Automated NGS Library Prep: Ligation Efficiency of NxSeq® Sample Preparation Using the Gilson PIPETMAX® 268

Application Note GEN01013

Keywords

Next Generation Sequencing, NGS, Sample Prep, DNA Library Prep, Automation, NxSeq, PIPETMAX

Introduction

This application note was performed by Erin Ferguson, Svetlana Jasinovica, Dr. Michael Lodes, and Curtis Knox, Lucigen Corporation, Middleton, WI, USA.

As the number of samples for sequencing increases, there is a need for library prep automation that matches the throughput of the next generation sequencing (NGS) instruments while not requiring an investment equal to the sequencer itself. By utilizing the Gilson PIPETMAX® 268, a novel NGS sample prep chemistry was automated to reduce the time to achieve the final prepared library, increasing the efficiency of the method and consistency of the resulting DNA library to be sequenced.

The NxSeq®DNA sample prep chemistry combines typical end-repair and A-tailing steps into one master mix step with buffers directly compatible with downstream ligation steps, eliminating the need for multiple cleanup steps throughout the process. The chemistry has also been optimized to drive higher A-tailing efficiencies, which reduces chimera formation from blunt fragments and loss of fragments tailed with nucleotides other than the necessary "A." Chemistries designed by Lucigen and automated on the PIPETMAX allow numerous DNA libraries to be prepared simultaneously, including incorporation of barcoded adapters for multiplex PCR and sequencing. In contrast to other small, dedicated automation platforms, the PIPETMAX is open and programmable, meaning users can choose to utilize the platform for a range of other applications, including qPCR and cell-based assays.

In this application, NGS libraries were prepared from *E. coli* DH10B genomic DNA (gDNA) and FAM-labeled adaptors using the NxSeq® DNA Sample Prep kit, comparing the manual method with an automated library prep protocol on the PIPETMAX®. This application note describes the results of the ligation efficiency of manual library preparation versus automated library preparation.

Materials & Methods

Materials

- *E. Coli* DH10B gDNA – Lucigen, Middleton, WI
- NxSeq DNA Sample Prep Kit – Lucigen, Middleton, WI
- FAM-Labeled Adaptors – Integrated DNA Technologies, Coralville, IA
- Qubit 2.0 Fluorometer – Life Technologies, Grand Island, NY
- Qubit dsDNA HS Assay Kit – Life Technologies, Grand Island, NY
- Agencourt AMPure XP Beads – Beckman Coulter, Indianapolis, IN
- Biorad DNA Engine Thermocycler – Biorad, Hercules, CA
- Agilent Bioanalyzer 2100 and High Sensitivity DNA Kit – Agilent Technologies, Santa Clara, CA
- Synergy 2 Multi-Mode Microplate Reader – Biotek, Winooski, VT
- PIPETMAX 268 with TRILUTION® micro software – Gilson, Middleton, WI

Methods – Sample Preparation

1) *E. coli* DH10B gDNA was sheared by sonication to a size range of 50-500 bp. The size was confirmed on the Agilent Bioanalyzer (Figure 1) and concentration readings were performed on the Qubit fluorometer.
2) End repair and A-tailing were performed for manual libraries by preparing a master mix of NxSeq 2X End Repair Buffer, NxSeq End Repair Enzymes, and Low TE added to 500 ng of DNA per reaction. The samples were incubated on a thermocycler for 20 minutes at 25˚C, 20 minutes at 72˚C, and then held at 4˚C.

3) For automated sample prep, end repair reagents, A-tailing reagents, and gDNA were placed on the bed of the Gilson PIPETMAX® 268 according to the NxSeq® Sample Prep protocol preloaded on the PIPETMAX TRILUTION micro software, as shown by the layout in Figure 2. Once samples, master mix reagents, and enzymes were combined, the samples were removed from the bed and placed on a thermocycler for 20 minutes at 25˚C, 20 minutes at 72˚C, and then held at 4˚C.

4) After incubation, FAM-labeled adaptors and high concentration DNA ligase were added to the manually prepared libraries. Libraries prepared on the PIPETMAX were placed back onto the bed where the samples, FAM-labeled adaptors, and high concentration ligase were combined using the PIPETMAX.

5) Both the manual and the PIPETMAX libraries were incubated at room temperature for 30 minutes.

6) During incubation, a stock of 1.7X sizing buffer was prepared by combining 30% PEG, 5M Sodium Chloride, and distilled water.

7) One replicate of both the manual and PIPETMAX libraries was size selected using 1.7X sizing buffer and AMPure XP beads. Another set of manual and PIPETMAX library replicates was size selected using a two-step bead cleanup. DNA was size selected with a 0.8X ratio of beads to DNA (ratio based on volume) for large fragment removal, followed by a 0.2X ratio of beads to DNA for small fragment removal. After size selection, 1 µL of each library and 1 µL of diluted 1:10 sheared gDNA were run on a High Sensitivity DNA Chip on the Agilent Bioanalyzer.

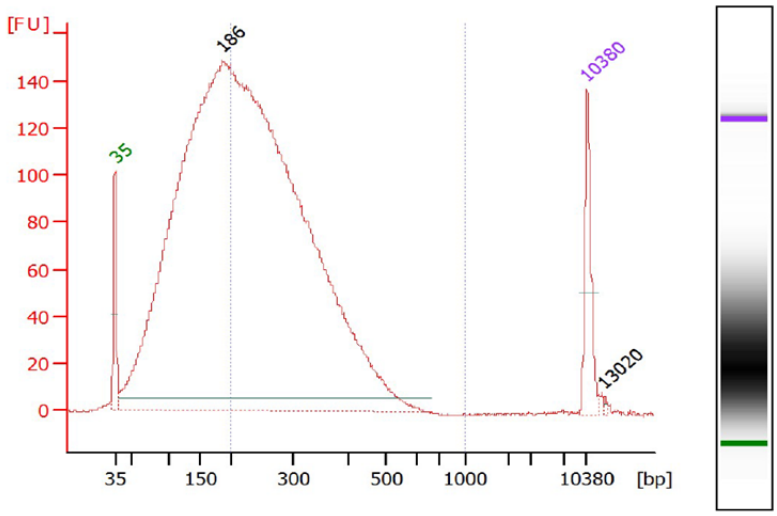

Figure 1.
Sheared DH10B gDNA to 50-500 bp, prior to NGS library preparation.

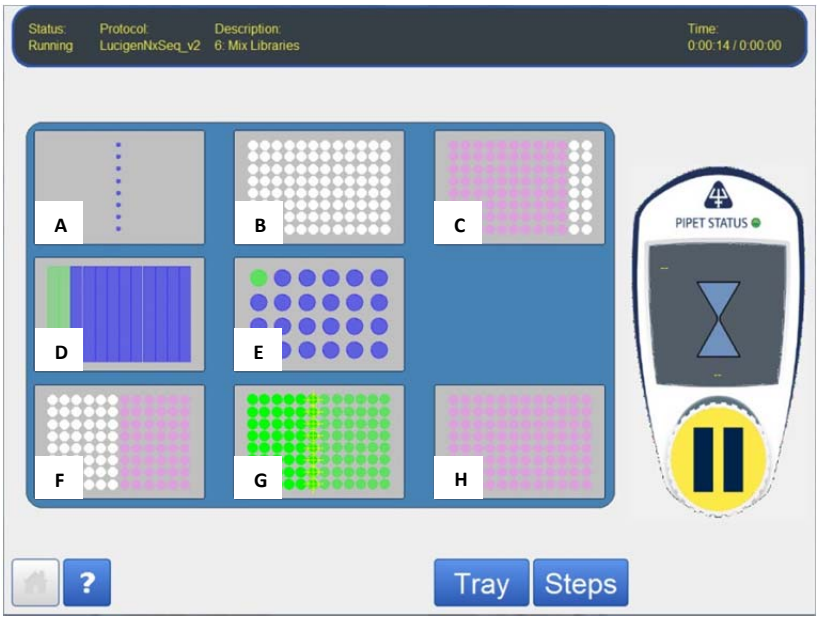

Figure 2.
TRILUTION® micro software setup to run the Lucigen NxSeq Sample Prep protocol on the PIPETMAX® 268. A) Tip waste bin; B) D200 tips; C) D200 tips; D) 12-column reservoir; E) Code 425 PT rack (cold block) with 1.5 mL tubes; F) DL10 tips; G) Code 496 PT rack with PCR strip tubes; H) DL10 tips.

A serial dilution of NxSeq FAM-labeled standards (Table 1) along with the samples were loaded on a 96-well plate in duplicate and read at excitation 485 nm and emission 528 nm. A standard curve was generated in Excel using the fluorescence readings as the Y-axis and the standard concentrations as the X-axis (Figure 3). The standard curve was used to determine the library concentration (molecules x 10^8 DNA/ng) for the manual and PIPETMAX prepared libraries.

Table 1. Serial dilution of NxSeq FAM-labeled standards.

Dilution tube number (dilution factor)	NxSeq FAM labeled Standard mol/µL x 10^8
1 (no dilution)	25
2 (2/3rd dilution of tube 1)	16.7
3 (2/3rd dilution of tube 2)	11.1
4 (2/3rd dilution of tube 3)	7.41
5 (2/3rd dilution of tube 4)	4.94
6 (2/3rd dilution of tube 5)	3.29
7 (2/3rd dilution of tube 6)	2.19
8 (2/3rd dilution of tube 7)	1.46

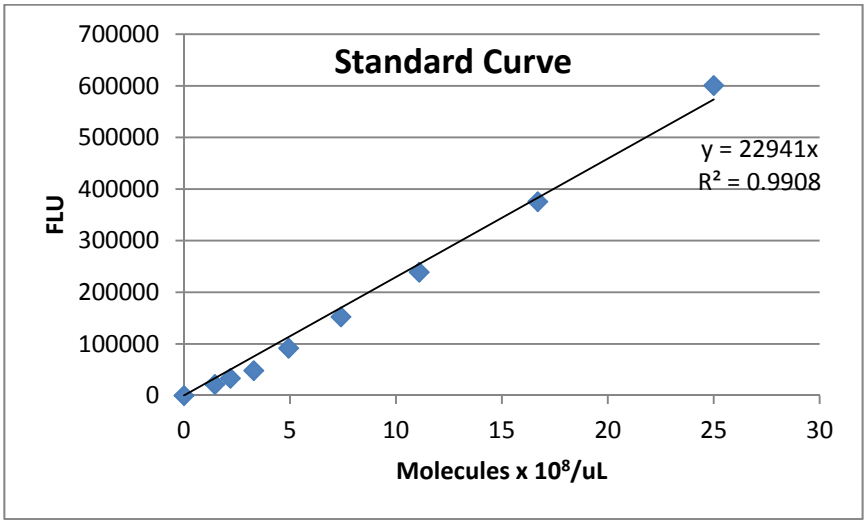

Figure 3. Scatter plot with linear trend-line with the library fluorescence reading as the Y-axis and serial dilution of standards as the X-axis.

DNA quantification readings (Qubit) and fluorescent molecule assay readings (Synergy) were taken in triplicate for each library and the average value was used to calculate the molecules x 10^8/ng (Table 2).

Results – Sample Analysis using Agilent Bioanalyzer

If the library has been correctly assembled with adapters ligated to both ends of the target fragments, there should be a difference of approximately 75 bp (+/- 20 bp) where the library samples are shifted approximately 75 bp to the right compared to the control non-ligated sheared gDNA sample (Figure 4). The expected library peaks started at 150 bp (+/- 20 bp) compared to the sheared gDNA. Although it wasn't observed in these experiments, a failed library would demonstrate no difference between overlaid samples either on one or both sides of the peaks.

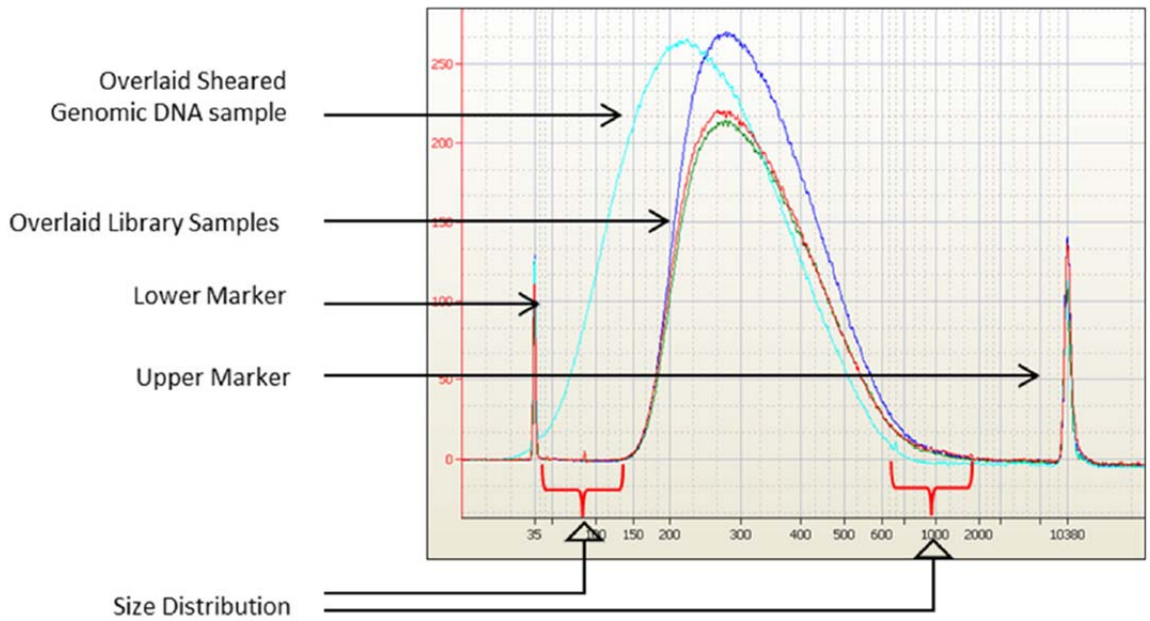

Figure 4. Comparison of Sheared Genomic DNA vs. prepared libraries using NxSeq® DNA Sample Prep chemistry and FAM-labeled adapters. Light blue = sheared gDNA. Dark blue, red, and green = examples of NxSeq FAM-labeled libraries. Note that all libraries demonstrated the expected size shift indicating libraries with adapters ligated to both ends.

Results – Size Selection

Using the bead sizing method, the samples from the PIPETMAX prepared libraries were more consistent and resulted in a greater yield relative to the manual preparation. Using the Agilent software navigation, the manually prepared and PIPETMAX prepared sample graphs were overlaid for comparison purposes (Figures 5 and 6).

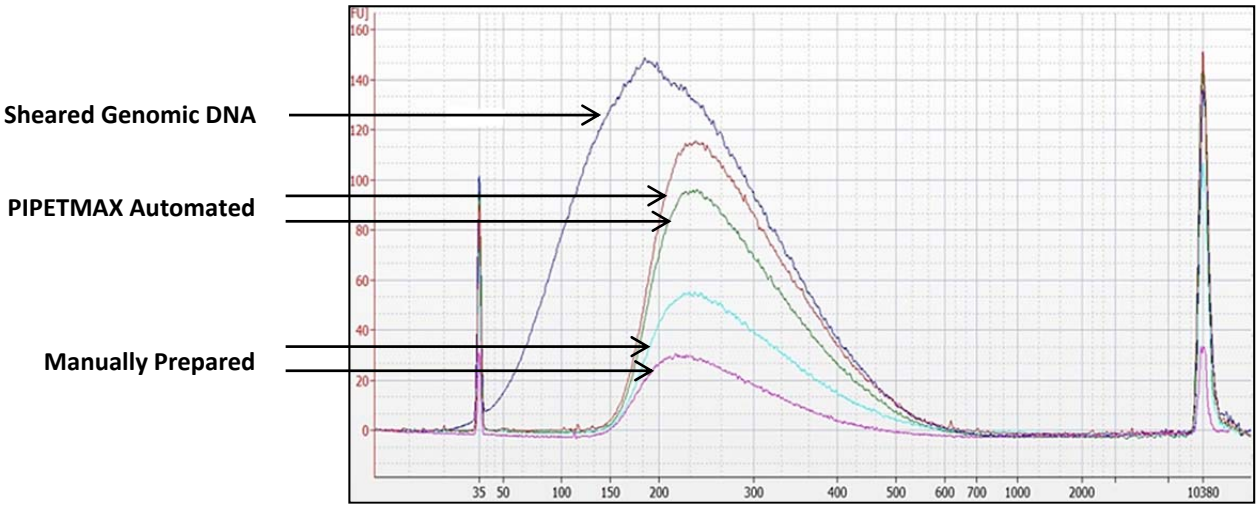

Figure 5. Comparison of Manual NxSeq® Libraries (light blue, pink) and PIPETMAX® NxSeq Libraries (red, green) using a 0.8:0.2 ratio of AMPure XP Beads for size selection.

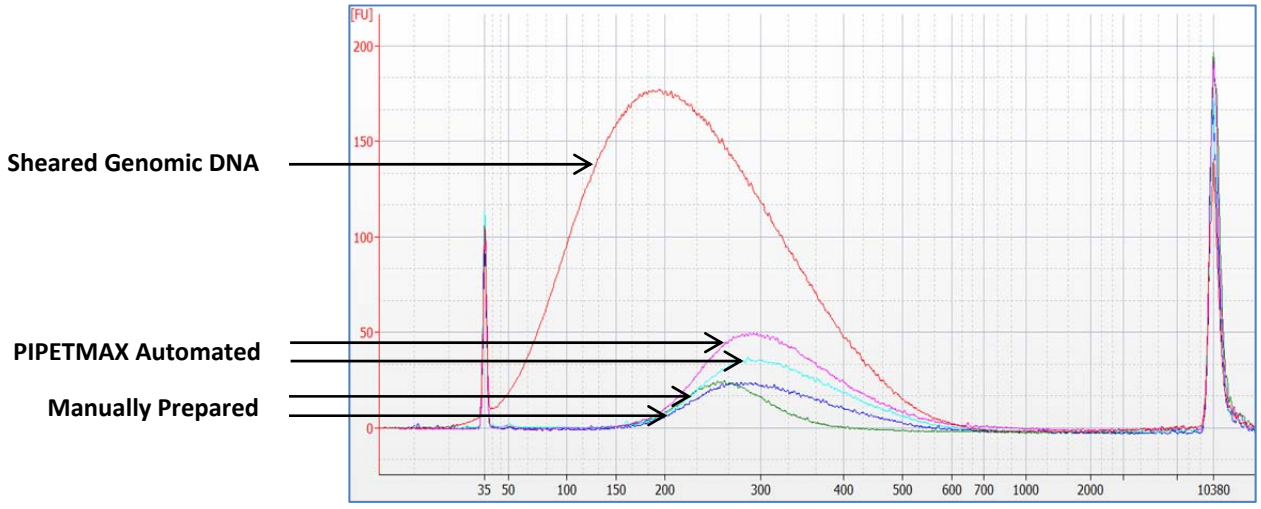

Figure 6. Comparison of Manual NxSeq® Libraries (green, dark blue) and PIPETMAX® NxSeq Libraries (pink, light blue) using 1.7X sizing buffer and AMPure XP beads for size selection.

Results – Sample Analysis using a Functional Assay

To assess the quantity and quality of each library, the amount of fluorescence was assayed using the Synergy 2 Multi-mode Microplate Reader. This result was compared to a dsDNA quantification standard (Qubit dsDNA High Sensitivity assay and Qubit 2.0 fluorometer) to measure the number of fluorescent molecules obtained per ng DNA (Figure 3; Table 2). Lucigen experiments using manual library preparation have previously shown that achievement of 3-6 molecules x 10^8/ng DNA demonstrates sufficient ligation efficiency for downstream NGS applications (data not shown).

Table 2. Quantity and quality of the manual and PIPETMAX prepared libraries using both methods for size selection.

Method	Molecules x 10^8 of DNA per ng
Manual Library Using Sizing Buffer (1.7X)	4.78
Manual Library Using 0.8:0.2 Beads	4.92
PIPETMAX Library Using Sizing Buffer (1.7X)	5.02
PIPETMAX Library Using 0.8:0.2 Beads	5.07

For downstream NGS amplification to be successful, it is essential to have efficient adaptor ligation occurring during library prep. Manual NxSeq® DNA prep methods have shown that the kit is capable of efficient library preparation, and the purpose of this comparison was to evaluate the efficiency of ligation achieved when performing the same methods on the PIPETMAX® automated platform. As indicated in Table 2, the library efficiencies obtained by the automated method were comparable to those obtained by the standard manual method.

Summary

Adapter ligation efficiency of the libraries prepared on the PIPETMAX® 268 is comparable to that of the NxSeq® libraries prepared manually, indicating that the overall quality of the library is not compromised when using the PIPETMAX. In addition, both sizing solution and beads for sizing perform as expected. It should be noted that overall yield, as measured by the Agilent High Sensitivity DNA kit, is greater for the automated method and bead-only sizing. More importantly, the use of an automated method for NxSeq sample prep enables easy preparation of multiple samples at one time with minimal effort and greater consistency.