Automated Illumina NGS Sample Preparation: TruSeq® Stranded mRNA on the Hamilton Microlab STAR

Application Note

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Abstract

The Illumina® TruSeq Stranded mRNA Sample Preparation kit provides reagents and a workflow for converting the mRNA in total RNA into a library of template molecules of known strand origin, which is then suitable for cluster generation and DNA sequencing. The kit has been verified for use on the Microlab STAR liquid handling workstation and the automation protocol has been Illumina-qualified*. Implementation of both the low throughput (LT) and high throughput (HT) sample preparation kits on the STAR have resulted in high yield and high quality library.

Introduction

Illumina's sample preparation kits produce high quality libraries for DNA and RAN sequencing. Hamilton offers the robust Microlab STAR line of robotic liquid handling workstations for automating sample preparation. Together, the automation of the TruSeq Stranded mRNA sample preparation protocol on the STAR gives the user hours of reliable, unattended library preparation.

Any number of samples between 1 and 96 can be processed. All steps of the protocol are done on-deck with the exception of one centrifugation step and the PCR thermal cycling. The programmed method is compatible with any index combination or pooling scheme.

To verify the capability of the STAR to perform the TruSeq Stranded mRNA sample preparation, two tests were run. One test ran the LT kit, with adapters in vials. The other test ran the HT kit, with adapters in the RNA Adapter plate (RAP). After library preparation, the sample were run on the HiSeq DNA sequencers. Positive results of the test have led to the designation of the ML STAR being Illumina-qualified for TruSeq Stranded mRNA sample preparation.

Figure 1:
The Hamilton Robotics Microlab® STAR Automated Workstation

HAMILTON

System Deck Layout:

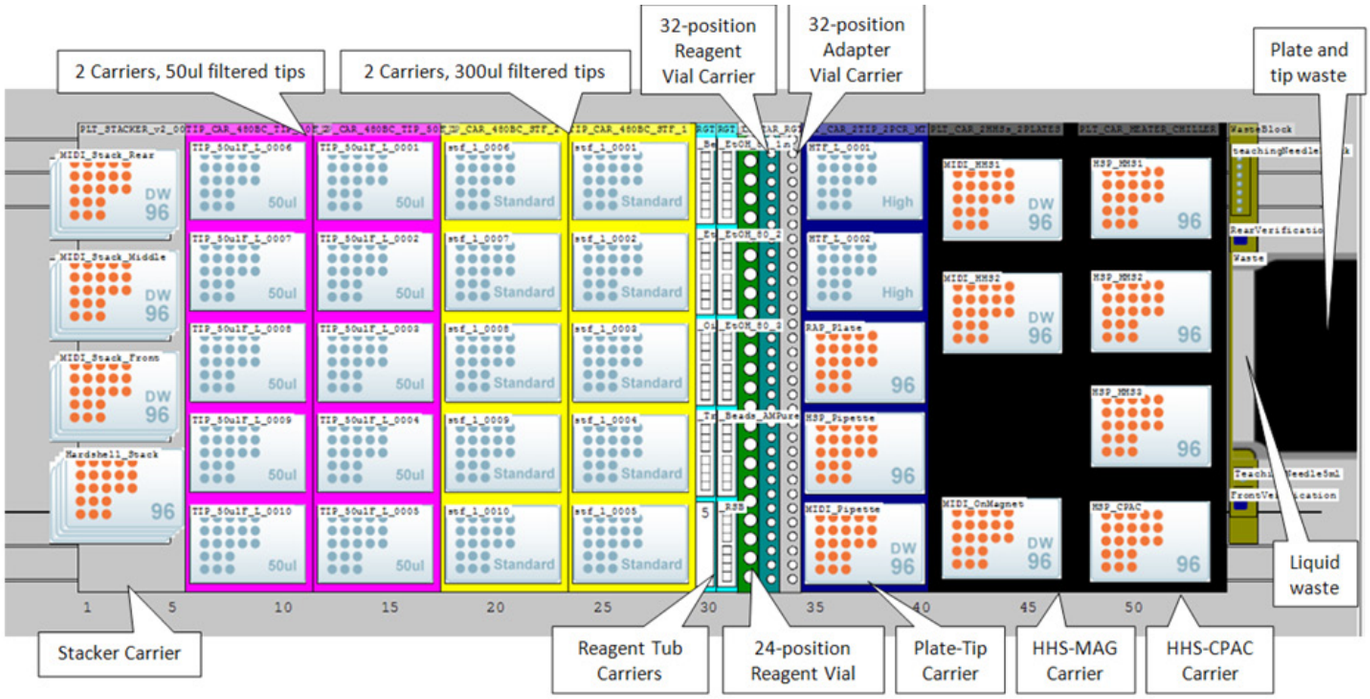

Figure 1: STAR deck layout for TruSeq Stranded mRNA Sample Preparation protocol.

System and Materials:

System Requirements

8 1ml channels	4 Tip carriers
iSWAP plate handler	2 5x50ml reagent carriers
Autoload for barcode reading	1 24-tube carrier (for kit reagents in 4ml tubes)
Liquid waste system	1 Multiflex carrier with 2 tip modules, 2 PCR96 modules, and 1 MIDI plate module
5 Hamilton heater shakers (HHSs) for on-deck incubations and mixing ▶ **2 with custom PCR plate heat blocks** ▶ **2 with custom MIDI plate heat blocks** ▶ **1 with a flat surface for plate mixing**	2 32-tube carriers with vial inserts ▶ **1 for kit reagents in 0.5ml and 2ml vials** ▶ **1 for adapters in vials**
1 Inheco CPAC for plate cooling	Invitrogen magnetic stand 96 (Life Technologies, PN AM10027)
1 Stacker carrier for on-deck storage of the many plates used in the process	1 base plate for 2 MIDI HHSs and a custom module for the magnetic stand
	1 base plate for 2 PCR HHSs, 1 flat HHS, and the CPAC

Required Consumables	Hamilton Part Number
50ul filtered tips, Hamilton	235948
300ul filtered tips, Hamilton	235903
1000ul filtered tips, Hamilton	235905
50ml reagent tubs, Hamilton	56694-01
96-well storage plates (“MIDI” plates), Fisher Scientific	AB-0859
96-well PCR plates, Bio-Rad, PN HSP-9601	188061APE

Illumina Sample Preparation Kits	Illumina Part Number
TruSeq Stranded mRNA LT Sample Prep Kit – Set A, Illumina	RS-122-2101
TruSeq Stranded mRNA LT Sample Prep Kit – Set B, Illumina	RS-122-2102
TruSeq Stranded mRNA HT Sample Prep Kit, Illumina	RS-122-2103

Other Required Materials and Equipment	Part Number
Agencourt AMPure XP beads, Beckman Coulter Genomics	A63881
Ethanol, absolute, for molecular biology, Sigma-Aldrich	E7023
SuperScript II Reverse Transcriptase, Invitrogen	18064-014
Mineral oil, molecular biology grade, Sigma	M8662
96-well thermal cycler with heated lid	

Sample Prep Workflow

Figure 2 illustrates the steps of the workflow. In addition to the indicated automation steps, qPCR set-up is also automated. The start and stop points are indicated in the diagram, and these correspond to the optional stopping points in the Illumina protocol.

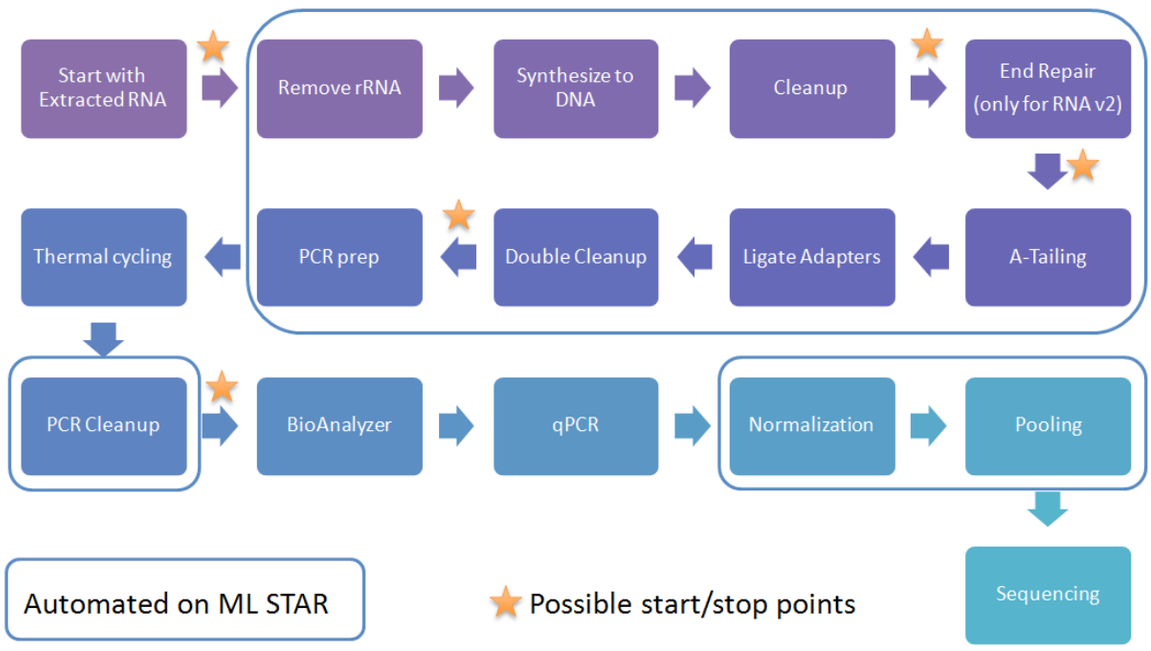

Figure 2: The steps of the workflow are shown here. The automated steps are circled. The method allows the user to start/stop the protocol at the points indicated by a star.

Automated Method

The automated protocol allows for run customization. The user selects which sections of the workflow to run, whether to run the HT or LT protocol (adapters in RAP or vials, respectively), whether controls are to be used, the fragmentation time (to allow for different fragment sizes), and a worklist that specifies how many samples and which adapters to use for each sample. See the Figure 3.

All incubations are done on-deck with the Hamilton heater-shakers (HHSs) and the Inheco CPAC. The HHSs are fitted with custom adapters for optimal heat exchange. For the 94C incubation, mineral oil is used to prevent evaporation.

The automated method is based on the "High Sample (HS)" portion of the Illumina sample prep protocol, regardless how many samples are to be processed (1 to 96).

All reagents required for the desired run process(es) are loaded onto the deck at the start of the run. The reagents are not temperature-controlled, with no adverse affects.

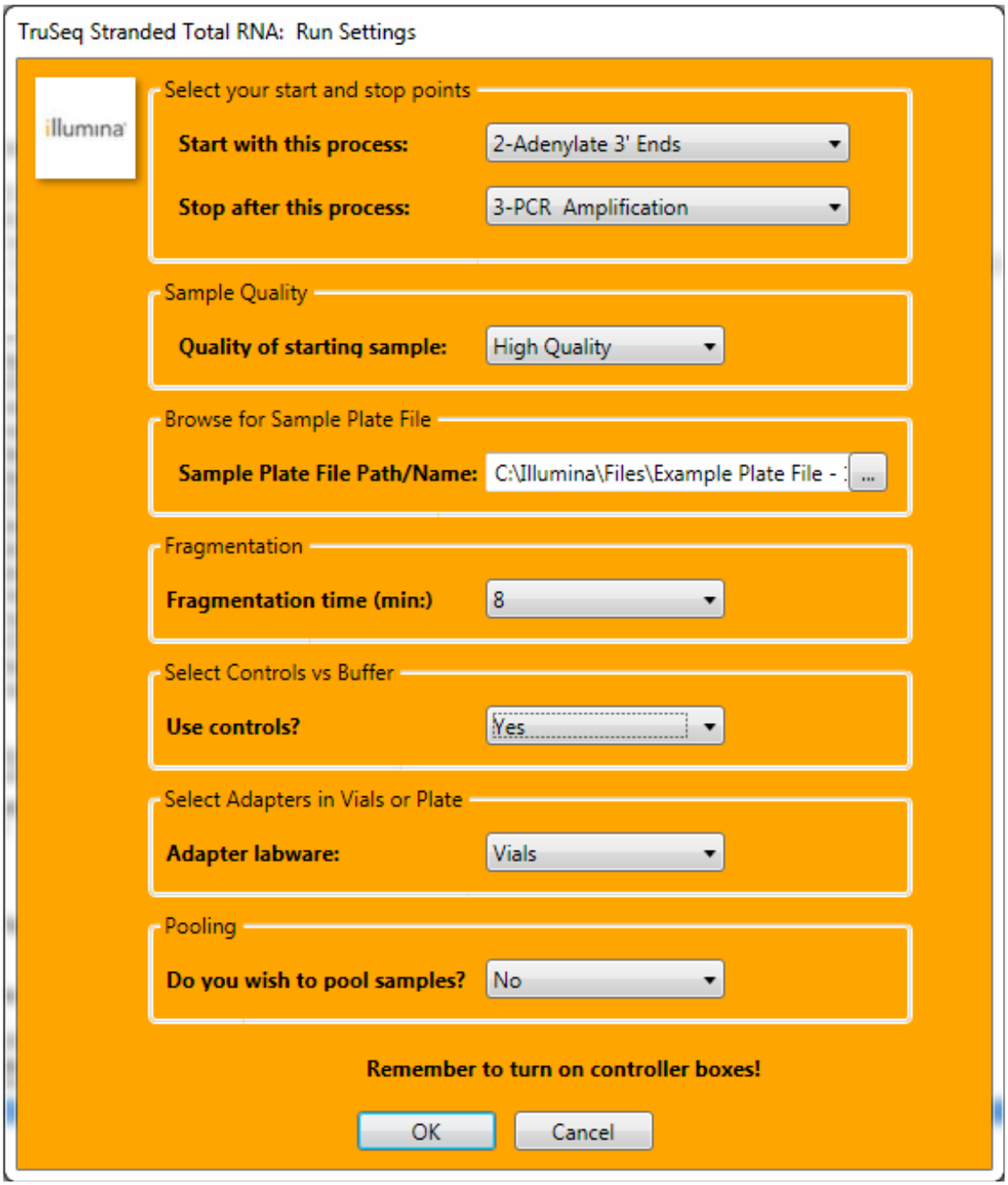

Figure 3: This dialog appears at the start of the run. The user selects the desired options.

Verification Run Setup

Two runs were performed to qualify the STAR. Table 1 shows the plate layouts. For both runs, controls were used and the fragmentation time was 8min.

Table 1. Sample plate layout

Run 1

Row/Column	1	2	3
A	1ug UHR	100ng UHR	100ng Brain
B	1ug UHR	100ng UHR	100ng Brain
C	1ug UHR	100ng UHR	100ng Brain
D	1ug UHR	100ng UHR	100ng Brain
E	100ng UHR	1ug Brain	100ng Brain
F	100ng UHR	1ug Brain	100ng Brain
G	100ng UHR	1ug Brain	100ng Brain
H	100ng UHR	1ug Brain	100ng Brain

Run 2

Row/Column	1	2	3	4	5	6
A	1ug UHR	100ng MUR	100ng RUR	100ng MUR	100ng RUR	1ug Brain
B	1ug UHR	100ng MUR	100ng RUR	100ng MUR	100ng RUR	1ug Brain
C	1ug UHR	100ng MUR	100ng RUR	100ng MUR	100ng RUR	1ug Brain
D	1ug UHR	100ng MUR	100ng RUR	100ng MUR	100ng RUR	1ug Brain
E	100ng UHR	100ng MUR	100ng RUR	100ng MUR	100ng RUR	100ng Brain
F	100ng UHR	100ng MUR	100ng RUR	100ng MUR	100ng RUR	100ng Brain
G	100ng UHR	100ng MUR	100ng RUR	100ng MUR	100ng RUR	100ng Brain
H	100ng UHR	100ng MUR	100ng RUR	100ng MUR	100ng RUR	100ng Brain

Table 1: Plate layouts for the 2 verification runs.

Results and Discussion

Throughput

The STAR-automated parts of the workflow were done over 3 days. Typical total hands-on time is 3hr 45min, to prepare samples and reagents, load and unload the deck, put away samples and reagents at the end of the run, and perform very minimal (10 minutes) daily maintenance. The method is fully automated, with the exception of these offline steps: one centrifugation step near the end of cDNA synthesis cleanup, PCR thermal cycling, BioAnalyzer and qPCR validation. The table below lists the STAR and thermal cycling run time requirements for the 48-sample run, as well as for typical 16- and 96-sample runs.

Number of Samples	Day 1: Purification, Fragmentation, and Synthesis	Day 2: Adenylation, Ligation, and PCR	Day 3: Normalization and Pooling	Total Run Time
16	4hr 30min	5hr 5min	10min	9hr 45min
48	5hr 40min	6hr 15min	15min	12hr 10min
96	6hr 45min	7hr 15min	20min	14hr 20min

Table 2: STAR run times for the workflow processes, including off-line thermal cycling.

Sample Quality and Yield

Samples were run on the Agilent 2100 BioAnalyzer to determine quality. See Figure 3. In addition, qPCR results demonstrated consistent yields. For example, the average yield was 292nM for the 8 replicates of UHR and 270nM for the 8 replicates of Brain.

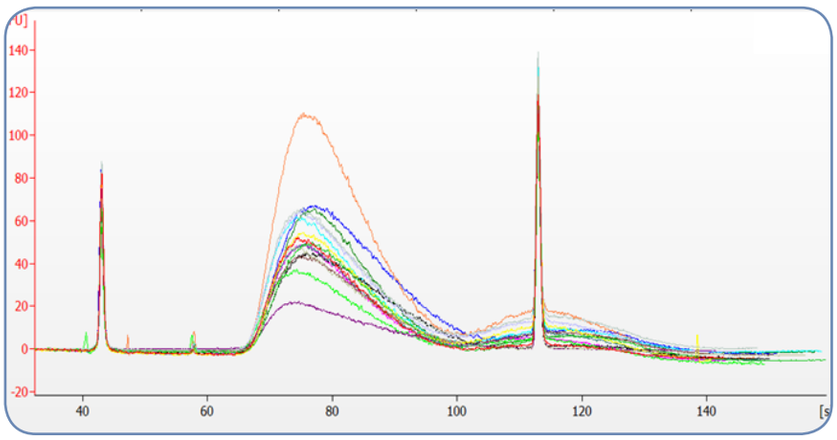

Figure 4: BioAnalyzer results.

Sequencing Results

The following figures and table represent data from the sequencing runs.

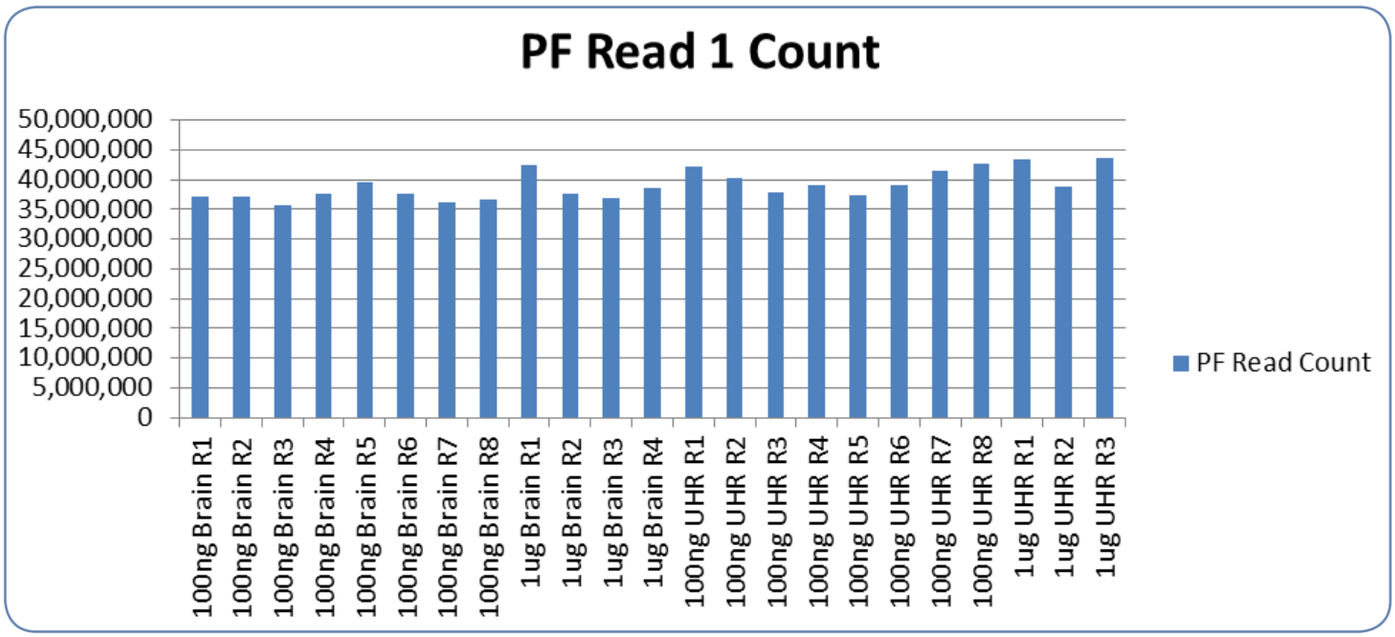

Figure 5: Pass filter reads.

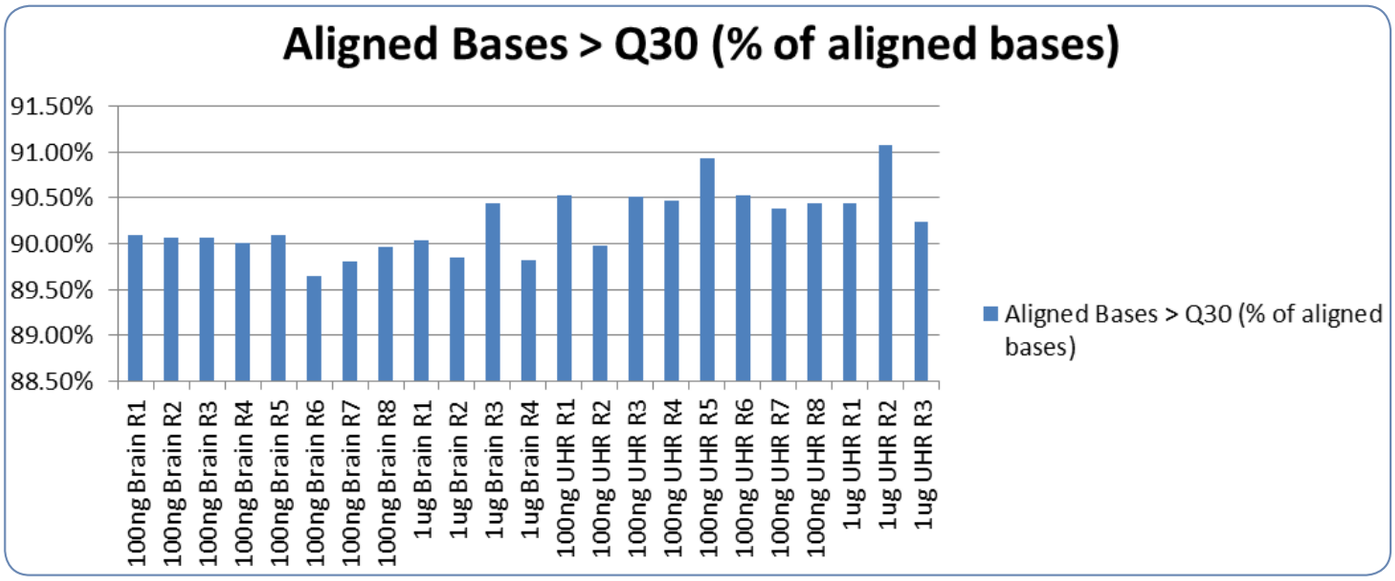

Figure 6: Percent of Bases with Q score >30.

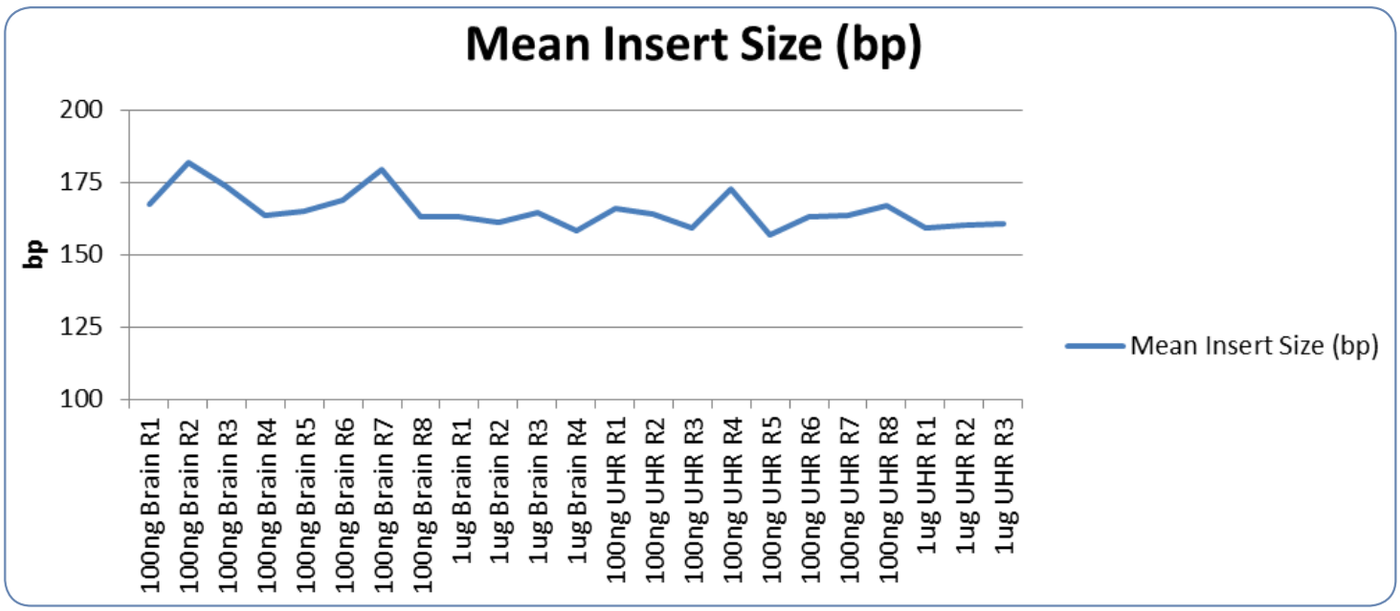

Figure 7: Targeted mean insert size.

Libray	Aligned	Unaligned	Human Ribosomal	Adapter Dimer	% Median CV of Coverage	% Stranded
100ng Brain R1	95.4%	4.6%	3.3%	0.1%	55.0%	99.3%
100ng Brain R2	95.2%	4.8%	3.7%	0.1%	53.0%	99.3%
100ng Brain R3	95.4%	4.6%	3.3%	0.1%	55.0%	99.3%
100ng Brain R4	95.1%	4.9%	3.7%	0.1%	55.0%	99.3%
100ng Brain R5	95.2%	4.8%	3.5%	0.1%	54.0%	99.3%
100ng Brain R6	94.9%	5.1%	5.0%	0.1%	54.0%	99.3%
100ng Brain R7	95.4%	4.6%	4.6%	0.1%	53.0%	99.3%
100ng Brain R8	94.6%	5.4%	2.9%	0.2%	54.0%	99.3%
1ug Brain R1	95.8%	4.2%	1.8%	0.1%	58.0%	99.3%
1ug Brain R2	95.9%	4.2%	2.0%	0.2%	58.0%	99.3%
1ug Brain R3	95.5%	4.5%	1.7%	0.2%	60.0%	99.2%
1ug Brain R4	95.8%	4.2%	1.8%	0.2%	56.0%	99.3%
100ng UHR R1	95.3%	4.7%	2.8%	0.1%	54.0%	99.4%
100ng UHR R2	95.2%	4.8%	2.8%	0.1%	54.0%	99.5%
100ng UHR R3	95.6%	4.4%	2.6%	0.1%	57.0%	99.4%
100ng UHR R4	95.3%	4.7%	3.0%	0.1%	54.0%	99.5%
100ng UHR R5	95.4%	4.6%	2.8%	0.1%	60.0%	99.3%
100ng UHR R6	95.1%	4.9%	3.0%	0.1%	55.0%	99.3%
100ng UHR R7	94.9%	5.1%	3.0%	0.1%	54.0%	99.2%
100ng UHR R8	95.1%	4.9%	2.7%	0.1%	54.0%	99.3%
1ug UHR R1	96.0%	4.0%	1.8%	0.1%	60.0%	99.2%
1ug UHR R2	95.2%	4.8%	1.5%	0.1%	64.0%	99.3%
1ug UHR R3	95.8%	4.3%	2.1%	0.1%	59.0%	99.4%
Average	95.3%	4.7%	2.8%	0.1%	56.1%	99.3%

Table 3: TruSeq Stranded mRNA Sequence Quality Metrics.

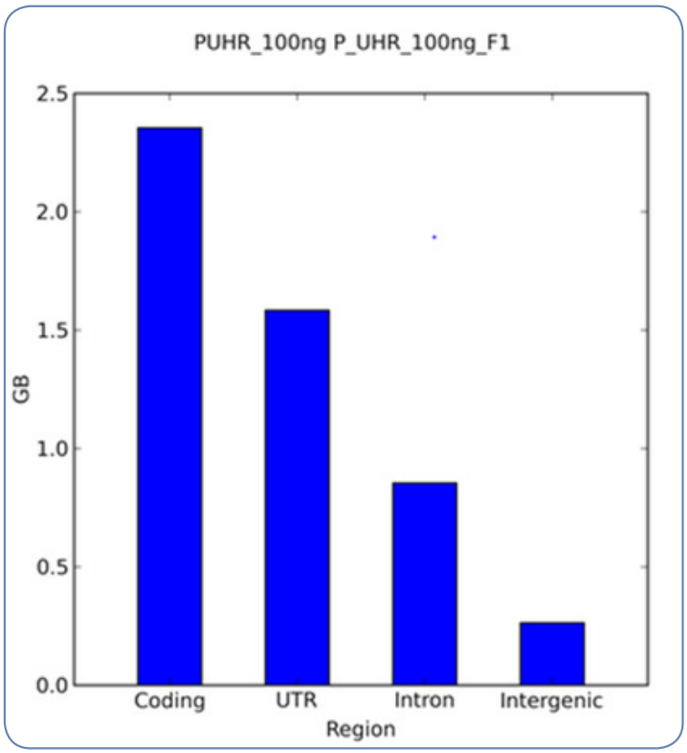

Figure 8: Sequence alignment distribution

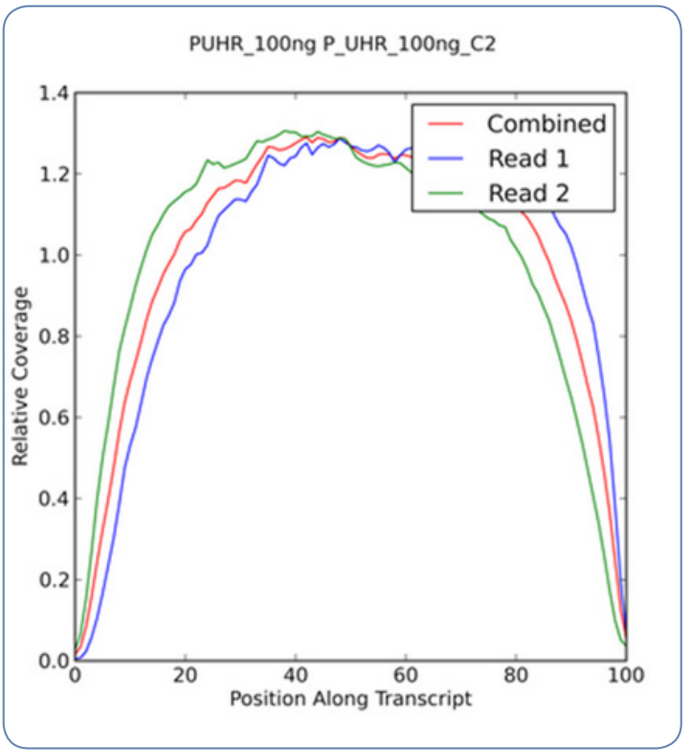

Figure 9: Uniformity of coverage across the entire transcript.

Cross-contamination

Analysis of the sequencing data showed no evidence of sample cross-contamination.

Conclusion

The long and labor-intensive TruSeq Stranded mRNA sample preparation protocol has been successfully automated on the Hamilton Microlab STAR. The Illumina-qualified automated method yields high quality libraries, ready for HiSeq or MiSeq sequencers.

*"Illumina-qualified" means Illumina has confirmed that the protocol produces comparable results when compared to the manual process.

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