preparation workflow to that provided by current Illumina RNA-Seq methods. T-EPICENTRE workflow of this study flow in the overall reaction and hands-on time, and number of steps required. No intermediate clean-up steps are required for preparing rRNA-depleted RNA to synthesis of di-tagged cDNA fragments.

**Results and Discussion**

**Significant reduction of rRNA background and improvement in uniquely mappable reads**

Intact and partially fragmented Universal Human Reference RNA (UHRR) (2 x 2.5 µg) each were treated with either the Ribo-Zero Kit or a competitive RNA removal kit. T the respective Ribo-Zero di-tagged cDNA fragments were pooled and, for each, RNA-Seq libraries were prepared in triplicate following the ScriptSeq procedure outlined in Fig. 1, using rRNA-depleted RNA from the equivalent of 1 µg total RNA. Replicates of the respective RNA-Seq libraries were pooled and sequenced to perform Illumina GAIIx sequencing with 36 nt reads. T-Replicate data were analyzed using Illumina Pipeline Eland_rna Module and CASAVA software as were as the TopHat library (http://tophat.cbcb.umd.edu/index.html). T-mapping results showed that RNA background in each library was significantly reduced by the Ribo-Zero Kit (Table 3). Further, for fragmented samples, the Ribo-Zero Kit considerably outperformed the competitor kit, both in terms of reducing rRNA background and obtaining uniquely mappable sequences.

**High-quality ScriptSeq libraries prepared from rRNA-depleted or poly(A)-enriched RNA samples**

We used UHRR, BrRR, and total RNA isolated from FFPE breast cancer tissue as starting material. T-expected samples (Figure 2) were treated with either the Ribo-Zero Kit, a competitive RNA removal kit (Company A), or a commercial oligo(dT)-based mRNA enrichment Kit. For UHRR and BrRR, ScriptSeq libraries were prepared from 50-ng aliquots of the resulting rRNA-depleted or poly(A)-enriched RNA, as outlined in Fig. 1. For FFPE samples, the entire amount of rRNA-depleted RNA recovered from 500 ng total RNA input was used to prepare the libraries. T-di-tagged cDNA reactions were amplified by PCR for each of 10 cycles (UHRR and BrRR) or 12 cycles (FFPE) followed by Exo I digestion. Each RNA-Seq library was purified using MinElute (Qiagen) and recovered in 15 µl of Elution Buffer. Replicate reactions were pooled and examined using a Bioanalyzer (Agilent). Single-lane, 54-nt unidirectional sequencing reads were obtained for each library using an Illumina GAIIx sequencer, and sequence analysis was performed using Bowtie.

**Conclusions**

**Ribo-Zero rRNA Removal**

- Highly efficient removal of rRNA from both intact and fragmented RNA samples (50 ng to 5 µg total RNA).
- Single-pass rRNA removal process.
- Enables sequencing of degraded RNA samples by significantly lowering the rRNA background.
- Enables recovery of both poly(A)− and non-poly(A) transcripts.
- Kits for human/mouse/rat (mammalian) and Gram-negative bacteria currently available.
- Kits for Gram-positive bacteria and plants in development.

**ScriptSeq Library Preparation**

- Simple, ligation-free, and directional RNA-Seq library preparation workflow with no need for gel purification; compatible with Illumina GAII and Roche FLX-Titanium chemistry.
- High-quality libraries from rRNA-depleted total RNA, poly(A) RNA, or FFPE RNA.
- Cluster generation-ready amplimers in under 4 hours from rRNA-depleted RNA (10 ng).
- Equal 5'-3' representation of transcripts.
- Detects both poly(A)+ and poly(A)− transcripts with use of random-primed cDNA synthesis.
- High correlation (~92%) with MAQC microarray data set.
- Barcoding option available for Illumina GAIIx libraries.