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A rapid, directional RNA-seq library preparation workflow for Illumina® sequencing

Most current RNA-seq library preparation methods are time-consuming, multistep processes. We describe a workflow that includes the Ribo-Zero™ and ScriptSeq™ v2 Kits that enables researchers to go from total RNA to cluster-ready RNA-seq libraries in less than 1 d. The RNA-seq libraries produced are virtually free of contaminating ribosomal RNA (rRNA) and provide for directional paired-end and multiplex sequencing on Illumina® sequencing platforms.

Massively parallel sequencing of cDNA produced from RNA (RNA-seq) has become a widely accepted alternative to microarrays for transcript profiling and analysis of new transcripts, new isoforms, alternative splice sites, rare transcripts and cSNPs. Current RNA-seq library preparation methods comprise preparing rRNA-depleted or poly(A)-enriched RNA followed by RNA fragmentation, cDNA synthesis, adaptor ligation and multiple cleanup steps. These methods are generally time consuming, requiring about 1.5 d and significant hands-on time. They typically produce nondirectional libraries.

We describe a greatly improved RNA-seq library preparation workflow that overcomes challenges associated with conventional methods. The workflow includes highly efficient rRNA removal (Ribo-Zero technology) followed by a rapid, ligation-free cDNA synthesis procedure for preparing directional RNA-seq libraries (ScriptSeq v2 technology).

rRNA removal

Ribo-Zero kits use a hybridization-capture process that removes >99% of cytoplasmic rRNA (and optionally, mitochondrial rRNA) from 1 µg to 5 µg of intact, partially degraded or formalin-fixed paraffin-embedded (FFPE) total RNA samples (Benes, V. *et al*. Ribo-Zero Gold Kit: improved RNA-seq results after removal of cytoplasmic and mitochondrial ribosomal RNA. *Nat. Methods* Application Note, 8, iii-iv, November 2011). The single-pass procedure can be completed in 1.5 h.

Rapid RNA-seq library preparation

The ScriptSeq v2 RNA-Seq Library Preparation Kit uses a patented terminal-tagging process (**Fig. 1**) to generate directional RNA-seq libraries in approximately 4 h. Briefly, 500 pg to 50 ng of Ribo-Zero–treated or poly(A)$^+$ RNA is fragmented and reverse transcribed using random primers containing a 5′-tagging sequence. The 5′-tagged cDNA is then tagged at its 3′ end by the terminal-tagging reaction to yield di-tagged, single-stranded cDNA. Following purification, the di-tagged cDNA is amplified by limited-cycle PCR, which completes the addition of the Illumina adaptor sequences, amplifies the library for subsequent cluster generation and adds an optional Illumina Index or user-defined barcode. The amplified RNA-seq library is purified and is ready for cluster generation and sequencing.

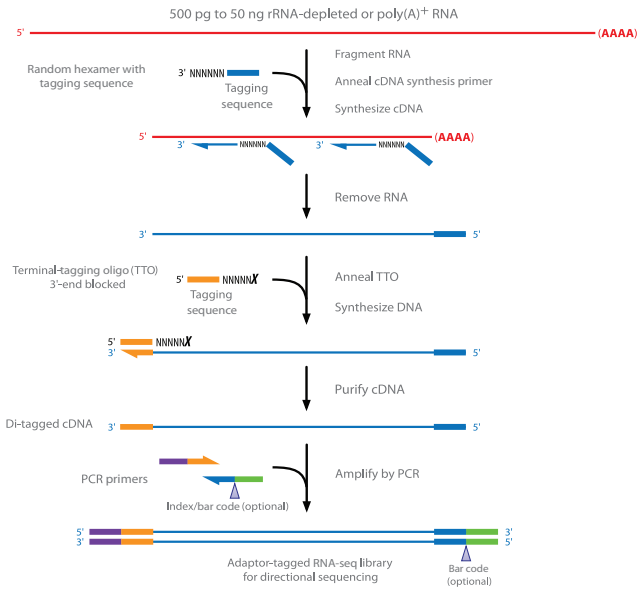

Figure 1 | Overview of the ScriptSeq v2 RNA-seq library preparation method.

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APPLICATION NOTES

Table 1 | Summary of RNA-seq metrics from ScriptSeq v2 libraries

RNA Source	RNA treatment method	Reads (M)	Reads passing filter (%)	Reads >Q30 (%)	Directionality (%)	rRNA content (%)	Total mapped reads (%)
Ribo-Zero vs. poly(A) treatment							
Rhesus macaque blood, 500 pg[a]	Ribo-Zero	73.27	88.85	90.8	98.8	0	ND
Rhesus macaque blood, 5 ng[a]	Ribo-Zero	49.64	94.16	95.6	99.2	0	ND
UHRR, 500 pg[a]	Poly(A)	48.64	93.22	95.6	99.1	3.83	96.57
UHRR, 5 ng[a]	Poly(A)	47.06	91.20	94.9	99.1	3.80	97.45
Intact vs. fragmented RNA							
UHRR, intact[b]	Ribo-Zero	42.83	95.53	95.1	99.06	0.70	97.94
UHRR, fragmented[b]	Ribo-Zero	47.54	95.17	95.6	98.48	0.81	98.59
BrRR, intact[b]	Ribo-Zero	41.93	96.70	97.0	98.87	0.55	98.31
BrRR, fragmented[b]	Ribo-Zero	52.35	95.11	95.8	98.42	0.54	98.46

[a]Indicated amount of RNA after Ribo-Zero treatment or poly(A) enrichment. [b]Starting amount of total RNA was 100 ng for each sample. UHRR, universal human reference RNA; BrRR, brain reference RNA; ND, not determined.

Directionality and coverage of ScriptSeq v2 libraries

We prepared ScriptSeq v2 libraries using total RNA from various sources after treatment with the Ribo-Zero Kit (Human/Mouse/Rat) or after poly(A) enrichment. In addition, we compared intact and fragmented RNA samples. All libraries were sequenced on an Illumina GAIIx sequencer. As shown in **Table 1**, the number of reads passing filter and Q30 scores[1,2] for ScriptSeq v2 libraries are within normal limits for Illumina sequencing.

The random-primed cDNA synthesis and terminal-tagging steps employed by the ScriptSeq v2 procedure add unique sequence tags to the 5′ and 3′ ends of the di-tagged cDNA that is synthesized. These unique tags permit >98% directional sequencing reads (**Table 1**). Using the Illumina sequencing primers, a single-read ScriptSeq v2 library generates the sequence corresponding to the sense strand of the original RNA molecule and a paired-end (reverse-end) read generates the antisense sequence of the original RNA molecule.

Figure 2 shows the sequence coverage of 600 transcripts in a ScriptSeq v2 RNA-seq library produced from poly(A)$^+$ universal human reference (UHR)RNA and sequenced using single-end reads on an Illumina GAIIx. The data demonstrate consistent sequence coverage using 500 pg to 50 ng of input RNA.

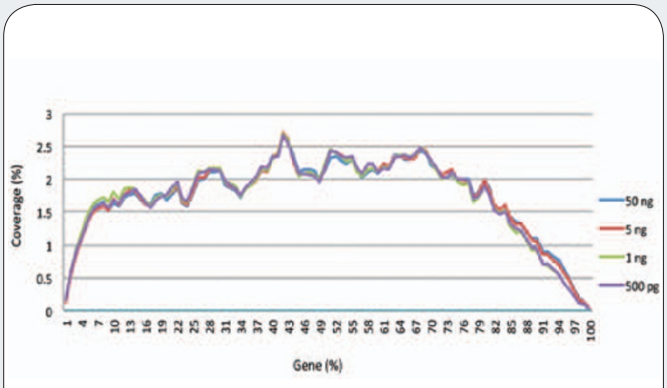

Figure 2 | Consistent coverage of ScriptSeq v2 RNA-seq libraries from varying amounts of input RNA.

Conclusions

The RNA-seq library preparation workflow presented here enables researchers to go from intact or fragmented total RNA samples to cluster-ready RNA-seq libraries in less than 1 d. Ribo-Zero technology provides highly efficient removal of rRNA from both intact and fragmented RNA samples, including FFPE RNA. ScriptSeq v2 library preparation technology generates ligation-free, directional RNA-seq libraries for single-read, paired-end read and multiplexed Illumina sequencing. The RNA-seq libraries produced exhibit high quality, strong directionality and good transcript coverage.

ACKNOWLEDGMENTS

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1. Ewing, B. *et al*. Base-calling of automated sequencer traces using phred. I. Accuracy assessment. *Genome Res.* **8**, 175–185 (1998).
2. Ewing, B. & Green, P. Base-calling of automated sequencer traces using phred. II Error probabilities. *Genome Res.* **8**, 186–194 (1998).