TECHNICAL NOTE

Automated NGS Library Preparation

Automated Small RNA Library Preparation with Reduced Bias and High Detection/Discovery Rates

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Introduction

Small RNAs are short (18 to 30 nucleotides), non-coding RNA molecules that can inhibit the expression of target genes via post-transcriptional gene silencing, chromatin-dependent gene silencing, and RNA activation. Small RNAs include several variations such as short interfering (siRNA), micro (miRNA), and others. Small RNAs play important roles in diverse cellular processes, and research also indicates small RNAs are involved in the pathogenesis of many diseases.

Small RNA library preparation is one of the most difficult types of NGS library preparation. Two primary drawbacks researchers have historically grappled with are:

- The need to manually gel-purify the final library, which limits start-to-finish automation and throughput capabilities
- The introduction of severe bias during the ligation steps, resulting in sequencing data does not accurately reflect the original small RNA abundance

The NEXTflex® Small RNA Sequencing Kit v3 (Bioo Scientific, a PerkinElmer company) is a gel-free, fully-automatable protocol using a dual adapter-dimer reduction approach. The kit uses a combination of bead-based excess 3′ adapter depletion and enzymatic excess 3′ adapter inactivation to provide a completely gel-free protocol that reduces bias through its use of adaptors with randomized ends.

Here the results of two studies on the efficacy of the NEXTflex Kit are presented. The first study compares miRNA libraries prepared with the NEXTflex Kit on the PerkinElmer Sciclone® G3 NGS Workstation to libraries prepared manually and using a standard gel-purification protocol. The second study compares libraries constructed using the NEXTflex Small RNA-Seq Kit v3 to those prepared using competitors' kits. The results of these comparisons demonstrate that the NEXTflex Small RNA Sequencing Kit v3 workflow on the Sciclone G3 NGS Workstation provides:

- High miRNA mapping and detection rates that are comparable to manually prepared libraries
- Sequencing metrics that are comparable to manually prepared libraries
- Reduced bias and greater miRNA detection rates compared to competitors' kits

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Automated Small RNA Library Preparation Using the NEXTflex Small RNA Sequencing Kit v3 on the Sciclone G3 NGS Workstation

Materials and Methods

Small RNA libraries were prepared from 500 ng of human brain total RNA. Thirty-two (32) libraries were prepared using the NEXTflex Kit on the Sciclone G3 NGS Workstation. Six (6) libraries were prepared manually using standard gel-purification methods. The LabChip® GX Touch™ platform was used to quantify and fragment-size the libraries. Figure 1 shows the automated library preparation workflow.

The final libraries were sequenced using an Illumina® MiSeq® platform in a 1x75 bp run at a depth of approximately 150,000 reads per sample. The Cutadapt command-line tool was used to trim 3' adapter sequences, random bases, adapter-dimer reads, and reads with inserts less than 15 nucleotides. The trimmed libraries were aligned to miRBase sequences using the Bowtie alignment tool. The aligned library was then analyzed with the miRUtils tool to determine miRNA group read counts.

Figure 1. **Automated NEXTflex Small RNA-Seq Kit v3 Workflow.** After setting up the reagents, the only manual intervention required is moving the sample plate to and from a thermocycler for reverse transcription and PCR.

Results and Discussion

The libraries prepared with the automated NEXTflex Small RNA Sequencing Kit v3 workflow on the Sciclone G3 NGS Workstation show high miRNA mapping and detection rates and low contamination of adapter-dimer products. Figure 2 presents electropherogram tracings for a representative manual preparation library and a representative automated preparation library. Both show a tight monolithic peak at approximately 150 bp which represents the miRNA library products.

Table 1 presents sequencing metrics for the manual and automated preparation libraries. The libraries prepared with the automated NEXTflex Small RNA Sequencing Kit v3 workflow on the Sciclone G3 NGS Workstation show read filter rates, alignment rates, and miRNA detection levels that are comparable to the manually prepared libraries. These results demonstrate that the automated NEXTflex Small RNA Sequencing Kit v3 workflow on the Sciclone G3 NGS Workstation protocol generates small RNA libraries with sequencing metrics equivalent to manual protocols.

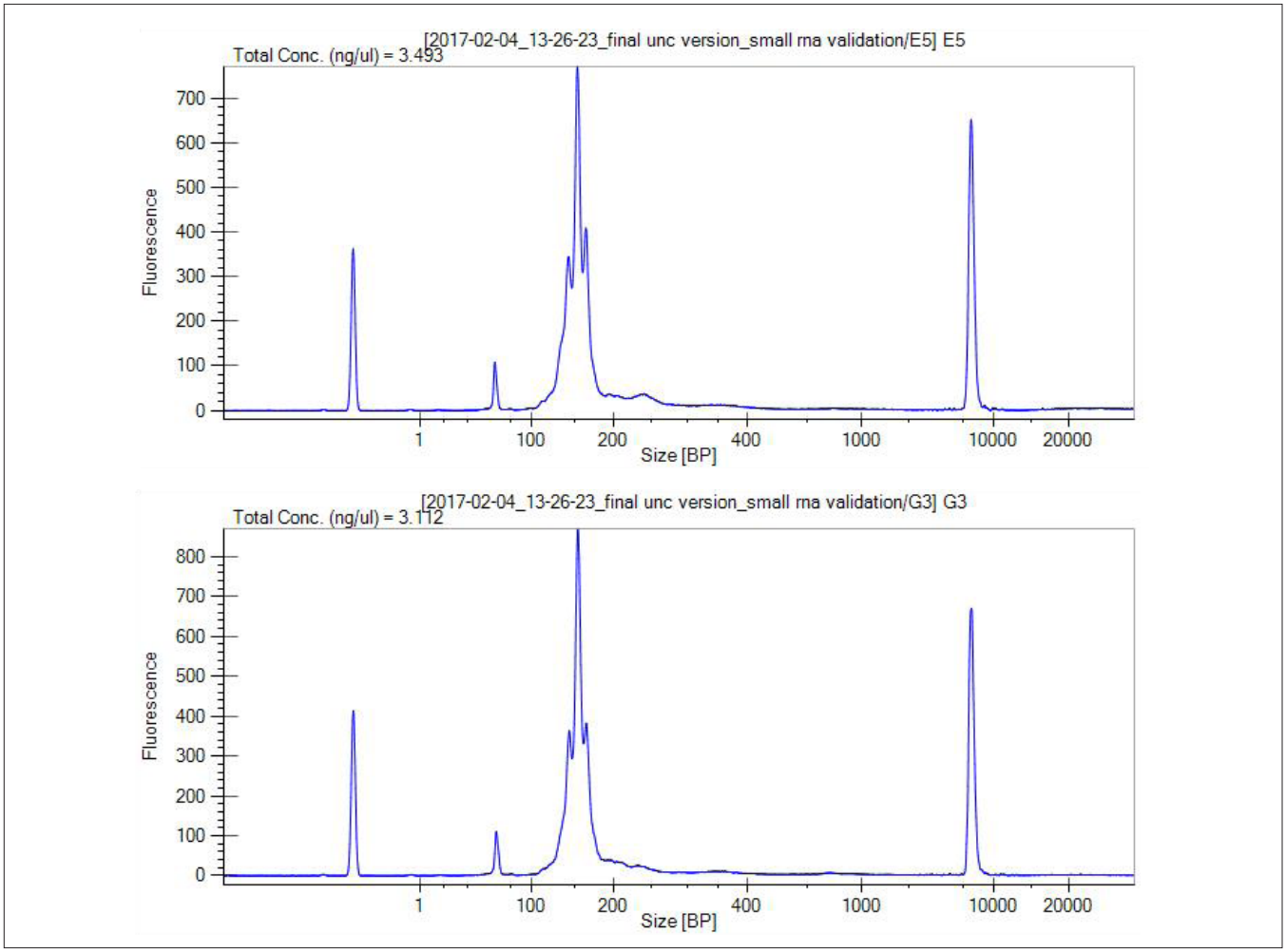

Figure 2. **LabChip GX Touch system Traces of Final Libraries.** Representative traces of final libraries created manually (top) and on the Sciclone G3 NGS Workstation (bottom). Both show a tight miRNA peak at ~150 bp, demonstrating a high level of correlation between the two protocols.

Table 1. **Correlation of miRNA expression in libraries created manually and on the Sciclone G3 NGS Workstation.** The libraries prepared with the automated protocol have rates of miRNA sequence alignment and detection that are comparable to manually prepared libraries.

	Reads Processed	Insert <15 bp (%)	Reads Passing Filter	miRBase Alignment Rate of Filtered Reads (%)	miRBase Alignment Rate of All Reads (%)	miRNA Groups Detected
Manual Average	143287.0	23.9	108982.5	61.9	47.1	376.2
Manual SD	0.0	4.0	5736.2	2.7	3.5	14.4
Automated Average	143287.0	21.8	112116.8	60.1	47.0	392.6
Automated SD	0.0	3.4	4838.9	4.9	3.3	10.7

Reduced Bias and High Discovery Rates with the NEXTflex Small RNA Sequencing Kit v3

Materials and Methods

Small RNA libraries were manually prepared from 100 ng human brain total RNA using three different kits:

- NEXTflex Small RNA Sequencing Kit v3
- Illumina® TruSeq® Small RNA Library Prep Kit
- New England BioLabs® NEBNext® Multiplex Small RNA Library Prep Kit

The kits were also used to prepare control libraries from 1 ng of Miltenyi Biotec® miRXplore™ miRNA Universal Reference, which is an equimolar mixture of 963 miRNAs. Each library was prepared in either duplicate or triplicate.

The final libraries were sequenced on an Illumina® MiSeq® platform. The libraries were sequenced and analyzed as previously described. The libraries created from the miRXplore™ RNA were also sequenced and analyzed as previously described, but were mapped to a reference created from the miRNAs present in the miRXplore™ sample.

Results and Discussion

Figure 3 shows the miRNA detection levels and Coefficients of Variation (CV) for the miRXplore™ libraries. The NEXTflex Small RNA-Seq Kit v3 libraries show a higher level of detection with less bias than the other kits throughout the range of read thresholds sampled. Figure 4 shows the relationship between sequencing depth and miRNA detection rates for the human brain total RNA sample libraries. The NEXTflex Kit libraries require fewer reads to obtain high levels of miRNA detection compared to competitors' kits.

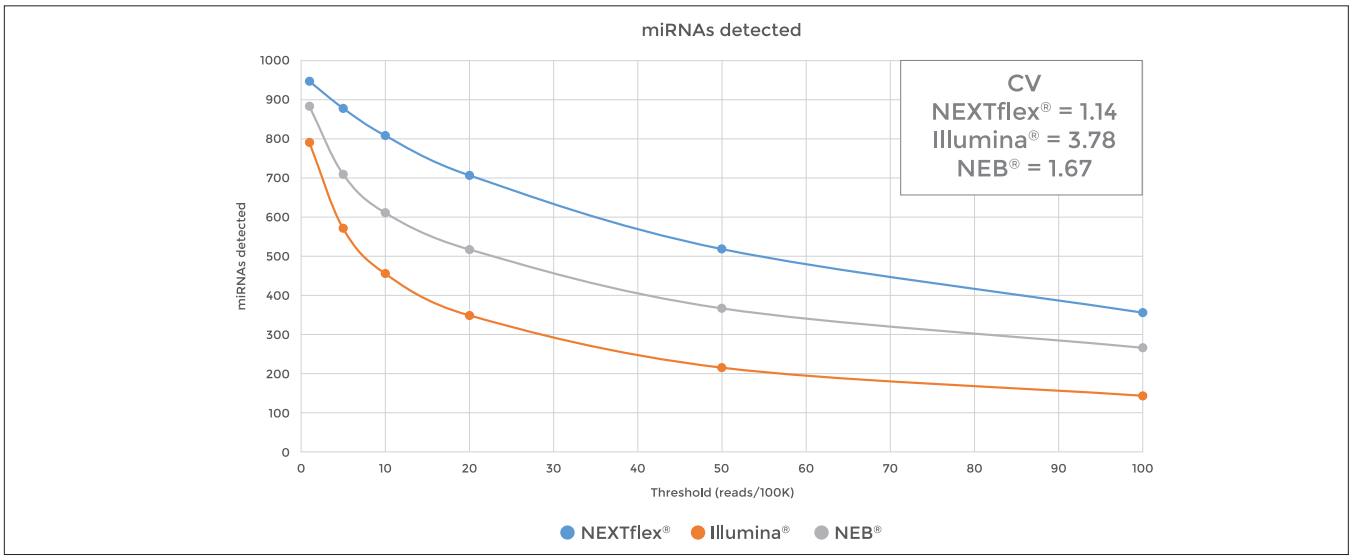

Figure 3. **Levels of Detection and Bias in Control Libraries.** The miRNA detection level for the NEXTflex Small RNA-Seq Kit v3-prepared miRXplore™ library is higher than the detection levels provided by the competitors' kits. The Coefficients of Variation demonstrate that the NEXTflex Kit produces libraries that have lower levels of bias.

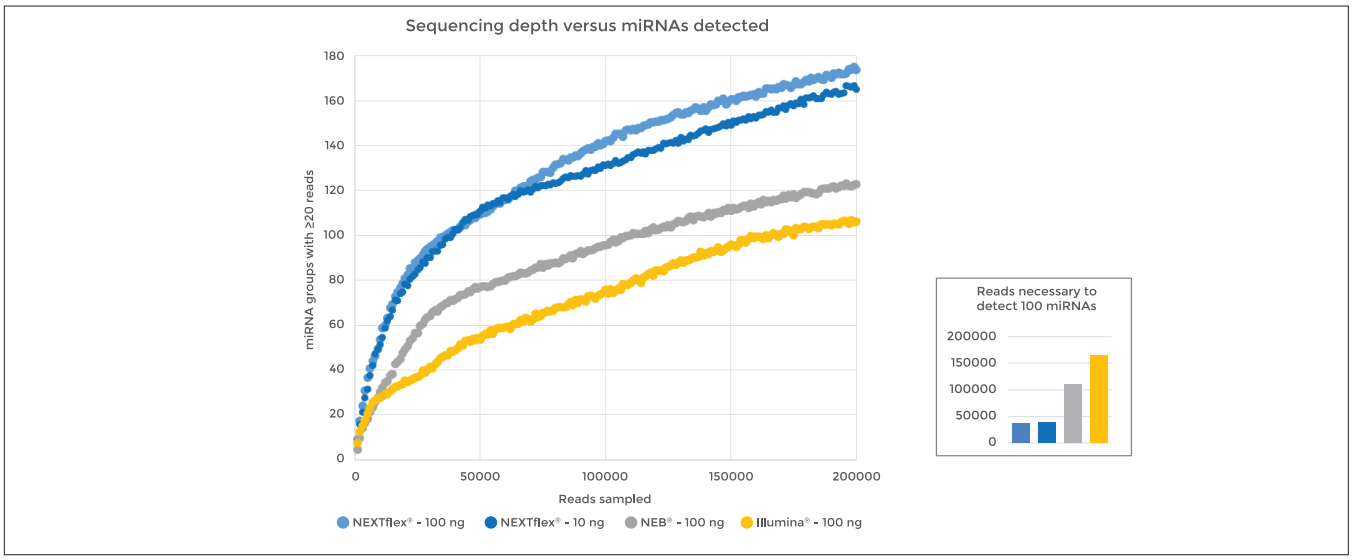

Figure 4. **Sequencing Depth vs. miRNAs Detected in Human Brain Total RNA Libraries.** The indicated number of reads was sampled from each library and the average number of miRNA groups with ≥ 20 reads determined. The libraries generated with the NEXTflex Kit require fewer reads to obtain high levels of detection.

Conclusion

The data demonstrate that using the NEXTflex Small RNA Sequencing Kit v3 on the Sciclone G3 NGS Workstation produces automated small RNA libraries with mapping rates, detection levels, and sequencing data that are comparable to manually prepared libraries. The advantage is that the automated protocol enables high sample throughput, generating more libraries in less time while maintaining data quality. Additionally, the NEXTflex Kit's dual approach to adapter-dimer reduction allows completely gel-free library preparation with reduced bias and greater miRNA detection rates than similarly tested competitor kits.

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