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Automation of NEBNext Directional Ultra II RNA Library Prep Kit for Next Generation RNA-sequencing

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Abstract

Sequencing of ribonucleic acid (RNA-seq) is essential for an unbiased analysis of the whole transcriptome in biological experiments, diseases and drug discovery research. An understanding of the gene expression levels across various disease backgrounds provides an opportunity to discover novel, disease-specific biomarkers whose downregulation or overexpression could help understand disease mechanisms and choose treatment approaches. To improve efficiency in next generation RNA-seq, it is vital to develop efficient workflows that reduce human error and increase time for data analysis.

To meet these demands, we developed an automated library preparation solution using the NEBNext Directional Ultra II RNA Library Prep Kit for RNA-seq. Efficient library preparation is key to producing high quality sequencing data but can vary based on user experience. Thus, we developed the automated workflow using the Beckman Coulter Biomek i7 hybrid workstation to minimize excessive hands-on time and reduce user-to-user variability. RNA from human cell lines (n=84) was used to benchmark our automated workflow against the manual processing.

There was a reduced workflow time from a 2-day manual to a 9-hour automated workflow time. The median library size for manual library was 397 base pairs (bp) with a 1.03 %CV, which was virtually identical to the automated library size (411 bp, 1.70 %CV). Overall transcript coverage exceeded 90% for both methods, where no differences in total gene counts and expression were observed. In summary, RNA-seq library preparation can be successfully automated and implemented on the Beckman Coulter Biomek i7 hybrid workstation yielding a hands-free workflow.

Introduction

Deep sequencing of RNA (RNA-seq) plays a major role in our understanding of diseases and provides an opportunity to develop novel treatments[1, 2, 3]. Data from RNA-seq is useful for viewing whole transcriptomes and uncovering minor gene variations that lead to various diseases. Such gene variations can be the basis for targeted drug discovery research to bring about unique treatment strategies[4]. Moreover, RNA-seq data can also yield a greater understanding of various biological and research manipulations. This includes knowledge of key processes such as cell lineage specification and differentiation, functional diversification and disease development[3]. The availability of deep sequencing allows researchers to combine various data (e.g., chromatin accessibility, histone modifications, DNA methylation, and transcription factor binding to RNA-seq) to understand how gene expression is regulated. These important insights can guide researchers to fully develop knowledge of the bigger picture underlying various biological changes and manipulations (e.g., disease versus normal, treated versus untreated), thus providing pathways to further investigate methods to improve treatment approaches.

The efficiency of deep sequencing is however impacted by cumbersome library preparation methods that are labor intensive and can contain user-introduced technical variabilities. While various biotechnology companies have attempted to improve the step-by-step protocols by designing specific kits, workflows are still usually long and can span multiple days. However, increasing knowledge and sequencing technology platforms are leading to better library construction methods that are essentially automatable. Among this is the NEBNext Directional Ultra II RNA Library Prep Kit for Illumina (NEB#E7765) that is designed to generate a streamlined library preparation method.

We evaluated the automation of the NEBNext Directional Ultra II RNA Library Prep Kit for Illumina using the Biomek hybrid workstation (Beckman Coulter Life Sciences). The automated workflow **(Figure 1)** was designed to reduce hands-on time while producing high quality library that is comparable to that generated by an experienced technician. The Biomek hybrid workstation contains a large deck size with sufficient room for integration of useful technology such as an automated thermocycler necessary for performing the library preparation in a totally hands-free environment. Thus, we compared data from the automated workflow to that performed manually.

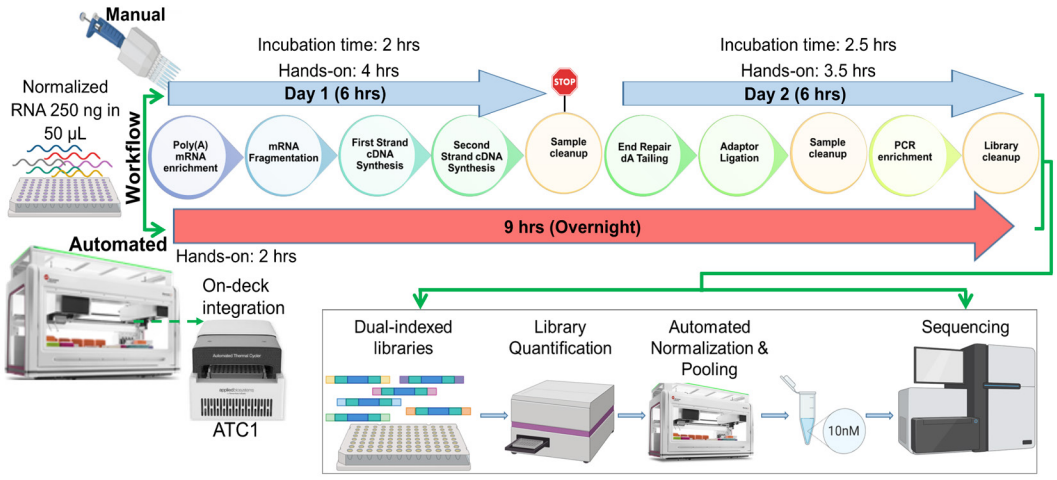

Figure 1. NEBNext Directional Ultra II RNA Library Preparation workflow.

Methods

Experimental design – comparison of manual versus automated workflows

We performed a side-by-side manual and automated library preparation workflow **(Figure 1)**. Total RNA isolated from human cell lines was normalized and subjected to a manual library preparation protocol that span two days **(Figure 1)**. When the same method was converted into automated workflow **(Figure 2A)** the overall workflow time reduced to an overnight automated 9-hour protocol time **(Figure 1)**. On day 1 of the manual protocol, the workflow included poly-mRNA enrichment, mRNA fragmentation, first and second strand synthesis and sample cleanup. On day 2, the sample was end-repaired using dA tailing, followed by adapter ligation, sample cleanup and PCR amplification **(Figure 1)**. The thermocycler used for the manual protocol was from Eppendorf and the final number of cycles in the amplification step was 12. The overall manual workflow included combined hands-on time of 7.5 hours and 4.5 hours of incubation time between day 1 and 2. The same steps were converted into an automated workflow using the Biomek 5 software. For the automated workflow, a 2-hour hands-on time was needed for reagent preparation, followed by a 9-hour hands-free protocol performed overnight on a Biomek i7 hybrid workstation with an integrated on-deck thermocycler from Fisher Scientific (catalog number A31489). The final number of cycles in the amplification step on the ATC1 was 13.

Method Setup on Biomek i7 hybrid Workstation

The automated library preparation method was performed through the demonstrated Biomek method interface containing step-by-step instructions for Biomek deck setup. Three modules were used to initiate the automated protocol and included:

- Biomek Method Option Selector (MOS) for selection of automated Biomek method and running parameters for RNA-seq.
- Automated RNA-Seq Biomek method **(Figure 2A)**.
- Biomek Guided Labware Setup (GLS) to automatically determine the required reagents and the amount of labware as per the number of samples to be processed **(Figure 2B)**.

Upon starting the automated method, the subsequent deck layout is set up in order to perform the hands-free protocol **(Figure 2B)**. Following the deck setup, the automated protocol is initiated and the Biomek i7 hybrid workstation performs the library preparation steps, including the incubation steps, without any user interaction, for a total time of 9 hours **(Figure 1)**. The dual indexed libraries generated both manually or through automation were then quantified, normalized and sequenced **(Figure 1)**.

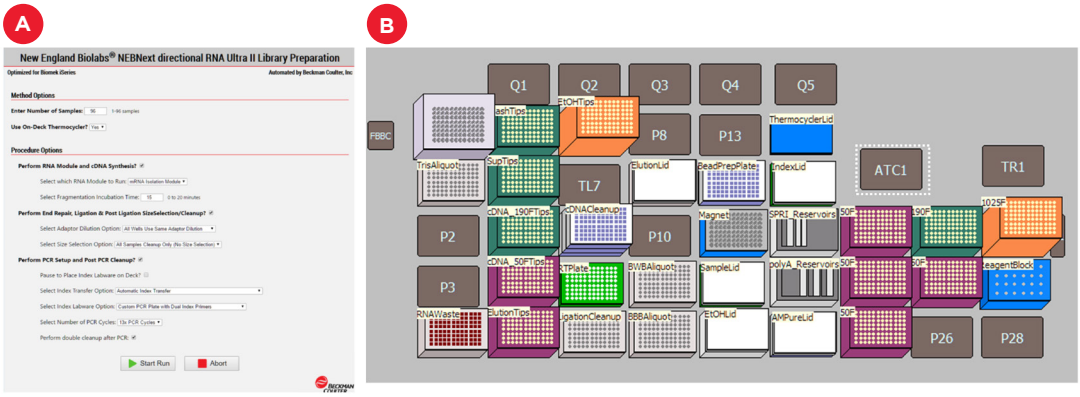

Figure 2. Automated workflow and Biomek deck layout. **A)** Graphical user interface chart. **B)** Deck layout for automation protocol.

Results

Quality control of libraries from manual and automated experiments

The overall library yield was compared by evaluating the distribution of library constructs from manual versus automated methods. The concentration of Biomek generated library (median 77nM, 19.1 %CV) were not significantly different from the concentration of the manual library yield (median 68nM, 22.5 %CV) and were within expected ranges as per manufacturer's recommendations (10nM – 120nM) **(Figure 3A)**.The size of fragments generated in the manual had a median of 397 bp **(Figure 3B)** compared to 411 bp for automated library. As expected, the fragment analyzer trace of the non-template control demonstrated a leftover adapter dimerization peaks at 140-150bp range (not shown), indicating that no amplification was present in the no-template controls (NTC). Importantly, cross-contamination checkerboard were NTC wells alternate with RNA template showed no sample-to-sample cross-contamination between adjacent wells (not shown).

Picard read assignment identified no major differences between both library preparation methods and there was a high overlap of mapping metrics for UTR, coding, ribosomal, intergenic and intronic regions **(Figure 4)**. Interestingly, there was a high percentage of coding regions (>50%) with inexistent rRNA contamination for both the Biomek and manual libraries.

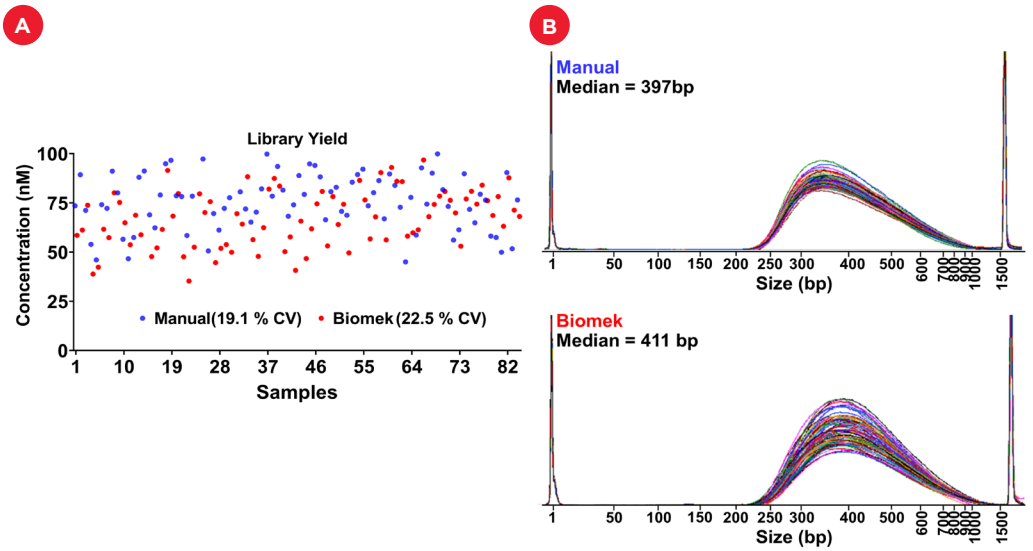

Figure 3. Library quality control for manual versus automated libraries. **A)** Overall library concentration for manual versus automated workflow. **B)** Fragment analyzer trace of the manual versus automated library.

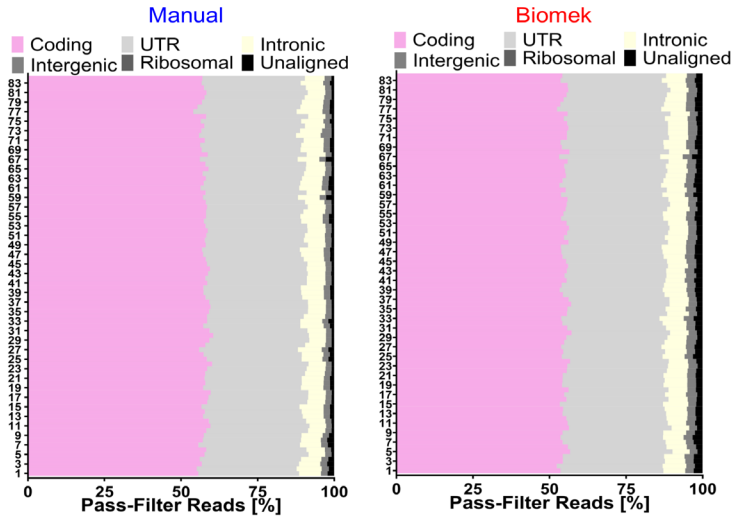

Figure 4. Sequencing quality control analysis of manual and automated libraries. Normalize gene coverage between manual versus Biomek libraries.

Summary

In our analysis of data from manual versus automated library preparation workflow, we found no major differences that were statistically quantifiable. These findings provide evidence that the workflow for NEBNext Directional Ultra II RNA Library preparation kit can be performed on the Biomek i7 hybrid workstation. This is because a comparison of mapped sequenced reads from both manual and automated library preparation workflows, was essentially similar, where manual library had an 8.4 %CV of read coverage (n=84) versus 7.86 %CV for automated workflow samples (n=84). The total number of gene counts generated was also similar in both library preparation methods, where the pairing of manual and automated expression data demonstrated a strong correlation coefficient (R^2=0.987). Importantly, there was a reduced workflow time for automated method from 2-day to 9-hour overnight protocol. While the manual workflow had a total hands-on time of 7.5 hours, the automated workflow only had 2 hours of hands-on time. We conclude that the automated workflow can be used in place of the manual workflow, providing the laboratory operator with increased walk-away time, reduced room for human introduced error, and increased throughput capacity.

Materials

Equipment/Material	Manufacturer
Biomek i7 hybrid automated liquid handler	Beckman Coulter Life Sciences
ATC1 Thermocycler	Thermo Fisher Scientific
Master Cycler X50	Eppendorf
NEBNext Directional Ultra II RNA Library kit	New England BioLabs
5300 Fragment Analyzer System	Agilent
Synergy HTX Multi-Mode Microplate Reader	Biotek
HiSeq 4000 Sequencing System, cBOT	Ilumina

References

1. Kozarewa I et al, "Amplification-free Illumina sequencing – library preparation facilitates improved mapping and assembly of (G+C) – biased genomes," Nature Methods, vol. 6, pp. 291-295, 2009.
2. Aird D, et al "Analyzing and minimizing PCR amplification bias in Illumina sequencing libraries.," Genome Biology, vol. 12, no. 2, p. R18, 2011.
3. Wang Z. et al, "RNA-Seq: a revolutionary tool for transcriptomics," Nature Review Genetics, vol. 10, no. 1, pp. 57-63, 2009.
4. Gulilat M. et al, "Targeted next generation sequencing as a tool for precision medicine," BMC Med Genomics., vol. 12, no. 81, p. epub, 2019.
5. Schlager S et al, "Inducible knock-out of BCL6 in lymphoma cells results in tumor stasis.," Oncotarget, vol. 11, no. 9, pp. 875-890, 2020.
6. Li B et al, "RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome.," BMC Bioinformatics, vol. 12, p. 323, 2011.
7. Liao Y et al, "FeatureCounts: an efficient general purpose program for assigning sequence reads to genomic features," Bioinformatics, vol. 30, no. 7, pp. 923-930, 2014.
8. Sayols S et al, "dupRadar: a Bioconductor package for the assessment of PCR artifacts in RNA-Seq data.," BMC Bioinformatics, vol. 17, no. 1, 2016 .
9. Ritchie ME et al, "limma powers differential expression analyses for RNA-sequencing and microarray studies.," Nucleic Acids Res., vol. 43, no. 7, p. e47, 2015.
10. Law CW et al, "voom: Precision weights unlock linear model analysis tools for RNA-seq read counts.," Genome Biol., vol. 15, no. 2, p. R29, 2014.

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