Next Generation Sequencing Method for Illumina TruSeq DNA Sample Preparation Protocol on the Hamilton STAR

Authors

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Method validation and testing performed by Oblique Bio, Inc. More information can be found at http://www.ObliqueBio.com/

Abstract

Next Generation Sequencing (NGS) has accelerated the sequencing of genomes and this technology has a major role to play in the biomedical research. The simplicity of NGS library preparation, the ability to make these libraries from tiny input amounts of DNA, the elimination of a bacterial cloning intermediate and the development of data-analysis approaches to support the increasing size of the sequence datasets have been invaluable in the advancement of NGS. This application note describes the automation of the Illumina TruSeq DNA sample preparation on the Hamilton STAR (Figure 1). The data presented includes PicoGreen assay and Sybr E-Gel results for comparison of library construction using automated and manual methods, Bioanalyzer and KAPA assay results and data from Illumina HiSeq. Automation allowed processing 96 samples simultaneously in less than 4 hrs. Sample recovery yields higher by 50% were obtained with automated compared to manual method. The sample preparation provided average 80% reads on target from Illumina HiSeq.
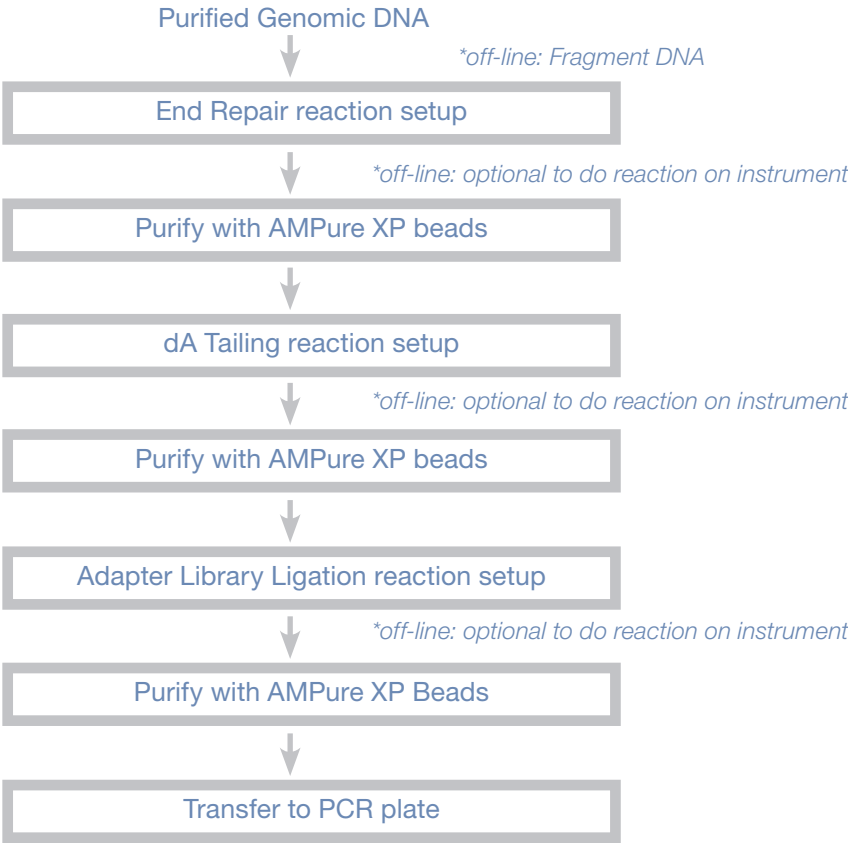
Introduction

The library preparation method and system setup are based on the low throughput portion of the Illumina TruSeq DNA Sample Preparation Guide (P/N 15005180 Rev A). Modifications to the low throughput protocol have been made to accommodate up to 96 samples. The 1000 µL independent channels are used for all bulk reagent liquid transfers on Hamilton STAR and CORE Grippers are used for all labware move steps. The 96 Channel CORE Multiprobe Head is used to pick up single rows of tips for all enzymatic and library oligos. The 96 Channel CORE Multiprobe Head is used for all paramagnetic bead wash and elution steps. The purpose of the TruSeq sample preparation protocol is to add adapter sequences onto the ends of DNA fragments to generate multiplexed sequencing libraries. Good liquid handling is crucial for pipetting precise volumes for example for Agilent Bioanalyzer. The protocol is optimized for 1 µg input DNA.

Figure 1:
The Hamilton Robotics Microlab®
STAR Automated Workstation

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Workflow

Purified Genomic DNA

↓ *off-line: Fragment DNA*

End Repair reaction setup

↓ *off-line: optional to do reaction on instrument*

Purify with AMPure XP beads

↓

dA Tailing reaction setup

↓ *off-line: optional to do reaction on instrument*

Purify with AMPure XP beads

↓

Adapter Library Ligation reaction setup

↓ *off-line: optional to do reaction on instrument*

Purify with AMPure XP Beads

↓

Transfer to PCR plate

Process Steps:

Process	Tip Usage
Perform End Repair	
Add Fragmented DNA to IMP PCR Plate	96 50µL tips
Add End Repair Control (or Resuspension Buffer) to IMP	96 50µL tips
Add End Repair Mix and mix	96 300µL tips
Clean Up IMP	
Add AMPure XP Beads to IMP (pre-mix if beads have settled) and mix	96 300µL tips
Incubate IMP at room temperature for 15 minutes	
Move IMP from plate carrier to magnetic plate stand and wait for 15 minutes	
Remove supernatant from IMP and transfer to waste trough	96 300µL tips
Add 80% Ethanol to IMP	96 300µL tips
Incubate IMP at room temperature for 30 seconds	
Remove from IMP and transfer to waste trough	
Add 80% Ethanol to IMP	96 300µL tips
Incubate IMP at room temperature for 30 seconds	

Remove from IMP and transfer to waste trough	
Add 80% Ethanol to IMP	96 300µL tips
Incubate IMP at room temperature for 30 seconds	
Remove from IMP and transfer to waste trough	
Move IMP from magnetic stand to plate carrier and wait for 15 minutes	
Add Resuspension Buffer to IMP and mix	96 50µL tips
Incubate IMP at room temperature for 2 minutes	
Move IMP from plate carrier to magnetic plate stand and wait for 5 minutes	
Add supernatant from IMP to ALP PCR plate	
Discard IMP to waste and move ALP to original IMP plate carrier position	
Adenylate 3' Ends	
Add A-Tailing Control (or Resuspension Buffer) to bottom of ALP wells	96 50µL tips
Add A-Tailing Mix to bottom of ALP wells and mix	96 50µL tips
Add Ligate Control (or Resuspension Buffer) to ALP	96 50µL tips
Add Ligate Mix to ALP	96 50µL tips
Add DNA Adapters to ALP and mix	96 50µL tips
Add Stop Ligate Mix to ALP and mix	96 50µL tips
Clean Up ALP	
Similar to Clean Up IMP	
Clean Up CAP	
Similar to Clean Up IMP	
Enrich DNA Fragments - Make PCR	
Add PCR Primer Cocktail to PCR plate	96 50µL tips
Add PCR Master Mix to PCR plate and mix	96 50µL tips
Prompt user to remove PCR plate and Amplify offline	
Clean Up PCR	
Similar to Clean Up IMP	

Method Overview

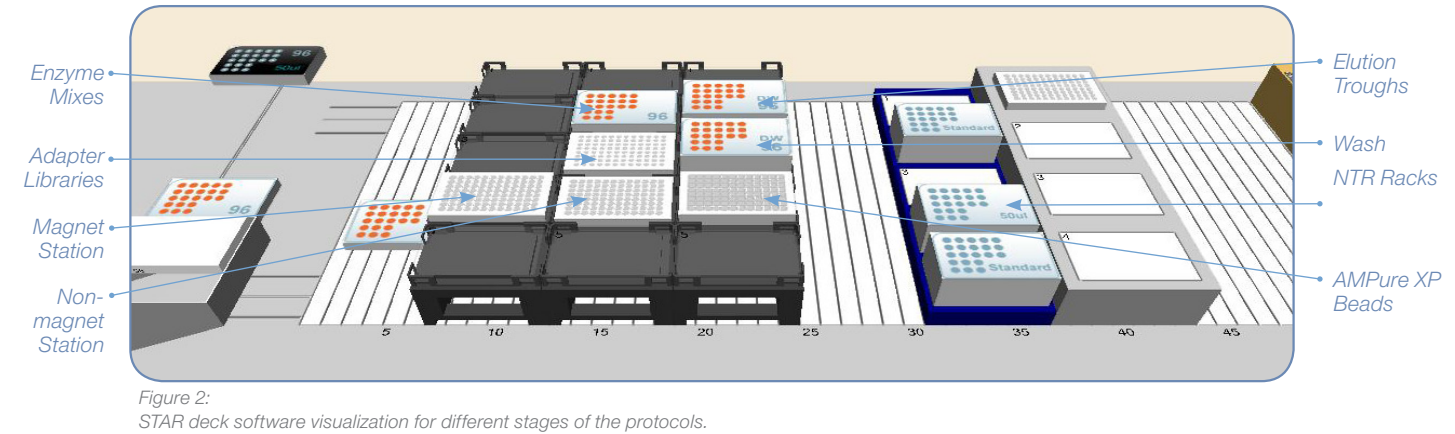The Hamilton Microlab STAR had been configured with the following components:

- ▶ **(8) 1000 µL independent pipetting channels**
- ▶ **96 channel CORE Multi Probe Head**
- ▶ **CORE GRIP 1 mL (PN 184089)**
- ▶ **PLT_CAR_L5AC (PN 182090)**
- ▶ **TIP_CAR_NTR (PN 182074)**
- ▶ **300 µL CORE tips without filter in nestable tip racks (PN 235950)**
- ▶ **50 µL CORE tips without filter in nestable tip racks (PN 235947)**

- ▶ **Cooling option comprising of Inheco CPA kit quantity 2 (PN 59146-01), Inheco Multi-TEC Control (PN OTP-8900030) and carrier baseplate for up to 4 heater-shakers (PN 187001) is optional**
- ▶ **24 Post Magnetic Separator (PN OTP-5-0020)**

The list of consumables required is:

- Axygen 500uL V bottom Assay Plate (PN P-96-450V-C)
- Axygen 12 columns Low Profile Reservoir (PN RES-MW12-MP)
- Axygen Single Well, 96 tip High Profile Reservoir (PN RES-SW96-HP)
- 96 Well 0.2mL Full Skirted PCR Plate
- Adhesive backed aluminum foil seal
- TruSeq DNA Sample Preparation Kit, Box A[1] (PN 15012999)

- TruSeq DNA Sample Preparation Kit, Box B[1,2] (PN 15013001)
- TruSeq DNA Sample Preparation Kit, PCR Prep Box[1] (PN 15012995)
- Agencourt AMPure XP 60 ml kit (PN A63881)

[1]Certain universally available materials in these kits were sourced from alternate vendors.

[2]Custom adapter oligos were synthesized by Oblique Bio.

The deck layout used for the method is given in Figure 2 below.

Figure 2:
STAR deck software visualization for different stages of the protocols.

The library preparation starts with off-line Covaris shearing that generates dsDNA fragments with 3' or 5' overhangs. The fragmentation process was optimized to obtain 300-400 bp average insert size for genomic sequencing (Table 1).

Volume	50uL
Duty Cycle	20%
Intensity	5
Cycles per burst	200
Time	165 seconds
z-height offset	6 mm

Table 1:
Covaris Parameters

This is followed by End Repair reaction which converts the overhangs into blunt ends using an End Repair Mix. The dA Tailing reaction results in adding a single 'A' nucleotide to the 3' ends of the blunt fragments. Finally ligate multiple indexing adapters to the ends of the DNA fragments, and transfer them to PCR enrichment plate, thus preparing them for hybridization onto a flow cell of HiSeq. There are intermittent clean up steps using the AMPure XP beads.

Results

The yield from sample preparation was determined by using PicoGreen assay (Figure 3) and the PCR amplification product was run on a gel (Figure 4). The quality of the samples was verified with Bioanalyzer and KAPA assay (Figures 5 and 6; Table 2). The sequencing data is shown from Illumina HiSeq in Table 3.
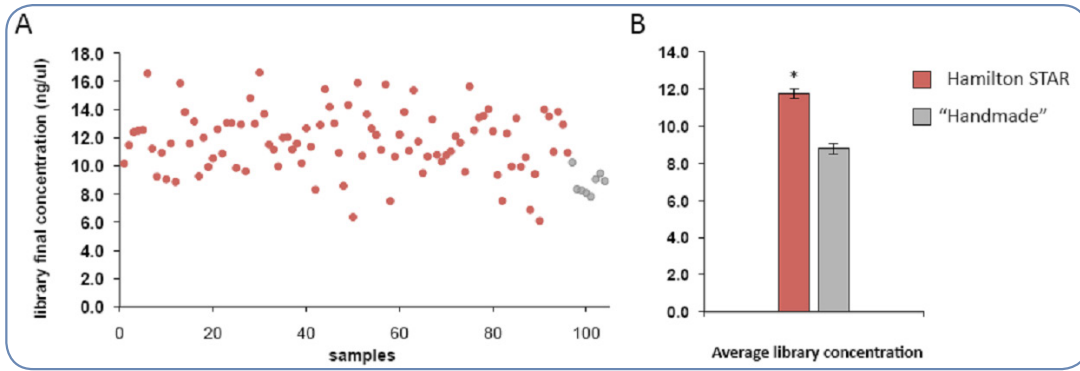
Figure 3:
Paired-end Illumina libraries constructed from 1μg of genomic DNA using the Hamilton STAR 96 well automation protocol compared to manual preparation by multichannel pipetor and paramagnetic bead cleanups between each enzymatic step. (A) Final concentrations of libraries prepared using the automation protocol (red) or manual (gray) were determined by PicoGreen assay using a stock 100ng/μL standard sheared by Covaris to approximately the same size as genomic DNAs used for library input. (B) Average library concentrations of samples prepared by automation show a significantly higher yield than those prepared manually.
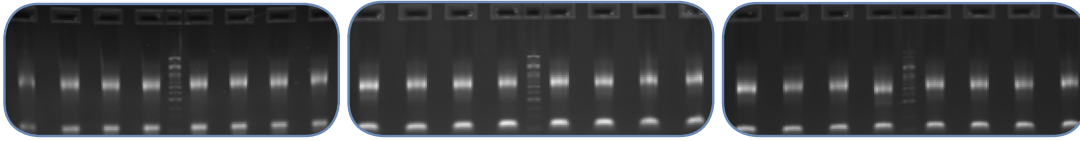

Figure 4:
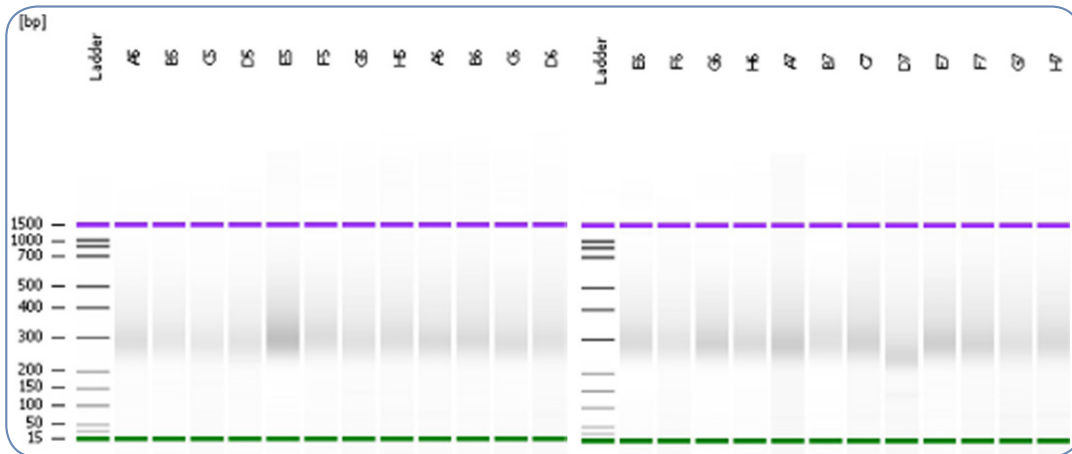*Post 4-plex pool & PCR enrichment results. 2% Sybr E-Gel.*

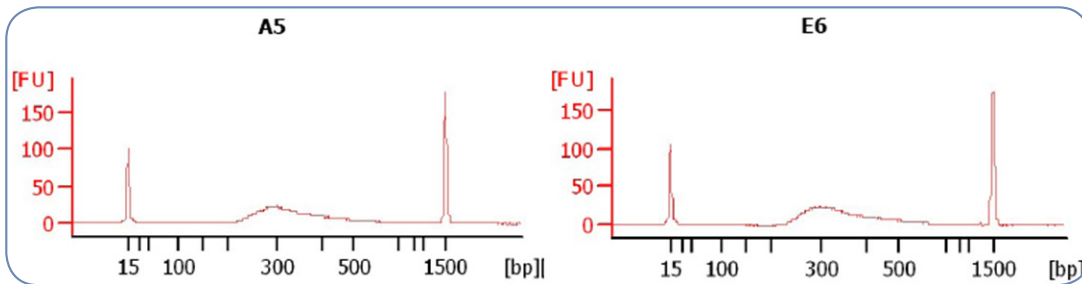*Figure 5:*
Post 4-plex Bioanalyzer 2100 summary results.

Figure 6:
Post 4-plex Bioanalyzer 2100 representative results.

Hamilton STAR plate Analysis

	Bioanalysis results				KAPA Assay Results	
4 plex	ng/ul	size bp	ug DNA	nM Yield	pM KAPA result	total lib(15nM)
A5	8.99	290	0.35	46.97	0.77	25.05
B5	5.36	290	0.21	28.00	1.18	22.80
C5	4.97	290	0.19	25.97	1.32	23.78
D5	6.76	290	0.26	35.32	1.09	26.66
E5	15.05	290	0.59	78.63	0.34	18.49
F5	7.47	300	0.29	37.73	0.74	18.74
G5	7.23	290	0.28	37.77	0.87	22.83
H5	7.21	300	0.28	36.41	0.93	22.67
A6	8.49	290	0.33	44.36	0.92	28.30
B6	7.92	295	0.31	40.68	0.94	25.96
C6	6.89	290	0.27	36.00	0.90	22.32
D6	6.95	295	0.27	35.70	0.84	20.43
E6	7.78	295	0.30	39.96	0.80	21.74
F6	5.31	295	0.21	27.27	0.98	18.18
G6	11.09	290	0.43	57.94	0.64	25.59
H6	7.68	290	0.30	40.13	0.90	25.10
A7	10.72	290	0.42	56.01	0.62	23.96
B7	6.58	300	0.26	33.23	0.91	20.31
C7	10.55	295	0.41	54.19	0.58	21.36
D7	8.25	255	0.32	49.02	0.81	31.44
E7	11.49	295	0.45	59.01	0.66	26.41
F7	8.99	290	0.35	46.97	0.61	19.83
G7	6.51	290	0.25	34.01	0.90	21.30
H7	7.69	295	0.30	39.50	0.93	24.88

Table 2:
Bioanalyzer and KAPA Assay results

samples A1-H12 PE 50, HiSeq 4 lanes 24-plex						
	total pf PE reads	PE reads alignedv1m1	% aligned	total PE reads on target	% reads on target	mean coverage of target (X)
Average	8019665	6961491.9	87.0%	4764654.359	80.0%	388.1564038
% StDev	30%	30%	2%	34%	15%	34%

Table 3:
Analyzed data from Illumina HiSeq

Conclusion

Automation of NGS sample preparation on Hamilton Microlab STAR was shown to be a successful alternative to the laborious manual approach. Hamilton Robotics provides a highly customizable and flexible automation platform that enables library preparation resulting in throughput, precision and accuracy.

Features and Benefits:

▶ **Process 96 samples simultaneously in less than 4 hours.**

▶ **50% increased sample recovery yields compared to manual method.**

▶ **Average 80% "Reads on Target"**

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