Streamlined Library Construction for Quantitative, Directional, and Standard RNA-Seq

Introducing: NEXTflex™ Rapid RNA-Seq Kit

Authors: Bioo Scientific Corporation. 7050 Burleson Road, Austin, TX 78744, USA

Introduction

RNA Sequencing (RNA-Seq) is a valuable tool for a broad range of clinical, environmental, and basic research. Producing high quality RNA-Seq libraries can be challenging for several reasons, including isolation of pure RNA, efficiently converting RNA to cDNA, and loss of material incurred during the series of enzymatic steps and cleanups required for library construction. Here we introduce Bioo Scientific’s family of Illumina compatible NEXTflex™ Rapid RNA-Seq kits, all of which include the thermostable NEXTflex™ Rapid Reverse Transcriptase enzyme. The NEXTflex Rapid RNA-Seq Kits provide affordable and unique technology, some of the shortest RNA-Seq library preparation times of any kits on the market, include all enzymes required for library preparation, and produce the highest quality RNA-Seq library.

Streamlined Library Construction Protocol

The NEXTflex™ Rapid RNA-Seq Kits allow the end user to complete library construction in less than 1 day. In contrast, the previous generation of NEXTflex™ RNA-Seq Kits, as well as the current Illumina TruSeq RNA-Seq Kits, require over 7 hours for completion (Table 1). By combining second strand synthesis and end repair into the same reaction, the need for a separate end repair step and cleanup is eliminated. This improvement is time efficient and reduces loss of material during cleanup.

<table>
<thead>
<tr>
<th>Kit</th>
<th>Time to Completion</th>
<th>Steps Required</th>
<th>Bead Cleanups Required</th>
<th>Reverse Transcriptase Included?</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEXTflex™ Rapid RNA-Seq Kit</td>
<td>5 hours 50 min</td>
<td>6</td>
<td>3</td>
<td>Yes</td>
</tr>
<tr>
<td>Illumina TruSeq™ RNA V2</td>
<td>7 hrs 10 min</td>
<td>7</td>
<td>4</td>
<td>No</td>
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</table>

Table 1. Comparison of RNA-Seq library preparation kit protocols and time to completion. Note that times include bead cleanups and account for time required from RNA fragmentation to the final bead cleanup after PCR.
Thermostable NEXTflex™ Rapid Reverse Transcriptase for Improved First Strand Synthesis and Library Yield

A critical step in RNA-Seq library construction is the conversion of RNA to cDNA. All NEXTflex Rapid RNA-Seq Kits include NEXTflex™ Rapid Reverse Transcriptase (RT) enzyme, in contrast to our previous RNA-Seq kits as well as to the Illumina TruSeq kit (Table 1). The NEXTflex Rapid RT is a thermostable, RNaseH minus enzyme that functions optimally at 50˚ C, a higher temperature than standard Moloney-Murine Leukemia Virus (M-MLV) reverse transcriptases, which function at 42˚ C. This elevated temperature allows for reduced secondary structure in RNA templates and therefore increased efficiency of first strand synthesis.

To examine the efficacy of NEXTflex Rapid RT in library construction, we compared library yields using cDNA produced by different enzymes. The same pool of Poly (A)+ selected mRNA isolated from murine Ag8 cells was used in first strand synthesis with either NEXTflex Rapid RT or SuperScript® III (Figure 1). Higher library yields were obtained using the NEXTflex Rapid RT coupled with the NEXTflex Rapid RNA-Seq Kit. Similar results were obtained using the NEXTflex™ Rapid Directional and NEXTflex™ Rapid Directional qRNA-Seq™ Kits. These results demonstrate improved library yields as a result of optimized first strand synthesis using NEXTflex Rapid RT.

High-quality RNA-Seq Data

Further analysis of NEXTflex Rapid RNA-Seq library quality was carried out using Illumina Sequencing, as library yield is only one metric of library quality. Libraries were prepared with the NEXTflex Rapid RNA-Seq Kit using 10 ng aliquots of a single murine Ag8 cell Poly (A)+ RNA sample, so as to disentangle biological variation from technical variation. Libraries were sequenced on a HiSeq2500 using a 67 bp single end RAPID run. Resulting reads were trimmed based on a quality score moving window using sickle and mapped to the UCSC mm10 assembly using TopHat 2.0.10. We obtained a total of 31,949,336 reads for Rapid RT libraries and 31,720,922 for Superscript® III libraries (Table 2). For transcript representation purposes, only reads mapping to exons, specifically all annotated 5’ UTRs, coding sequences, and 3’ UTRs, were further considered. The number of total reads as well as unique reads mapping to exons were similar between the two enzymes; however, a greater number of transcripts, 12,418 vs. 12,129, were represented in the NEXTflex Rapid RT libraries vs. SuperScript III libraries, respectively.

<table>
<thead>
<tr>
<th>Rt Enzyme</th>
<th>Total Reads</th>
<th>Reads After Quality Trimming</th>
<th>Unique Reads Mapping to Exons</th>
<th>Unique Reads in Exons (%)</th>
<th>Number Transcripts Represented</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEXTflex™ Rapid RT</td>
<td>31,949,336</td>
<td>31,891,608</td>
<td>17,411,368</td>
<td>54.60</td>
<td>12,418</td>
</tr>
<tr>
<td>Superscript® III RT</td>
<td>31,720,922</td>
<td>31,663,364</td>
<td>17,487,086</td>
<td>55.23</td>
<td>12,129</td>
</tr>
</tbody>
</table>

Table 2. Read counts in NEXTflex™ Rapid RNA-Seq libraries and unique reads after mapping to exons.
A further analysis of mapped reads was performed. Read quality before and after mapping was visualized using FastQC and GC content as a function of reads examined (Figure 2). Read quality is excellent in the total library and is slightly higher in the remaining set of trimmed, mapped reads, which is expected given a quality-aware trimming step was carried out. Furthermore, GC content is very similar to the theoretical distribution, indicating accuracy in transcript representation. Visual inspection of several genes demonstrates read coverage across all exons (Figure 3A and B). Furthermore, a metagene analysis of read coverage across all genes divided into 100 bin segments, demonstrates even read coverage across the 5', gene body, and 3'-ends of transcripts (Figure 3C).

Figure 2. Metrics of NEXTflex Rapid RNA-Seq data determined by FastQC. Quality score plots for (top) and mean GC content (bottom) for (A) all reads or (B) reads mapping to exons. Plots shown correspond to NEXTflex Rapid RNA-Seq libraries constructed using NEXTflex Rapid RT.

Figure 3. Read coverage across gene bodies. Mapped reads at the (A) Trp53 and (B) Eif5b loci scaled to read density as indicated. (C) Metagene plot of read density across all annotated loci. All gene bodies and mapped read densities are scaled to 100 bin segments; mean read density is shown in reads per kilobase per million mapped reads (RPKM; solid line) +/- standard error across replicates (faded bands). Shown is read signal corresponding to libraries made with either NEXTflex Rapid RT (red) or SuperScript III (blue).
Finally, we examined library consistency across protocols. We compared RNA-Seq read counts in exons from libraries constructed using the NEXTflex Rapid RNA-Seq Kit to longer traditional protocols. Indeed, libraries constructed using the two methods were highly similar ($r = 0.9976$; Figure 4). These data demonstrate that the NEXTflex Rapid RNA-Seq protocol provides significantly faster library construction with enhanced reverse transcriptase performance.

**Figure 4.** Correlation between Rapid and Standard RNA-Seq data. Pearson's correlation between the average log2 of normalized counts per million (CPM) of all genes across all replicates of Rapid RNA-Seq vs. Standard RNA-Seq, $r = 0.9976$.

Conclusions

The present study demonstrates the utility of the NEXTflex Rapid RNA-Seq Kits as a method to improve speed of library preparation without any compromise in library quality. All NEXTflex Rapid RNA-Seq Kits include NEXTflex Rapid RT, a high performance thermostable enzyme, thus providing all components required for library construction within a single kit. NEXTflex Rapid RNA-Seq Kits produced high-quality sequencing data with improved transcript representation. In addition, the increased library yield using NEXTflex™ Rapid RT is an important improvement for users with low-input amounts. For researchers wishing to multiplex libraries, Bioo Scientific continues to offer our full range of up to 96 NEXTflex™ RNA-Seq adapter barcodes. The NEXTflex Rapid RNA-Seq, Rapid Directional RNA-Seq, and Rapid Directional qRNA-Seq™ Kits provide a streamlined workflow for users to create high-quality RNA-Seq libraries with less hands-on time.