APPLICATION NOTE #1

Mondrian™ SP Library Preparation Method 1

DNA Library Construction for Illumina SBS Sequencing Platforms

I. Introduction

This application note describes the materials and methods for constructing DNA sequence libraries utilizing NEBNext® DNA Sample Prep library construction reagents on the Mondrian™ SP digital microfluidics system. The protocol requires 100 nanograms of fragmented DNA as input and produces indexed or non-indexed libraries ready for quantitation and normalization prior to cluster generation.

The system processes eight samples at a time on a single Mondrian SP Cartridge. Reagent master mixes and samples are prepared by the user and loaded into the cartridge. After placing the cartridge into the Mondrian SP Workstation, the protocol is selected on the touchscreen user interface. The system processes the samples through each enzyme step and purification steps with no further intervention by the user. At the end of the approximately three and a half hour run, the processed sample is extracted from the cartridge, added to PCR master mix and placed into a thermal cycler for library enrichment. Following library enrichment the library is quantitated and normalized for cluster generation. The libraries created are suitable for sequencing on the Genome Analyzer IIx/IIe (GAII), HiScan SQ, HiSeq and MiSeq systems.

II. Materials

Instrumentation and Consumables

- Mondrian SP Workstation (NuGEN Part No. 8000)
- Mondrian SP Universal Cartridge (NuGEN Part No. 8010).

  The Universal Cartridge package includes:
  - Digital microfluidics cartridge
  - Filler Fluid
  - Sample Concentration Solution
  - Bead Binding Solution
  - Bead Wash
  - Elution Solution
  - Reagent Additive
  - Cartridge Loading Guide

Reagents

- NEBNext® DNA Sample Prep Master Mix Set 1 (NEB Cat. #E6040S)
- PfuUltra II Hotstart PCR Master Mix (Agilent Cat. #600850)
- AMPure XP magnetic bead DNA purification system (Beckman Coulter Genomics Cat. #A63881)
- Appropriate adaptor mix and PCR enrichment primers must be obtained separately (see http://www.currentprotocols.com/protocol/hg1802 for reference).

Additional Equipment

- NanoDrop 8000 Spectrophotometer or equivalent for the quantification of DNA by UV absorbance
- Thermocycler for PCR amplification
- 0.5–10 µL pipette, 2–20 µL pipette, 20–200 µL pipette, 200–1000 µL pipette
- Vortexer
- Microcentrifuge for individual 1.5 mL and 0.5 mL tubes and 0.2 mL PCR tubes
- Magnetic separation device options:
  - Agencourt® SPRIPlate® Ring Super Magnet Plate (Beckman Coulter Genomics, Cat. #A32782)
  - Invitrogen™ DynaMag -96 Side (Invitrogen, Cat. #123-31D

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- Invitrogen DynaMag -96 Side Skirted (Invitrogen, Cat. #120-27)
- Promega MagnaBot II Magnetic Separation Device (Promega, Cat. #V8351)
- Agencourt SPRIStand (Beckman Coulter Genomics, Cat. #A29182)

Supplies and Labware

- Ethanol (Sigma-Aldrich, Cat. #E7023), for purification
- 10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0
- Water, nuclease-free
- Nuclease-free pipette tips
- 1.5 mL and 0.5 mL RNase-free microcentrifuge tubes
- 0.2 mL individual thin-wall PCR tubes
- Disposable gloves
- Kimwipes
- Ice bucket
- Cleaning solutions such as DNA-OFF™ (MP Biomedicals, Cat. # QD0500) for preparing the work space

Samples

The DNA samples used in this study were genomic DNA from *E. coli* and *H. sapiens*. gDNA samples were sonically sheared using the Covaris S220 ultrasonicator system according to manufacturer's instructions to generate DNA fragments of approximately 150 to 200 bases.

III. Protocol

A. Overview

The approximate time to complete a library run is summarized below:

Time required for each step of a library preparation run.

Preparation of master mixes and cartridge set up	0. 5 hours
Running the protocol	3.25 hours
PCR enrichment, purification & quantitation	2.25 hours
Total time to prepare and analyze amplified library	**~6 hours**

B. Sample Concentration

Prepare DNA samples as follows:

Sheared DNA (100 ng)	xx µL*
Sample concentration solution	25 µL
AMPure XP magnetic beads	3.6 µL
Water	xx µL*
Total volume	**50 µL**

* Note: adjust the DNA volume and water to ensure a final sample volume of 50 microliters.

Mix thoroughly and incubate at room temperature for at least 10 min. The additional required master mixes (below) can be prepared during this time. **DO NOT PLACE SAMPLES ON ICE.**

C. Preparation of Master Mixes

1. End Repair Mix (prepare and keep on ice)

COMPONENT	VOLUME
End Repair Reaction Buffer (10X)	1.5 µL
End Repair Enzyme Mix	0.75 µL
Reagent additive	1.5 µL
Water	3.75 µL
Total volume	**7.5 µL**

2. dA Tailing Mix (prepare and keep on ice)

COMPONENT	VOLUME
dA Tailing Reaction Buffer (10X)	1.5 µL
Klenow Fragment (3´→ 5´ exo–)	0.9 µL
Reagent additive	1.5 µL
Water	3.6 µL
Total volume	**7.5 µL**

3. Ligation Mix (prepare and keep on ice)

COMPONENT	VOLUME
Quick Ligation Reaction Buffer (5X)	4.5 µL
Quick T4 DNA Ligase	2.25 µL
Reagent Additive	0.75 µL
Total volume	**7.5 µL**

4.a. Indexed Adaptors (one mix prepared for each library. Prepare and keep at room temperature)

COMPONENT	VOLUME
50 µM adaptor mix	1.5 µL
Reagent Additive	1.0 µL
Total volume	**2.5 µL**

4.b. Non-indexed Adaptors (a single mix prepared for all eight libraries. Prepare and keep at room temperature)

COMPONENT	VOLUME
50 µM adaptor mix	12 µL
Reagent Additive	8 µL
Total volume	**20 µL**

D. Loading the Cartridge

Refer to the Mondrian SP Universal Cartridge User Guide for instructions on how to prepare, load reagent and mount the Universal Cartridge on the Mondrian SP Workstation. Pipet 50 µL of each sample mix into S1–S8 (ensure that the 10-minute incubation step as described above is performed prior to loading onto the cartridge).

1. Pipet 50 µL of Bead Wash into E7.
2. Pipet 50 µL of Elution Solution into E5. (NOTE: Well E6 is intentionally left empty.)
3. Pipet 50 µL of Bead Binding Solution into E4.
4. Pipet 6 µL of End Repair mix into D7.
5. Pipet 6 µL of dA Tailing mix into D6.
6. Pipet 6 µL of Ligation mix into D5.
7. Pipet 1.5 µL of Adaptor 1 into A1, 1.5 µL of Adaptor 2 into A2, and so forth until adaptors are loaded in all eight adaptor wells.

E. Starting the Run

1. Refer to the Mondrian SP Workstation User Manual for instructions on how to turn on the workstation and select and run a protocol.
2. Run the protocol "Library Preparation Method 1."
3. If desired, fill in user and sample information for the run as described in the Mondrian SP Workstation User Manual.
4. The run will be completed in 3.25 hours.

F. Collecting the Library

1. When the run is completed the screen displays "Run Successful." Press the "Done" button and turn off the instrument by pressing the Power button below the touch screen. (Note: If the screen indicates there were issues with the run, continue to the Home screen and press the "LOG" button. On the following screen select the current run and export the log (.csv) file to a USB drive inserted on the right side of the Touch Screen.)
2. Open the lid if closed.
3. Collect samples from the appropriate wells as described in the Mondrian SP Universal Cartridge User Guide.
4. After all the prepared libraries have been extracted from the cartridge, push the deck lever to disengage the cartridge and remove the cartridge from the instrument deck by gently pulling it toward the front of the instrument. Dispose the cartridge as appropriate and use a Kimwipe to clean up any spilled filler fluid on the deck.
5. Add 20 µL of TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) to each tube containing the library droplet and

filler fluid. Mix and allow the aqueous (lower) phase to separate from the filler fluid.

G. PCR Enrichment

1. Remove the PfuUltra II Hotstart PCR Master Mix and PCR primers and allow them to thaw.
2. Prepare a PCR reaction Master Mix according to the following table. Keep on ice:

COMPONENT	VOLUME
2X PfuUltra II Master Mix	225 µL
PCR Primer 1 (25 µM)	18 µL
PCR Primer 2 (25 µM)	18 µL
Water	27 µL

3. Set up each library for enrichment according to the following table, carefully recovering the 18 µL sample from the lower aqueous phase:

COMPONENT	VOLUME
PCR Master Mix	32 µL
Sample	18 µL
Total volume	**50 µL**

4. Mix each PCR reaction setup thoroughly.
5. Process the samples in a thermocycler running the following program:

   95°C – 1 min, 10 cycles (95°C – 30 sec, 65°C – 30 sec, 72°C – 1 min), 72°C – 10 min, hold at 4°C

H. Library Purification and Quantification

1. Remove the Agencourt® AMPure® XP magnetic beads from the refrigerator and allow to warm to room temperature before proceeding.
2. Resuspend beads by inverting and tapping the bottle. Ensure beads are fully resuspended before adding to sample.
3. Prepare a 70% ethanol wash solution. It is critical that this solution be prepared fresh on the same day of the experiment from a recently opened stock container. Measure both the ethanol and the water components carefully prior to mixing. Failure to do so can result in a higher than anticipated aqueous content that may reduce yield.
4. Allow the PCR-enriched libraries to come to room temperature.
5. Add 50 µL (1 volume) of the AMPure XP bead suspension to each sample. Mix thoroughly by pipetting up and down several times.
6. Incubate the samples at room temperature for 10 minutes.
7. Transfer tubes to a recommended magnet device and allow to stand 5 minutes or until the solution is completely cleared of beads.
8. Carefully remove 85 µL of the binding buffer from each sample and discard. (Leaving some of the volume behind minimizes bead loss at this step.)

   Note: The beads should not disperse; instead, they will stay on the walls of the tubes. Significant loss of beads at this stage will impact the yield of DNA from PCR amplification, so ensure beads are not removed with the binding buffer or the wash.
9. With the tubes still on the magnet, add 200 µL of freshly prepared 70% ethanol and allow to stand for 30 seconds.
10. Remove the 70% ethanol wash using a pipette.
11. Repeat the 70% ethanol wash one more time, for a total of two washes.

    Note: With the final wash, it is critical to remove as much of the ethanol as possible. Use at least two pipetting steps and allow excess ethanol to collect at the bottom of the tubes after removing most of the ethanol in the first pipetting step.
12. Air-dry the beads on the magnet for 5–10 minutes. Inspect each tube carefully to ensure that all the ethanol has evaporated. It is critical that all residual ethanol be removed prior to continuing.
13. Remove tubes from magnet.
14. Add 22 µL TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) to the dried beads. Mix thoroughly to ensure all the beads are resuspended and let stand on the bench top for 3 minutes.
15. Transfer tubes to the magnet and let stand for 3 minutes for the beads to clear the solution.
16. Carefully remove 20 µL of the eluate, ensuring as few beads as possible are carried over, transfer to a fresh set of PCR tubes and place on ice. It is better to leave

some of the solution behind rather than to carry over the beads.

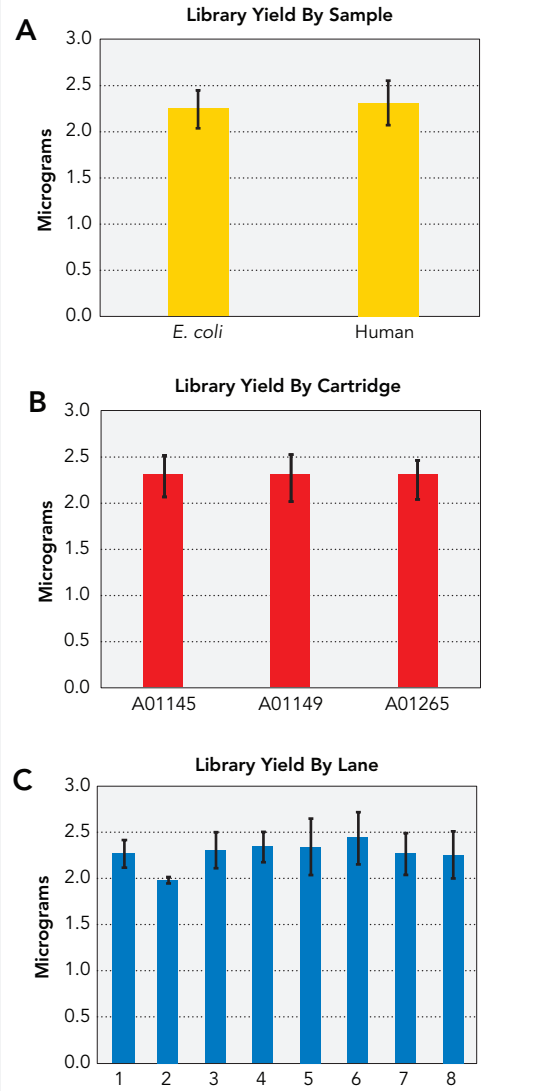
17. Remove 1.5 µL of the purified library and determine its concentration on a NanoDrop 8000 or equivalent.

IV. Results

The system produces sufficient material for library quantitation, evaluation and cluster generation. Figures 1A–C show the average library yield obtained for samples across multiple cartridges. Consistent and sufficient PCR yield was determined by Nanodrop measurement.

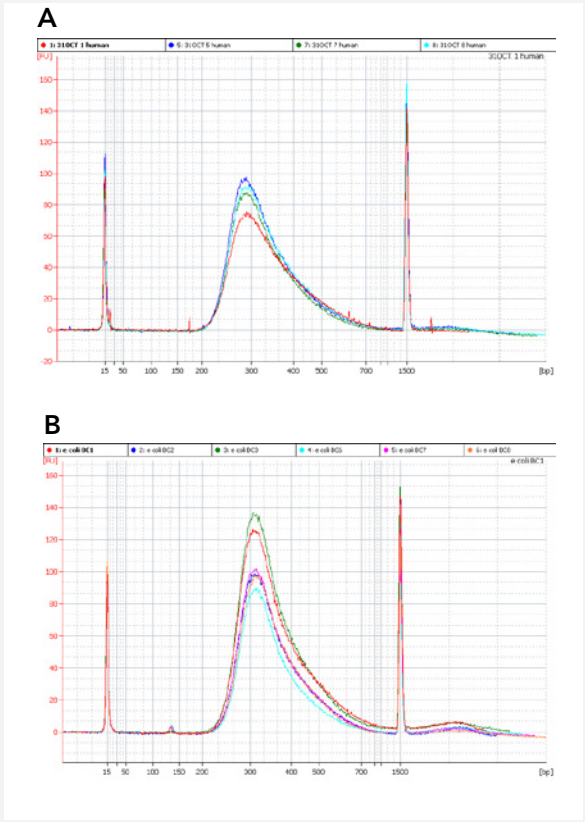
The libraries were further characterized by Bioanalzyer to determine size distribution and the presence or absence of adaptor dimer. As seen in Figure 2, libraries from both human and *E. coli* genomic DNA were produced of the expected size with little or no adaptor dimer.

FIGURE 1. **Enriched library yields plotted by sample type (A; N=12), cartridge number (B; N=8) and lane number (C; N=3)**



Average is shown with standard deviation.

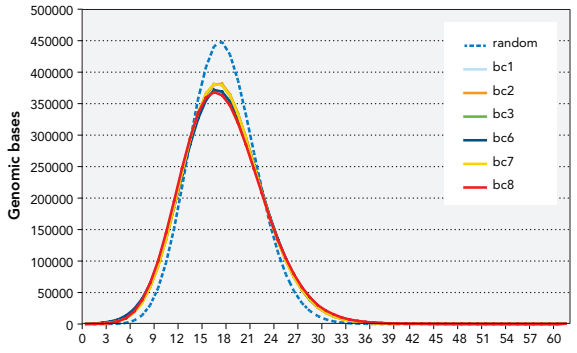
FIGURE 2. **Bioanalyzer traces of purified libraries from human (A) and *E. coli* (B) genomic DNA**



Sequencing of barcoded *E. coli* gDNA libraries show even coverage with no discernable bias in representation (Figure 3). These results are similar to in-house results with libraries processed manually.

The distribution of a theoretical randomly generated library is shown as the dotted line. The distribution plots for six actual libraries (2.35 million reads per library) are shown as an overlay, illustrating very even coverage of the genome with no discernable regions of over- or under-representation.

FIGURE 3. **Plot illustrating depth of coverage for a series of libraries generated from 100 ng *E. coli* gDNA on the Mondrian SP Workstation using Library Preparation Method 1**



V. Conclusions

The Library Preparation Method 1 protocol on the Mondrian SP Workstation is a robust, hands-free system for producing high-quality libraries for sequencing on the Illumina SBS Sequencing Systems. The first in a series of enabling molecular methods to be ported to the Mondrian SP digital microfluids platform, this method allows for a substantial savings in hands-on lab time while providing efficiencies of scale in reagent use and reproducibility by reducing reaction volumes and automating the most time-consuming and effort-intensive tasks.

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Portions of this method utilize Broad proprietary technology.

The Mondrian SP Workstation and Cartridges were developed and manufactured for NuGEN by Advanced Liquid Logic.

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