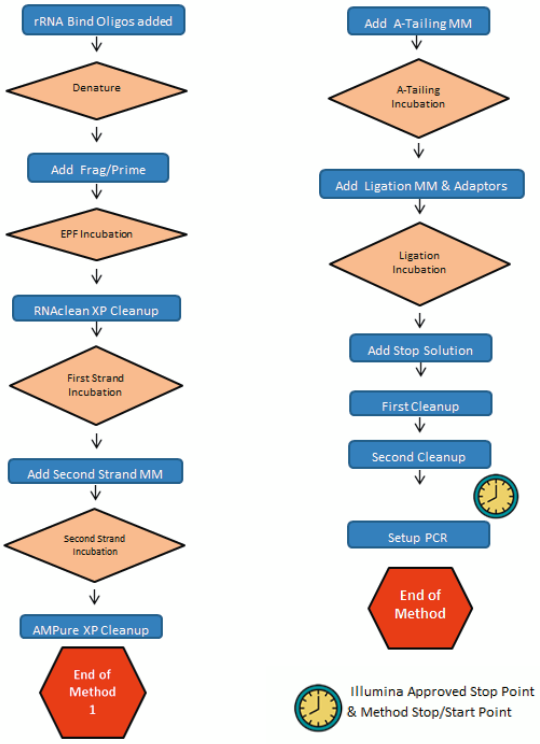
High-Throughput TruSeq® Stranded Total RNA Library Construction from FFPE Samples on the Biomek FX^P Dual-Arm Multi-96 and Span-8 Workstation

Abstract

The manual preparation of large numbers of sequencing libraries, which can be labor-intensive and time-consuming, is a bottleneck for many laboratories. Manual methods also carry a higher risk of human error and inconsistency. The Biomek FX^P Laboratory Automation Workstation puts every aspect of liquid handling required for automation of NGS sample preparation—including optimized pipetting for reagents and samples, cooling, shaking and thermocycler integration to maintain protocol-defined environmental conditions—into a single, automated system, while limiting manual handling of any potentially hazardous chemicals. It has the capability to provide high-quality template libraries at a throughput needed to take advantage of the high capacity of NGS, while providing a system flexible enough to meet a user's changing needs.

TruSeq Stranded Total RNA Sample Preparation kits incorporate Ribo-Zero™ rRNA removal chemistry to capture and remove ribosomal RNA (rRNA) while leaving behind both mRNA and multiple forms of non-coding RNA to enable whole-transcriptome sequencing by generating strand information. This streamlined workflow offers a gene expression quantification tool with multiplexing, enabled by the availability of 96 unique index pairs using the Illumina® RNA adapter plate or the Illumina adapter tubes for lower throughput. This application note describes the automation of the Illumina TruSeq Stranded Total RNA kit on the Biomek FX^P Dual-Arm Multi-96 and Span-8 Workstation (BFX^P) for whole-transcriptome library construction. The automated method enables processing of various input types such as total RNA from FFPE. It consists of 2 sample preparation modules, one to cover cDNA synthesis and another to prepare libraries all the way through PCR[1] enrichment. One to 96 samples can be run simultaneously, and setup requires only a few clicks of the mouse and 1-time pipetting of the required kit components into the reservoirs and tubes. The pipetting tools and software improve the ease and speed of reaction setup. The end result is as many as 96 sequence-ready libraries in just 2 days, from as little as 0.1 µg of total RNA[2]. The resulting high-quality libraries display consistent insert sizes, minimal adapter dimers, low numbers of ribosomal sequences, and high fidelity to

Biomek FX^P Workstation.



TruSeq Stranded Total RNA Workflow per Illumina Published Protocol (PN RS-122-2203).

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the reference genome—enabling a detailed analysis of the transcriptome under investigation. All of this was accomplished with minimal hands-on time (see Table 1) and no detectable sample cross-contamination.

Table 1. Major Process Description.

Automated/Hands-On Time			
	24 Samples	48 Samples	96 Samples
cDNA Synthesis: Day 1			
Prepare Reagants/ Set up Inst.	25 min	27 min	30 min
Method Run	3 hrs 27 min	3 hrs 41 min	4 hrs 17 min
Total	3 hrs 52 min	4 hrs 8 min	4 hrs 47 min
Library Construction			
Prepare Reagants/ Set up Inst.	25 min	27 min	30 min
Method Run	2 hrs 30 min	2 hrs 40 min	3 hrs 5 min
Total	2 hrs 55 min	3 hrs 7 min	3 hrs 55 min

Timing does not include thawing of reagents and PCR thermocycling.

Method Details

Setting Up Library Construction

Setup for the automated library construction requires making a few selections in the Biomek FX^P user interface (Figures 1 and 2). The automated method is designed to closely follow the Illumina protocol while maintaining the flexibility that Biomek software provides. Built into the user interface are all the Illumina-approved start and stop points to allow the user: (1) to select which steps of the process to perform; (2) configure how many samples from 1 to 96 to prepare; and (3) indicate how to handle incubations. Users are also given multiple options on how to configure adapters. The user interface enables users to select the adapter labware from a list that includes the Illumina RNA adapter plate (RAP), Illumina RNA adapter tubes, or a custom plate as defined by the user. Users may choose which adapters to use with which samples by selecting their own transfer files or by using the default setup. Setting up the PCR reaction to enrich for fragments with the proper adapters is also optional and allows for the volume of sample to change based on user needs (Figure 2).

Once all of the method parameters have been entered, the Biomek FX^P software provides a reagent calculator that tells the users the volume of each reagent needed, as well as how to prepare the reagents, when necessary (Figure 3). The volume required is updated based on the number of samples selected in order to minimize reagent loss.

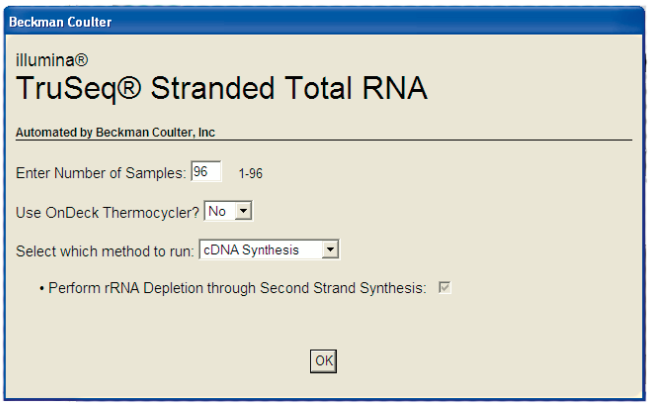

Fig. 1. User interface showing cDNA synthesis options.

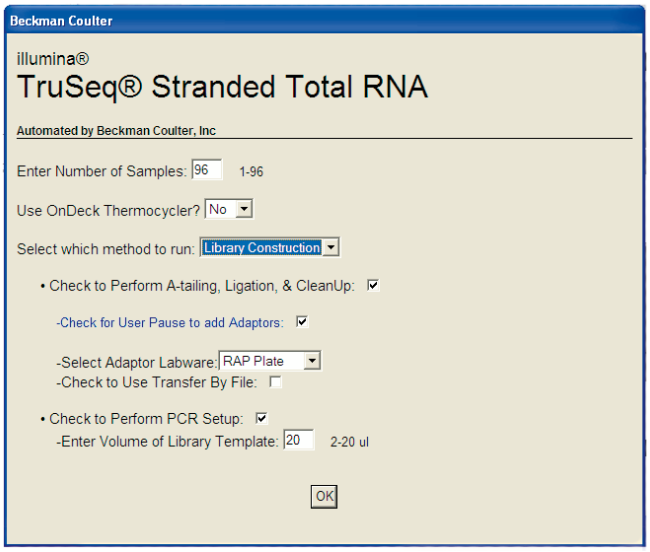

Fig. 2. User interface showing library construction options.

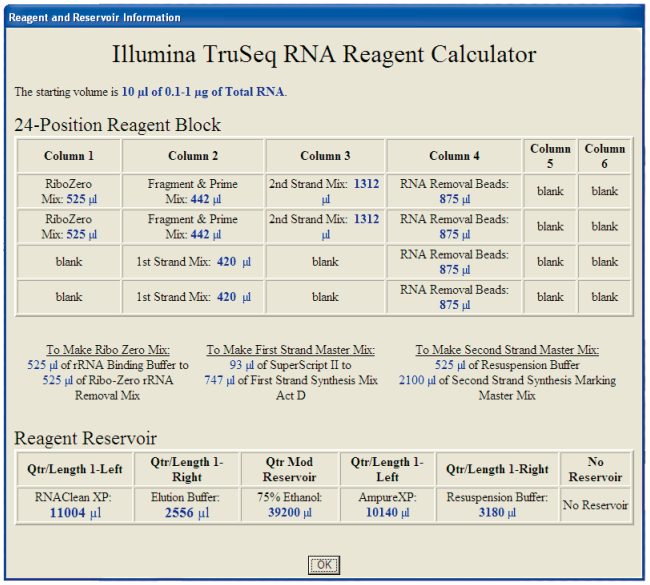

Fig. 3. cDNA synthesis reagent calculator.

Results

Sample Layout and Results

Dana Farber Cancer Institute (DFCI) extracted total RNA from human prostate cancer samples from either Formalin Fixed Paraffin Embedded (FFPE) or Fresh Frozen (FF) tissues. The total RNA was extracted using the Biomek FX^P along with the Agencourt FormaPure kit. For library construction, the FFPE and FF samples were processed at either 50 ng or 100 ng input to test the lower input levels typically found for these sample types. Along with the samples provided by DFCI, Universal Human Reference RNA and Human Brain Reference RNA were used as controls and were also processed at various concentrations to fully test the automated workflow. The RNA samples to be processed were placed in a 96-well plate configured as shown in Table 2. Two FFPE samples and 2 of their corresponding FF samples (highlighted in blue in the sample table) were used for the initial sequencing run to test the quality of the libraries produced.

Table 2. Sample Plate Map for 96-Sample Run.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1986B FFPE 50ng	1973B FFPE 50ng	**1920B FF 50ng**	**Universal 700ng**	**Brain 700ng**	Universal 700ng	Universal 500ng	Brain 200ng	Universal 100ng	Universal 500ng	Brain 200ng	Universal 100ng
B	1986B FFPE 100ng	1973B FFPE 100ng	1920B FF 100ng	Universal 600ng	Brain 600ng	Universal 600ng	Universal 500ng	**Brain 200ng**	Universal 100ng	Universal 500ng	Brain 200ng	Universal 100ng
C	1986T FFPE 50ng	1973T FFPE 50ng	**1920T FF 50ng**	Universal 500ng	Brain 500ng	Universal 500ng	**Universal 500ng**	Brain 200ng	Universal 100ng	Universal 500ng	Brain 200ng	Universal 100ng
D	1986T FFPE 100ng	1973T FFPE 100ng	1920T FF 100ng	Universal 400ng	Brain 400ng	Universal 400ng	**Universal 500ng**	Brain 200ng	Universal 100ng	Universal 500ng	Brain 200ng	Universal 100ng
E	**1920B FFPE 50ng**	1986B FF 50ng	1973B FF 50ng	Universal 300ng	Brain 300ng	Universal 300ng	Universal 500ng	Brain 200ng	Universal 100ng	Universal 500ng	Brain 200ng	Universal 100ng
F	1920B FFPE 100ng	1986B FF 100ng	1973B FF 100ng	Universal 200ng	Brain 200ng	Universal 200ng	Universal 500ng	Brain 200ng	**Universal 100ng**	Universal 500ng	Brain 200ng	Universal 100ng
G	**1920T FFPE 50ng**	1986T FF 50ng	1973T FF 50ng	Universal 100ng	Brain 100ng	Universal 100ng	Universal 500ng	Brain 200ng	**Universal 100ng**	Universal 500ng	Brain 200ng	Universal 100ng
H	1920T FFPE 100ng	1986T FF 100ng	1973T FF 100ng	**Universal 50ng**	**Brain 50ng**	Universal 50ng	Universal 500ng	Brain 200ng	Universal 100ng	Universal 500ng	Brain 200ng	Universal 100ng

Note: Highlighted cells in blue were sequenced initially.
Universal = Universal Human Reference RNA
FF = Fresh Frozen Human Prostate Cancer
Brain = Human Brain Reference RNA

Both the FF and FFPE samples produced good quality libraries that were sequenceable. As shown in Figure 4, the Pass Filter (PF) read depth for the samples is consistently over 140 million reads. Figure 5 displays the transcript coverage and shows the 3' bias that is expected when comparing FFPE and FF samples. Both the FF and FFPE display high alignment to the reference genome with FFPE samples showing ~75% alignment and the FF samples showing ~95% alignment (Figure 6).

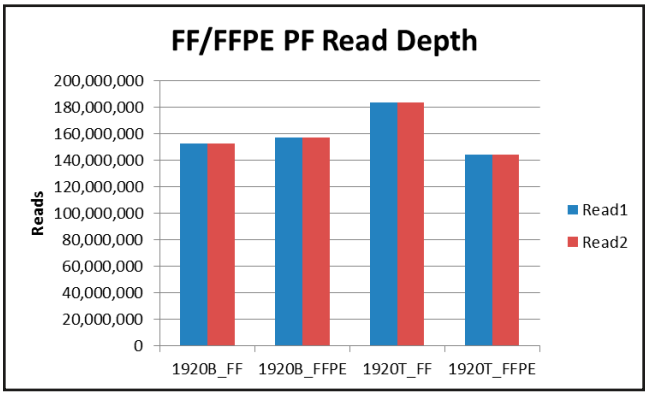

Fig. 4. Pass filter read depth for read 1 and 2 of the FF and FFPE samples on the NextSeq® 500.

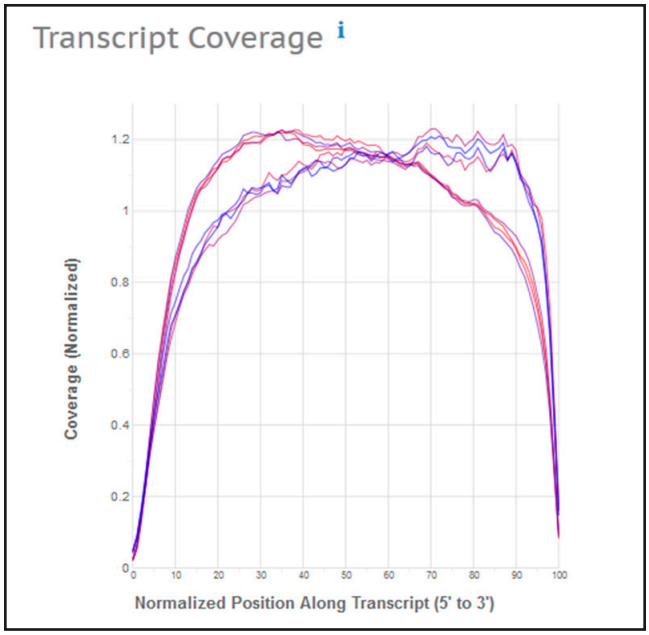

Fig. 5. Transcript coverage displaying typical 3' bias for the FFPE compared to FF samples.

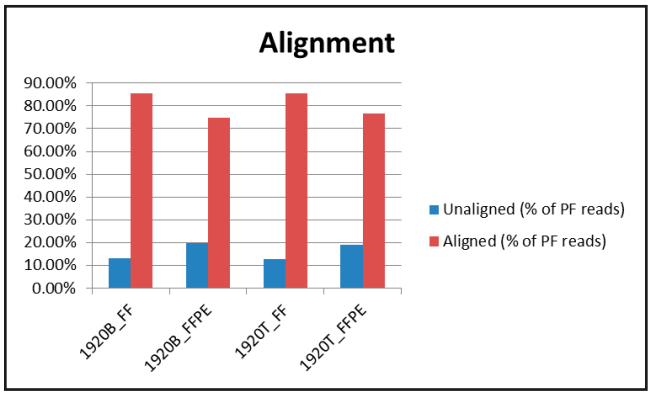

Fig. 6. Percent alignment of FF and FFPE samples show high alignment, with the FFPE samples being only ~10% lower than the FF samples.

Table 3. Key Sequencing Results.

Library	Mean Insert Size (bp)	% Duplicates	% Strand Aligned	Median CV Coverage
1920B_FF	138.34	16.41%	99.54%	0.48
1920B_FFPE	125.82	5.14%	97.51%	0.66
1920T_FF	152.01	14.65%	99.57%	0.48
1920T_FFPE	133.45	6.96%	97.66%	0.68

rRNA Depletion

The goal of Ribo-Zero depletion is to remove as much rRNA and other contaminants as possible while leaving behind the less abundant RNA types. FFPE samples can be more difficult than non-degraded sample types. The goal is to achieve less than 5% of reads mapping to contaminants such as rRNA. Figure 7 shows that the FFPE samples mapped to less than 5% to rRNA, and less than 2% for other contaminants. FF samples mapped to less than 2% for all contaminants.

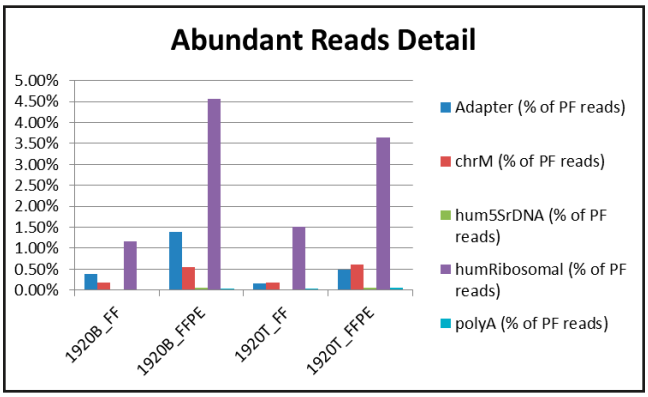

Fig. 7. Less than 5% of reads mapping to rRNA for FFPE samples; Less than 2% of reads mapping to rRNA for FF samples; Low percent of reads mapping to other contaminants.

Conclusion

Reliable and efficient automation solutions for NGS library construction enable laboratories to take full advantage of Illumina's powerful Next Generation Sequencing technology. Biomek Automation standardizes the process, reducing the potential for human error, which saves time and reagent cost and gives researchers valuable, worry-free, walk-away time. The Biomek FX^P automated method creates up to 96 TruSeq Stranded Total RNA sequenceable libraries in 2 days from various total RNA sample types and input concentrations as low as 50 ng.

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Dana Farber Cancer Institute, Boston, MA USA

Reagents Used		
Part Number	**Manufacturer**	**Description**
N/A	User-Preferred	Elution Buffer Nuclease-Free Water, TE Buffer
A63882	Beckman Coulter, Inc.	AMPure XP
A66514	Beckman Coulter, Inc.	RNAClean XP
AB00128-01000	American Bioanalytical	Ethanol
RS-122-2203	Illumina	TruSeq Stranded Total RNA HT kit
AM6050	Life Technologies	Human Reference Brain RNA
740000	Agilent Technology	Universal Human Reference RNA

Consumables Used			
Part Number	**Manufacturer**	**Description**	**Qty for 96-Sample Run**
717253	Beckman Coulter	Biomek AP96 P250 Tips, Pre-Sterile Barrier	2 Tip Boxes
379503	Beckman Coulter	Biomek Span-8 P250 Tips, Pre-Sterile Barrier	2 Tip Boxes
A21586	Beckman Coulter	Biomek P50 Tips, Pre-Sterile Barrier	8 Tip Boxes
717256	Beckman Coulter	Biomek AP96 P20 Tips, Pre-Sterile Barrier	2 Tip Boxes
B01124	Beckman Coulter	Biomek Span-8 P1000 Tips, Pre-Sterile Barrier	1 Tip Box
372790	Beckman Coulter	Quarter Modular Reservoir	3 Reservoirs
372788	Beckman Coulter	Quarter Reservoir, Divided by Length	2 Reservoirs
372795	Beckman Coulter	Reservoir Frame*	1 Frame
A32782	Beckman Coulter	SPRIPlate 96R—Ring Super Magnet Plate*	1 Magnet
A83054	Beckman Coulter	Tube Block*	1 Tube Block
373661	Beckman Coulter	24-Position Black Microfuge Tube Rack**	1 Tube Rack
373696	Beckman Coulter	Insert, Tube, 11 mm, White, for Microfuge Tubes**	24 Inserts
16466-042	VWR	2 mL SuperClear™ Screw Cap Microcentrifuge Tubes	19 Tubes
AB-1127	Thermo Scientific	ABgene 96-Well Plate, Square Well, 1.2 mL	5 Plates
HSP-9641	Bio-Rad	Hard-Shell Thin-Wall 96-Well Skirted PCR Plate	7 Plates
MSL-2022	Bio-Rad	Arched Auto-Sealing Lids***	1 Lid
MSP-1003	Bio-Rad	Microseal 'P' Replacement Pads***	2 Pads
4312063	Applied Biosystems®	MicroAmp® Splash-Free 96-Well Base***	1 Base

* One-time purchase.
** Dependent on adapter labware options.
*** Required for on-deck thermocycling.

QC Analysis Equipment Used		
Part Number	**Manufacturer**	**Description**
G2940CA	Agilent Technology	Agilent 2100 Bioanalyzer
5067-4626	Agilent Technology	High-Sensitivity DNA Kit

BFX^{P} Configuration Used[3]		
Part Number	**Manufacturer**	**Description**
A31844	Beckman Coulter	Biomek FX^{P} Dual Multichannel Span-8
719654	Beckman Coulter	Span-8 Wash ALP
719363	Beckman Coulter	96-Well Wash Station
379448	Beckman Coulter	Orbital Shaker ALP, Single Position
719590	Beckman Coulter	Span-8 Disposal ALP
719357	Beckman Coulter	Static 1x1 ALP Platform
719361	Beckman Coulter	Static Peltier ALP
719948	Beckman Coulter	4x3 ALP Kit
719366	Beckman Coulter	Biomek FX Device Controller
Request a Quote	Beckman Coulter	Biometra T-Robot Integration

References

1. The PCR process is covered by patents owned by Roche Molecular Systems, Inc. and F. Hoffman La Roche, Ltd.
2. Experiments conducted as part of method development demonstrated successful results with 50 ng input; however, please note that Illumina only supports 100 ng as the minimum input amount.
3. Contact a Beckman Coulter sales consultant for a system quotation at www.beckmancoulter.com.

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