GENOMICS

APPLICATION NOTES

DISCOVERY
in motion.

BECKMAN COULTER
Life Sciences

TABLE OF CONTENTS

Genomics Application Notes

Title **Page**

Biomek-Automated NGS Library Construction Methods

DEMONSTRATED METHOD		BIOMEK FX^P HYBRID	BIOMEK FX^P MULTI-CHANNEL	BIOMEK FX^P SPAN 8	BIOMEK NX^P SPAN 8	BIOMEK NX^P MULTI-CHANNEL	BIOMEK 4000
CANCER PANELS	Illumina TruSight® Cancer						■
	Illumina TruSight® Tumor 15						■
DNA SEQUENCING	Illumina Nextera® XT	●					
	Illumina TruSeq® DNA PCR-Free	■					■
	Illumina TruSeq® Nano DNA	■			■		■
	Illumina TruSeq® Custom Amplicon Low Input Kit	●					
	KAPA Hyper Prep Library Prep Kit for Illumina NGS	▲					
	KAPA HyperPlus Library Prep Kit for Illumina NGS	▲					
	NEBNext® Ultra DNA for Illumina NGS (ChIP-seq and HLA)	■					
	NEBNext® Ultra DNA II Kit for Illumina NGS	■					
	Rubicon Genomics ThruPLEX® Plasma-seq Kit for Illumina NGS	■					
	Swift Biosciences Accel - NGS® 2S Plus DNA Library Kit for Illumina NGS	■					
HLA TYPING	Illumina TruSight® HLA Sequencing Panel v2				●		
	Immucor Mia Fora NGS	▲			▲		▲
	Omixon Holotype HLA X2						■
RNA SEQUENCING	Illumina TruSeq® RNA v2	■					
	Illumina TruSeq® RNA Access	■					
	Illumina TruSeq® Stranded mRNA	●					
	Illumina TruSeq® Stranded Total RNA	●					
	NEBNext® Small RNA Kit for Illumina NGS						■
	NEBNext® Ultra Directional RNA Library Kit for Illumina NGS	■					
TARGET/EXOME CAPTURE	Agilent HaloPlex™ Target Enrichment - Ion Torrent	■			■		■
	Agilent SureSelect XT®	■					
	Epicentre ScriptSeq® Complete Gold Low Input	●					
	Illumina Nextera® Rapid Capture	●					
	Illumina TruSeq® Exome	■					
	Illumina TruSeq® Rapid Exome	■					
	Nimblegen SeqCap EZ® for Illumina NGS	■					

Biomek-Automated Nucleic Acid Sample Preparation Methods

DEMONSTRATED METHOD		BIOMEK FX^{P} HYBRID	BIOMEK FX^{P} MULTI-CHANNEL	BIOMEK FX^{P} SPAN 8	BIOMEK NX^{P} SPAN 8	BIOMEK NX^{P} MULTI-CHANNEL	BIOMEK 4000
DNA AND RNA PURIFICATION AND CLEAN UP/ SIZE SELECTION/ QUANTITATION/ NORMALIZATION/ POOLING	AMPure XP - PCR Purification	■	■		■	■	■
	CleanSEQ - Dye Terminator Removal	■	■			■	■
	KAPA Biosystems Library Quantification Kit - Illumina	■			■		
	qPCR setup 384	■			■		■
	qPCR setup 96	■			■		■
	Quantitation/Normalization	■			■		■
	RNAClean XP - Post cDNA Purification and Post-IVT cRNA Purification	■	■			■	■
	SPRIselect for DNA Size Selection	■			■		■
	Sample Pooling for Multiplexing	■					
NUCLEIC ACID ISOLATION	CosMCPrep – High/Low Copy Plasmid Extraction	■	■			■	
	DNAdvance - DNA Isolation from Mammalian Tissue	■	■			■	■
	FormaPure DNA - DNA Isolation from FFPE Tissue						■
	FormaPure - RNA Isolation from FFPE Tissue	■		■	■		
	Mo Bio PowerMag Soil DNA Isolation Kit						■
	RNAdvance Tissue	■		■	■		
	RNAdvance Cell v2 - RNA Isolation from Tissues	■	■	■	■	■	

Method Key

■ Demonstrated Method developed for a sample preparation kit following a vendor's published manual protocol. Each one is tested with relevant samples and has yielded results that meet the kit vendor's specifications either in a customer lab (customer demonstrated) or in a Beckman Coulter Life Sciences Lab (Beckman demonstrated). Beckman Coulter makes no claims regarding the use or performance of these methods.

■ Methods currently in the data generation phase of development.

● Illumina Qualified Method, which indicates that Illumina's analysis of libraries prepared with the Biomek-automated method has shown the libraries to perform comparably to those prepared manually. This method is not an Illumina product, and Illumina does not support this product. Illumina makes no representations or warranties with respect to this product.

▲ Method created and provided by kit vendor.

BECKMAN COULTER Life Sciences

For Beckman Coulter's worldwide office locations and phone numbers, please visit "Contact Us" at beckman.com.

B2014-14915 DS-18966B beckman.com PRINTED IN U.S.A.

Automated DNA Library Construction Using the NEBNext® Ultra™ DNA Library Prep Kit for Illumina® on the Beckman Coulter Biomek FX^P Automated Liquid Handler

Abstract/Introduction

The ability to construct DNA sequencing (DNASeq) libraries from a wide range of starting input masses is essential for many next generation sequencing (NGS) applications. The New England Biolabs® NEBNext Ultra DNA Library Preparation Kit for Illumina (Catalog # E3730) provides users with the ability to construct indexed DNASeq libraries from inputs ranging from 5 ng to 1 ug of starting DNA. In this technical note, we describe the automation of the New England Biolabs NEBNext Ultra DNA Library Preparation Kit for Illumina on the Beckman Coulter Biomek FX^P Dual-Arm Multichannel 96 and Span-8 automated liquid handler (Biomek FX^P). The automation method allows the user to prepare up to 96 individually indexed DNASeq libraries in approximately 4-1/2 hours. An intuitive HTML-driven user interface allows the user to specify the number of samples to process (up to 96 samples), and which size selection option to be used. The user interface also provides users with the option to utilize either off-deck incubations using an external thermocycler, or to perform incubations on-deck with a Biometra T-Robot thermocycler integrated to the Biomek FX^P liquid handler. To help simplify and reduce errors during system setup, an HTML-driven reagent calculator is presented to the user with information on the required reagents, their respective volumes, and their location on the instrument deck based on the user's input regarding number of samples and steps to be run. The method also incorporates all recommended stop points described in the NEBNext Ultra DNA Library Preparation Kit for Illumina protocol, allowing users the maximum amount of flexibility in planning their experiments.

Biomek FX^P Workstation

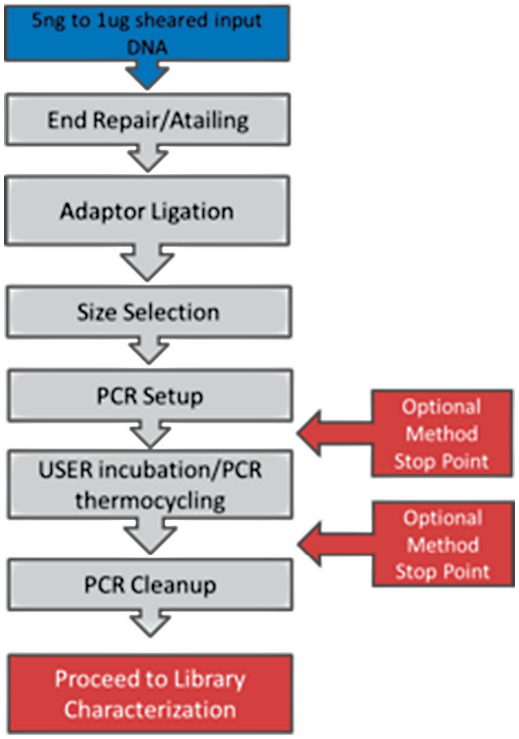


Fig. 1. NEBNext Ultra DNA automated method workflow.

Method Overview and Hardware Description

This automation method was developed on the Biomek FX^P liquid handler, equipped with low volume tubing and 1 ml syringes. The deck configuration of the instrument

BECKMAN COULTER
Life Sciences

is displayed in Figure 2. A list of automated labware position (ALP) hardware is presented in Table 1. The method employs individual Span-8 probes to deliver enzyme and reagent transfers while DNA cleanup, wash, and elution transfers are performed using the multichannel 96 pipetting head. A static Peltier unit ensures that enzyme master mixes are kept cool during the course of the method. For on-deck incubations, a Biometra T-Robot integration is required, otherwise the method prompts the user to remove the plate from the deck and perform the incubation in an off-deck thermocycler. Filter tips are employed throughout the method to reduce the possibility of instrument or sample contamination. A list of automation consumables and user-supplied reagents can be found in Tables 2 and 3, respectively.

Fig. 2. NEBNext Ultra DNA automated deck layout with integrated Biometra T-Robot.

The automation method follows NEBNext Ultra DNA Library Preparation Kit for Illumina protocol with a few modifications. Sheared gDNA is added to a 96-well plate by the user, along with the master mixes and reagents as directed by the reagent calculator. The NEBNext adapter is kept separate from the Ligation Master Mix to inhibit the formation of adapter dimers prior to the adapter ligation step. Another important modification to the NEBNext Ultra DNA Library Preparation Kit for Illumina protocol involves the USER enzyme. The USER enzyme has been integrated into the PCR[1] master mix, requiring a 15-minute, 37°C incubation added to the beginning of the PCR program outlined in the NEBNext Ultra DNA Library Preparation Kit for Illumina protocol. This reduces the number of tips required by the protocol in addition to offering time savings overall. Lastly, the automation method employs Beckman Coulter's SPRIselect reagent in place of AMPure XP to provide for more consistent size selection. The automation workflow is presented in Figure 1.

The automated NEBNext DNA Ultra method utilizes an HTML-driven User Interface (UI) that allows the user to customize their workflow by offering a number of different options. Some major options offered by the UI include the following:

1. Selecting any number of samples to process, between 1 and 96.
2. Selecting which procedures of the library construction workflow to run.
3. Selecting from an extensive list of size selection options to be used in the library construction procedure (150 bp, 200 bp, 250 bp, 300–400 bp, 400–500 bp, 500–700 bp inserts or no size selection at all).
4. Choosing to conduct on-deck or off-deck incubations.
5. User-defined transfer volumes for various steps to further increase the flexibility of the method.

An image of the user interface is presented in Figure 3.

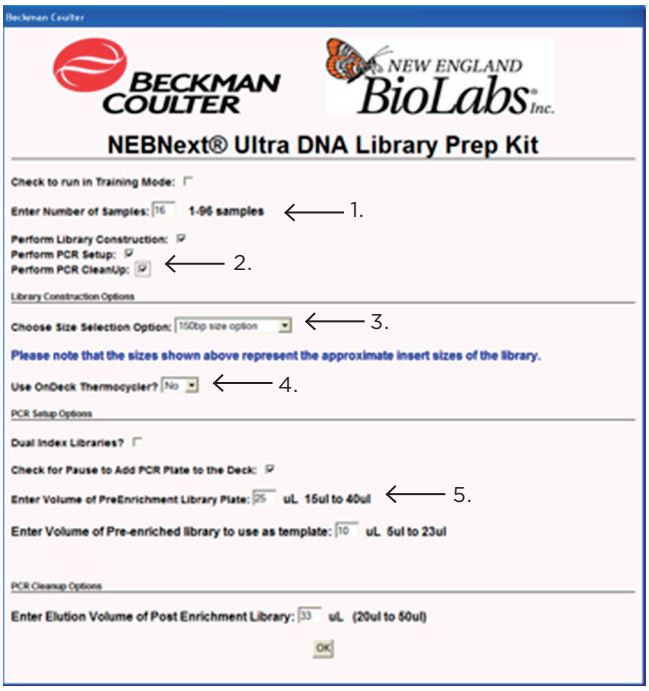


Fig. 3. NEBNext Ultra DNA user interface.

In addition to the user interface, the automated NEBNext Ultra DNA method provides the user with an HTML-driven reagent calculator that provides the user with the final volumes of all of the reagents and master mixes required on the deck, as well as instructions on how to generate the various master mixes—based upon the number of samples to be processed and the amount of the workflow that the user wishes to pursue. An image of the reagent calculator is presented in Figure 4.

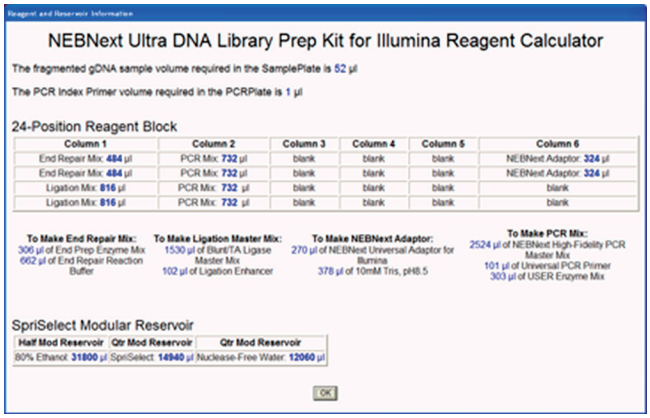


Fig. 4. NEBNext Ultra DNA reagent calculator.

Results

Sixteen genomic DNA (gDNA) samples from *A. thaliana* and *H. sapiens*, 2 *C. elegans* amplicon pools, 2 *H. sapiens* ChIP DNA, and 4 bacterial gDNA samples were supplied by various laboratories at Indiana University, Bloomington, for Biomek automated library construction at Indiana University. In addition, *E. coli* K12 gDNA was supplied by New England Biolabs as a positive control. The 200 ng aliquots for each of the gDNA samples were sheared to an average size of 400 bp (data not shown) and arrayed in the sample plate as shown in Figure 5.

Row/Column	1	2	3
A	*E.coli (S1)*	*H. sapiens*	bacteria
B	*A. thaliana*	*E.coli (S10)*	bacteria
C	*A. thaliana*	*A. thaliana*	bacteria
D	*A. thaliana*	*H. sapiens ChIP*	*E.coli (S20)*
E	*A. thaliana*	*H. sapiens*	bacteria
F	*E.coli (S6)*	*H. sapiens*	*C.elegans*
G	*A. thaliana*	*E.coli (S15)*	*C.elegans*
H	*A. thaliana*	*H. sapiens ChIP*	*E.coli (S24)*

Fig. 5. Automated library construction plate layout.

Libraries were constructed using the NEBNext Ultra DNA automation method utilizing the 400–500 bp insert size selection option. Eight cycles of PCR enrichment were used to amplify the libraries using an off-deck thermocycler. Following analysis on the Agilent 2200 TapeStation, all of the libraries processed were then sequenced on an Illumina MiSeq® using a 2x300 cycle paired end run. For the purposes of this document, we concentrated our analysis on the six *E. coli* K12 control libraries, which were assayed on the Agilent 2200 TapeStation using D1000 ScreenTape (PN# 5067-5582). The electropherograms for all 6 *E. coli* K12 control libraries are presented in Figure 6.

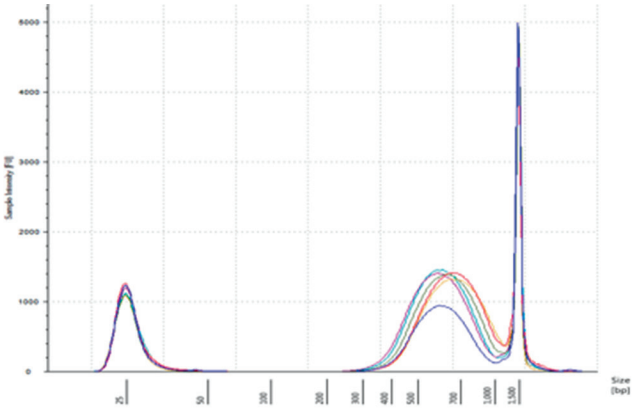


Fig. 6. *E. coli* K12 gDNA control libraries created with the NEBNext Ultra DNA automated method.

Data analysis was performed at New England Biolabs using a local instance of Galaxy. For each of the 6 *E. coli* K12 control libraries, over 1.4 million pass filter reads for each library were generated by the MiSeq run. Pass filter read counts are presented in Figure 7. Reads were then trimmed using SeqPrep prior to mapping back to the *E. coli* K12 MG1655 reference genome using Bowtie (version 2.1.10). Less than 1% of the reads from the *E. coli* K12 control libraries failed to map back to the *E. coli* K12 MG1655 reference genome. We also observed low percentages of chimeric reads, PCR duplicates, and mismatched reads. These metrics indicate that the libraries were high-quality libraries. Data on basic library quality metrics are presented in Figure 8. Additionally, we investigated the average size of the library inserts of the *E. coli* K12 control libraries utilizing the library mapping data. As shown in Figure 9, the average size inserts of the *E. coli* K12 control libraries was approximately 400 bp.

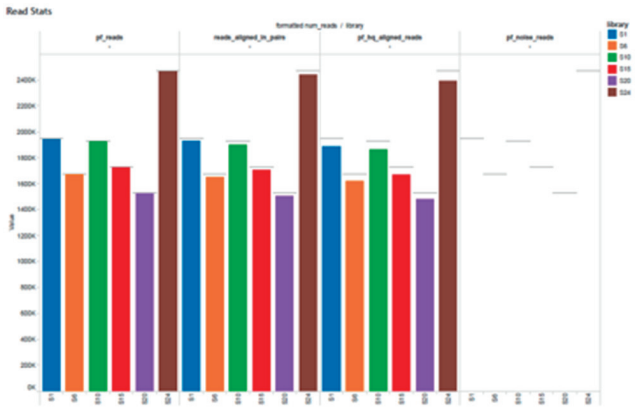


Fig. 7. *E. coli* K12 gDNA control library pass filter read counts.

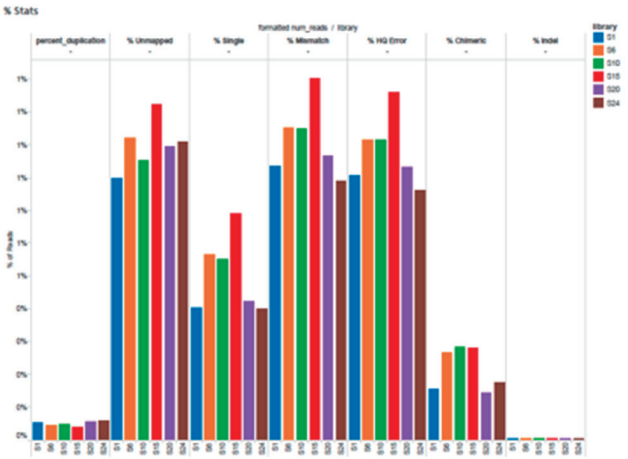


Fig. 8. *E. coli* K12 gDNA control library quality metrics.

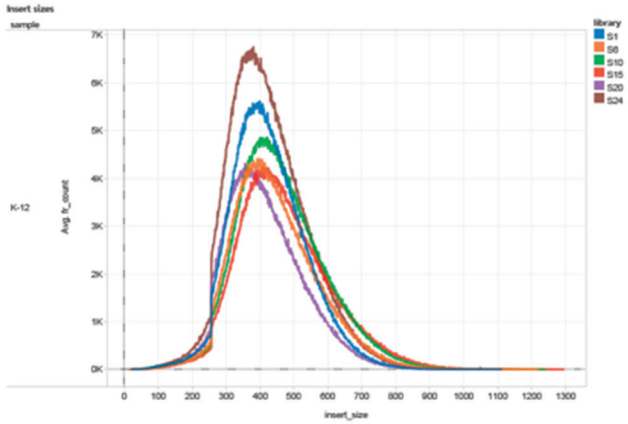


Fig. 9. *E. coli* K12 gDNA control library insert size distributions.

E. coli K12 control library reads were also mapped back to the *A. thaliana* (TAIR10) and *C. elegans* (ce10) reference genomes using Bowtie to check for the presence of contaminating sequences. As shown in Figure 10, the percentages of reads in the *E. coli* K12 control libraries that mapped back to the *A. thaliana* and *C. elegans* reference genomes were generally quite low, and reveal no particular pattern when the physical layout of the samples is considered. These results show that the libraries derived from the automated NEBNext Ultra DNA method on the Biomek FX^P show no discernible evidence of cross-contamination during library construction.

	E.coli Control Libraries					
Organism	S1	S6	S10	S15	S20	S24
A. thaliana	0.03%	0.07%	0.04%	0.00%	0.00%	0.00%
C.elegans	0.03%	0.01%	0.01%	0.01%	0.09%	0.01%

Fig. 10. Cross-contamination analysis of *E. coli* K12 gDNA control libraries.

Conclusion

As a result of a successful run on the Illumina MiSeq, we have shown that the libraries are suitable for sequencing on all Illumina sequencing platforms. Additionally, analysis of the sequenced libraries indicated that the libraries mapped well to the reference genome, and that the insert sizes targeted during the course of library construction were achieved. Finally, the libraries produced by this automated method show no evidence of cross-contamination as demonstrated by our MiSeq data. In conclusion, we have demonstrated that high-quality, sequence-ready, DNASeq libraries are generated from using the Biomek FX^P automated method in conjunction with the NEBNext Ultra DNA Library Kit for Illumina.

Table 1. Biomek FX^P Dual-Arm Multichannel 96 and Span-8 Configuration[2]

Part Number	Qty.	Manufacturer	Description
719948	1	Beckman Coulter, Inc.	ALP, High-Density, 12-Position, 4 x 3
379448	1	Beckman Coulter, Inc.	ALP, Shaking, Orbital, Single-Position
719357	3	Beckman Coulter, Inc.	ALP, Standard Single-Position
719361	1	Beckman Coulter, Inc.	ALP, Cooling/Heating, Single-Position
719590	1	Beckman Coulter, Inc.	Waste, Span-8, ALP
719356	1	Beckman Coulter, Inc.	Disposable Tip Loader ALP
719654	1	Beckman Coulter, Inc.	ALP, Tip Wash, 8-Channel
719363	1	Beckman Coulter, Inc.	Wash Station including Pump and Tubes
719366	1	Beckman Coulter, Inc.	Biomek FX^P Device Controller
Contact Beckman Coulter, Inc.	1	Biometra	(*Optional*) Biometra T-Robot for On-Deck Incubations

Table 2. Automation Consumables Required.

Part Number	Qty.	Manufacturer	Description
B01124	1	Beckman Coulter, Inc.	Biomek Span-8 P1000 Tips, Pre-Sterile with Barrier
379503	1	Beckman Coulter, Inc.	Biomek Span-8 P250 Tips, Pre-Sterile with Barrier
A21586	3	Beckman Coulter, Inc.	Biomek P50 Tips, Pre-Sterile with Barrier
717253	1	Beckman Coulter, Inc.	Biomek AP96 P250 Tips, Pre-Sterile with Barrier
372790	2	Beckman Coulter, Inc.	Quarter Reservoir
534681	1	Beckman Coulter, Inc.	Half Reservoir, Nonpyrogenic
C5064	1	Acme-Automation	Reactor Adapter 96 Flat*
372795	1	Beckman Coulter, Inc.	Frame for Reservoirs*
A32782	1	Beckman Coulter, Inc.	Agencourt SPRIPlate 96R—Ring Super Magnet Plate*
A83054	1	Beckman Coulter, Inc.	Blue Heater/Chiller 24-Well Block*
AB-1127	3	Thermo Scientific	ABgene 96-Well Storage Plate, Square Well, 1.2 mL
16466-042	10	VWR	2 mL SuperClear™ Screw Cap Microcentrifuge Tubes—Conical Bottom
HSP-9641	5	Bio-Rad	Hard-Shell® Thin-Wall 96-Well Skirted PCR Plates
MSL-2022	1	Bio-Rad	Arched Auto-Sealing Lids**

* One-time purchase.
** For on-deck thermocycling only.

Table 3. User-Supplied Reagents.

Part Number	Manufacturer	Description
N/A	User-Preferred	Elution Buffer Nuclease-Free Water, TE, 10 mM Tris, pH 8.5
B23318	Beckman Coulter, Inc.	SPRIselect Reagent Kit
AB00138-01000	American Bioanalytical	Ethanol
E7370S (24 rxns) or E7420L (96 rxns)	New England Biolabs	NEBNext Ultra DNA Library Prep Kit for Illumina
E7335L (Set 1, 96 rxns) or E7500L (Set 2, 96 rxns) or E7600	New England Biolabs	NEBNext Multiplex Oligos for Illumina (Index Primers Set 1 or 2) or NEBNext Multiplex Oligos for Illumina Dual-Index Primers Set 1

Software used in QC Testing

Bowtie (version 2.1.10) installed on New England Biolabs Galaxy instance.

SeqPrep (version 1.0) installed on New England Biolabs Galaxy instance.

Authors

Zach Smith, M.S.
Beckman Coulter Life Sciences, Indianapolis, IN USA

Bradley W. Langhurst, Ph.D.
New England Biolabs, Inc. Ipswich, MA USA

Scott Michaels, Ph.D. and James Ford
The Center for Genomics and Bioinformatics, Indiana University, Bloomington, IN USA

References

1. The PCR process is covered by patents owned by Roche Molecular Systems, Inc. and F. Hoffman La Roche, Ltd.

2. Contact a Beckman Coulter sales consultant for a system quotation at www.beckmancoulter.com.

Galaxy Citations

3. Goecks J, Nekrutenko A, Taylor J and The Galaxy Team. Galaxy: a comprehensive approach for supporting accessible, reproducible, and transparent computational research in the life sciences. *Genome Biol.* 11(8); R86: (Aug 25 2010).

4. Blankenberg D, Von Kuster G, Coraor N, Ananda G, Lazarus R, Mangan M, Nekrutenko A, Taylor J. Galaxy: a web-based genome analysis tool for experimentalists. *Current Protocols in Molecular Biology.* Chapter 19: Unit 19.10.1–21: (Jan 2010).

5. Giardine B, Riemer C, Hardison RC, Burhans R, Elnitski L, Shah P, Zhang Y, Blankenberg D, Albert I, Taylor J, Miller W, Kent WJ, Nekrutenko A. Galaxy: a platform for interactive large-scale genome analysis. *Genome Research.* 15(10); 1451–5: (Oct 2005).

BECKMAN COULTER
Life Sciences

For Beckman Coulter's worldwide office locations and phone numbers, please visit "Contact Us" at www.beckmancoulter.com

AAG-350TCH08.14-A

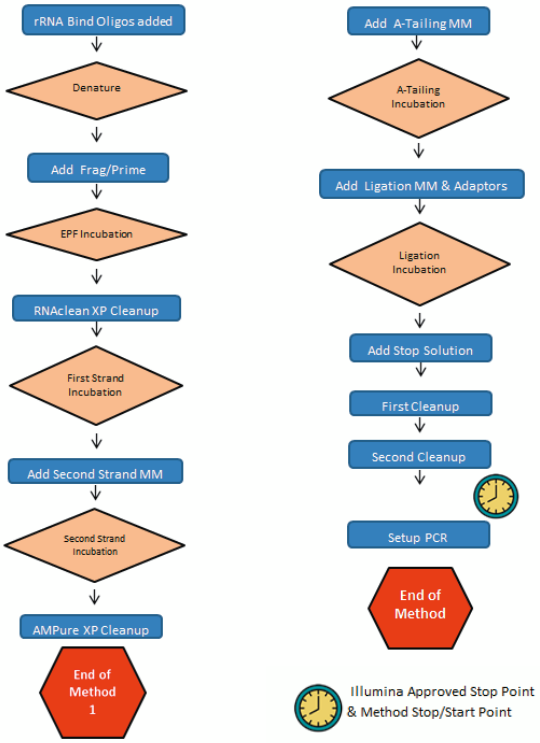
High-Throughput TruSeq® Stranded Total RNA Library Construction from FFPE Samples on the Biomek FX^P Dual-Arm Multi-96 and Span-8 Workstation

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).

BECKMAN COULTER
Life Sciences

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.

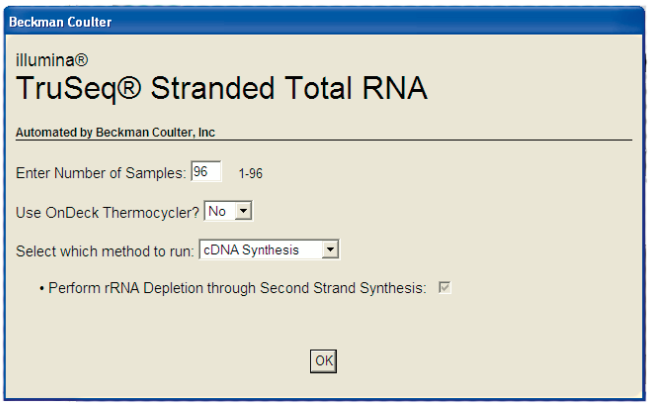


Fig. 1. User interface showing cDNA synthesis options.

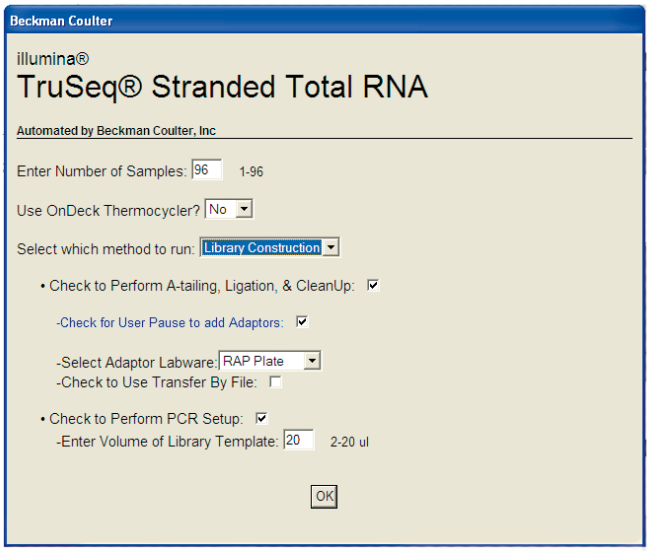


Fig. 2. User interface showing library construction options.

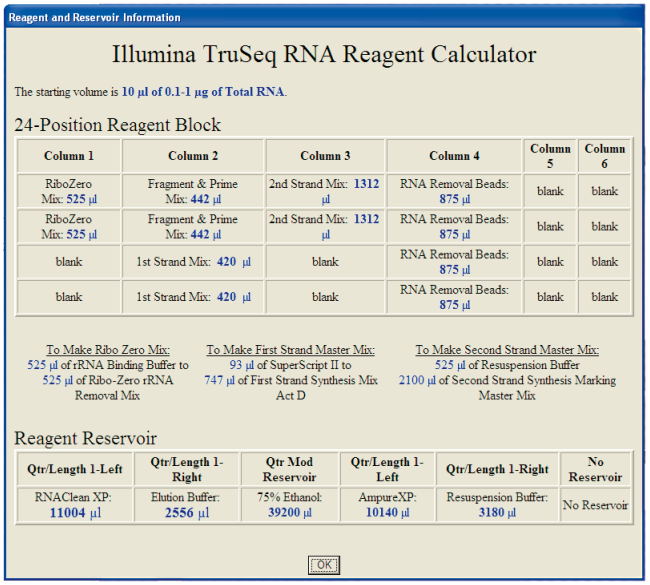


Fig. 3. cDNA synthesis reagent calculator.

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).

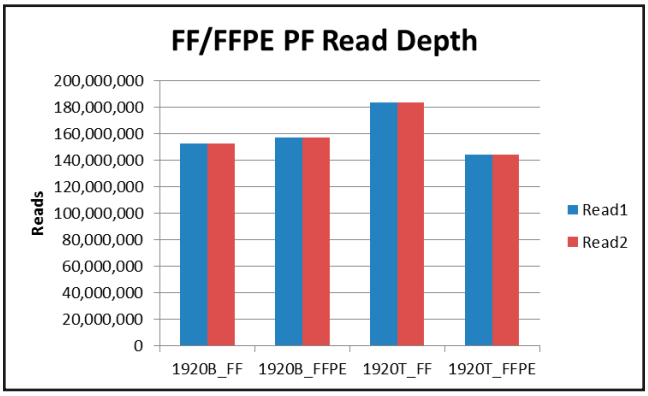


Fig. 4. Pass filter read depth for read 1 and 2 of the FF and FFPE samples on the NextSeq® 500.

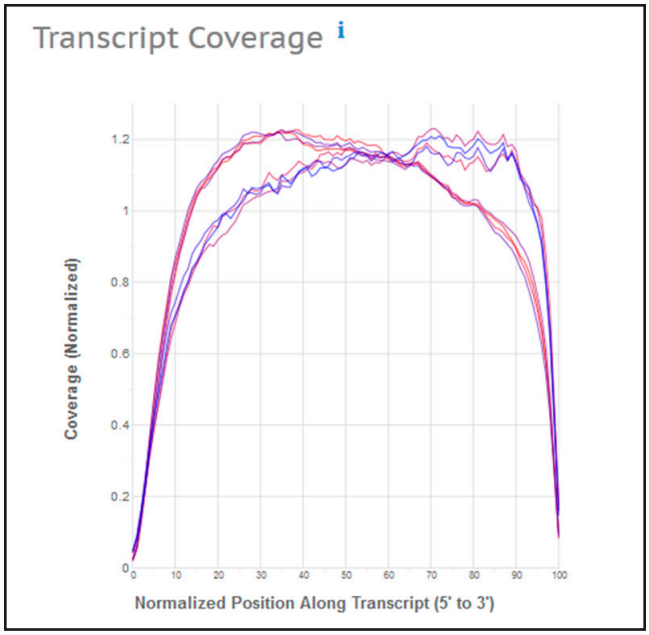


Fig. 5. Transcript coverage displaying typical 3' bias for the FFPE compared to FF samples.

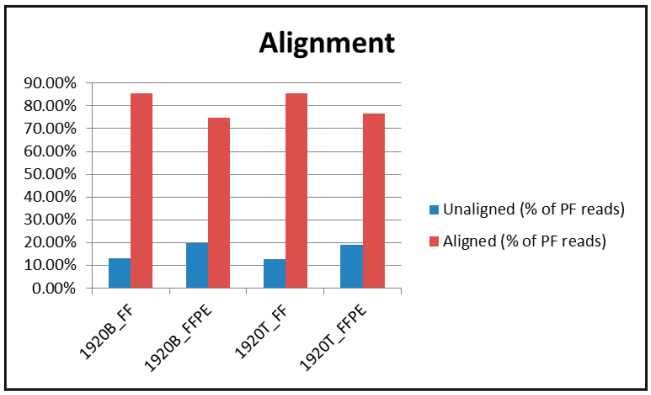


Fig. 6. Percent alignment of FF and FFPE samples show high alignment, with the FFPE samples being only ~10% lower than the FF samples.

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.

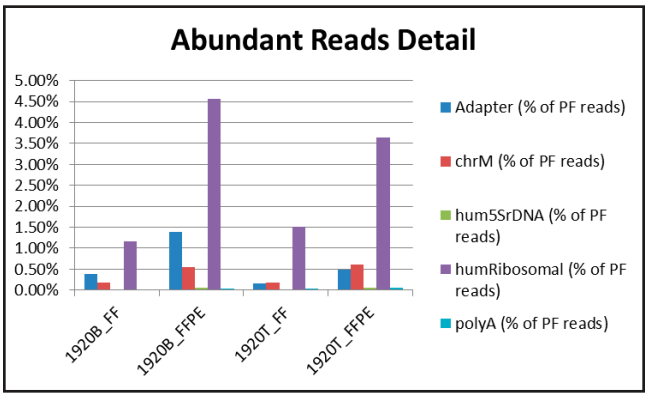


Fig. 7. Less than 5% of reads mapping to rRNA for FFPE samples; Less than 2% of reads mapping to rRNA for FF samples; Low percent of reads mapping to other contaminants.

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.

Authors

Mary Blair & Louie Lamorte
Beckman Coulter Life Sciences, Indianapolis, IN USA

Michaela Bowden, PhD & Melanie Prasol, PhD
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.

BECKMAN COULTER

Life Sciences

For Beckman Coulter's worldwide office locations and phone numbers, please visit "Contact Us" at www.beckmancoulter.com

AAG-314TCH07.14-A

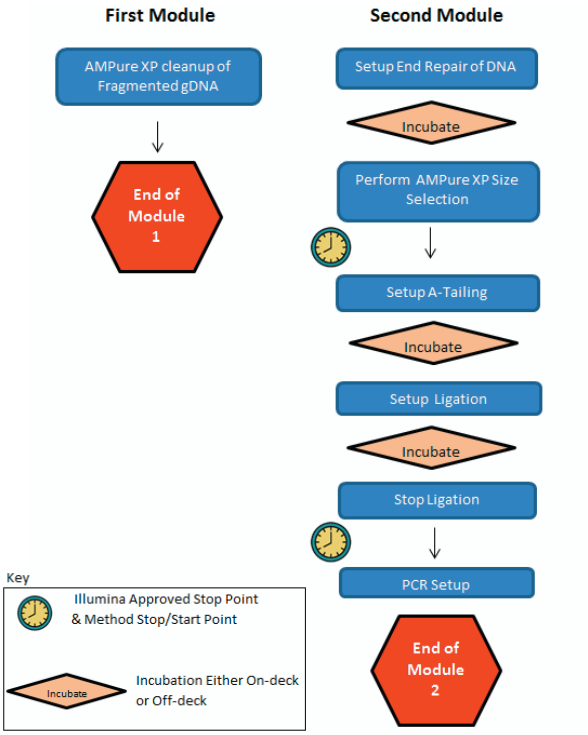
High-Throughput Illumina® TruSeq® Nano DNA Library Construction on the Biomek FX^P Dual-Arm Multi-96 and Span-8 Workstation

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 Dual-Arm Multi-96 and Span-8 Workstation (BFX^P) 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 consistently 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.

The Illumina TruSeq Nano DNA kit is a DNA library prep system for low sample inputs that provides a streamlined workflow that includes Solid Phase Reversible Immobilization (SPRI) bead-based size selection. This technical note describes the automation of the Illumina TruSeq Nano DNA kit on the Biomek FX^P for interrogation of low-input samples down to 100 ng. 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. The pipetting tools and software improve the ease and speed of reaction setup. The automated method consists of two modules: one for SPRI-based cleanup to purify sheared samples; and the other to prepare libraries all the way through PCR[1] enrichment. The end result is as many as 96 sequence-ready libraries from as little as 100 ng of DNA. The resulting high-quality libraries display consistent insert sizes, minimal adapter dimers, and low percent PCR duplication to provide efficient interrogation of samples with limited DNA. All of this was accomplished with minimal hands-on time (see Table 1).

Biomek FX^P T-Robot SPRIWorks



Workflow of the TruSeq Nano DNA method, highlighting the steps of the protocol and the Illumina-approved start and stop points built into the user interface and method.

BECKMAN COULTER
Life Sciences

Table 1. Major Process Description.

Automated/Hands-On Time			
	24 Samples	48 Samples	96 Samples
Shear Cleanup			
Prepare Reagants/ Set Up Inst.	15 min	15 min	15 min
Method Run	40 min	43 min	49 min
Total	55 min	58 min	1 hrs 4 min
Library Construction			
Prepare Reagants/ Set Up Inst.	25 min	27 min	30 min
Method Run	5 hrs 43 min	6 hrs 4 min	6 hrs 36 min
Total	6 hrs 8 min	6 hrs 31 min	7 hrs 6 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 TruSeq Nano DNA protocol while maintaining the flexibility that Biomek software provides (see workflow on previous page). 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) 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 DNA adapter plate (DAP), Illumina DNA 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 which reagents are required, what volume of each is necessary, 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.

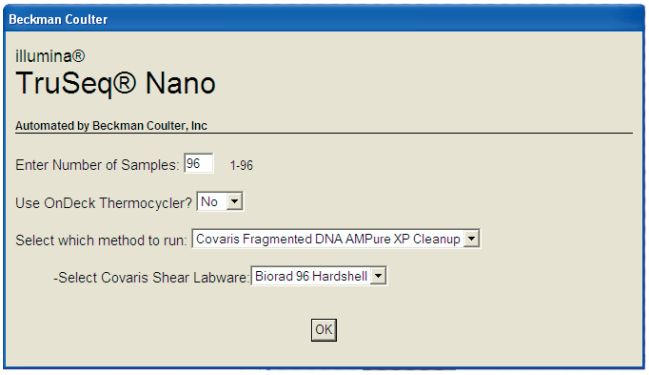


Fig. 1. User interface showing Shear cleanup option.

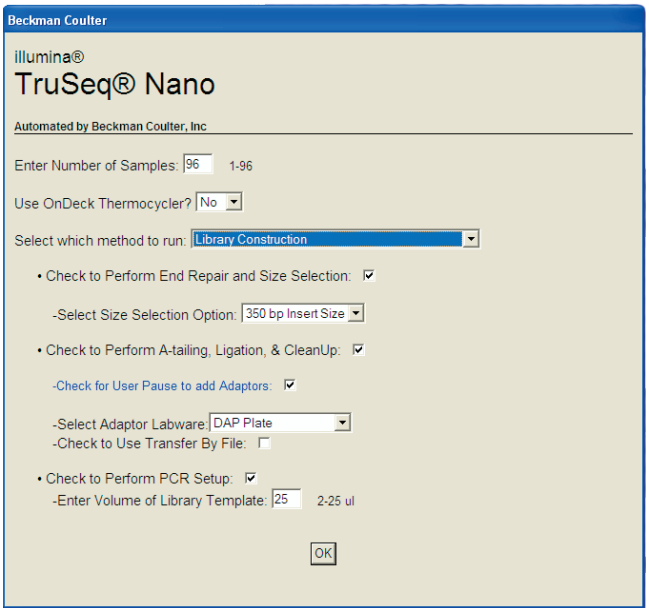


Fig. 2. User interface showing library construction options.

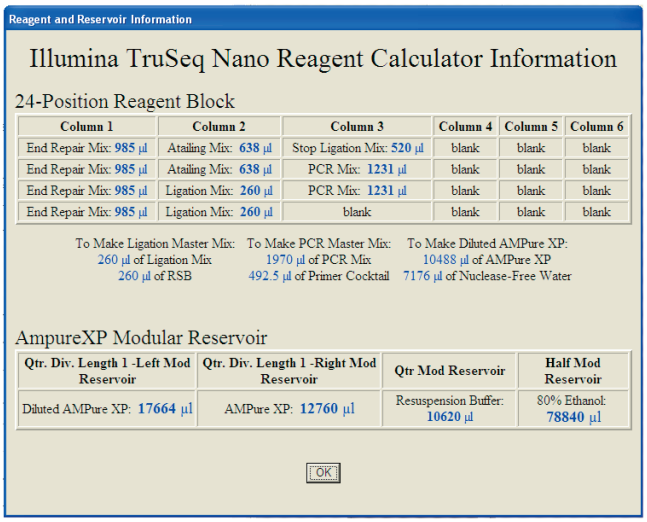


Fig. 3. Library construction reagent calculator.

Results

Sample Layout and Results

Genomic DNA from *E. coli* was sheared using a Covaris® protocol for 550 bp insert size. Twelve replicates of the sheared gDNA were distributed in a 96-well plate (as shown in Figure 4) and purified in a starting volume of 50 µl and an input concentration of 200 ng in a 96-well plate using AMPure XP.

	1	2	3	4
A	Rep 1	Rep 9		
B	Rep 2	Rep 10		
C	Rep 3	Rep 11		
D	Rep 4	Rep 12		
E	Rep 5			
F	Rep 6			
G	Rep 7			
H	Rep 8			

Fig. 4. Sample plate map for 12 samples run.

The cleaned gDNA were processed by selecting the options in the user interface for the Illumina adapter tubes and the 550 bp selection, along with off-deck incubation. For PCR enrichment, 20 µl of sample was used. The samples were purified and quantified using the AMPure XP and Kapa Biosystems Library Quant qPCR Setup method. To check the quality of the libraries, a 1:100 dilution of the first 11 libraries was checked using Agilent Bioanalyzer 2100 for size distribution. Figure 5 displays consistent library size and distribution of the 11 libraries and no detectable adapter dimer peak at ~120 bp. Amplification of the libraries resulted in an average yield of 269 nM resulting in more than enough library to process for sequencing (Figure 6).

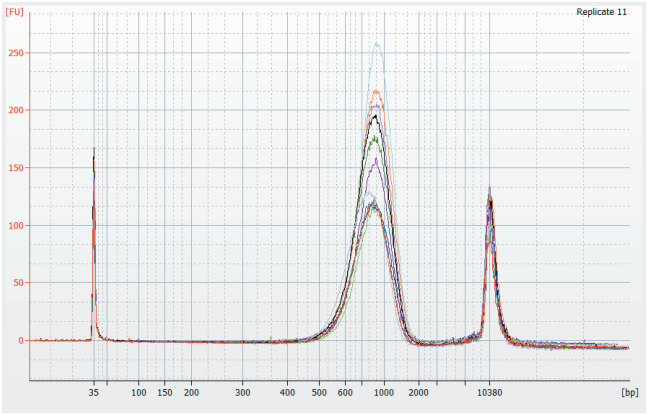


Fig. 5. Agilent Bioanalyzer 2100 trace of 1:100 dilutions of the prepped libraries.

	Source (Library) Plate Concentration				nM
	1	2	3	4	5
A	255.8	203.5	NA	NA	NA
B	284.7	298.3	NA	NA	NA
C	299.7	339	NA	NA	NA
D	289.9	325.5	NA	NA	NA
E	225.7		NA	NA	NA
F	278.9		NA	NA	NA
G	216.4		NA	NA	NA
H	216.1		NA	NA	NA

Fig. 6. Kapa qPCR quantitation results shown in nM concentration.

Table 2. Library-Quality Statistics Generated by the BaseSpace® Resequencing Analysis Pipeline (basespace.illumina.com) Set to Default Parameters.

Library	% Aligned Read 1	Read 1 Q30	% Aligned Read 2	Read 2 Q30	Frag Length Median	St.Dev Frag length	% Duplicate
Rep 1	88.43%	98.06%	87.86%	94.37%	567	144	0.2%
Rep 2	88.66%	98.04%	88.29%	94.82%	583	148	0.2%
Rep 3	88.61%	97.95%	88.17%	93.96%	616	154	0.2%
Rep 4	88.29%	98.02%	88%	94.84%	598	147	0.2%
Rep 5	89.04%	98.02%	88.75%	94.87%	594	148	0.2%
Rep 6	89.25%	98.02%	88.94%	94.97%	594	146	0.2%
Rep 7	89.08%	98%	88.79%	94.79%	597	149	0.2%
Rep 8	89.04%	97.99%	88.50%	94.18%	603	150	0.2%
Rep 9	89.14%	98.10%	88.60%	94.14%	554	142	0.2%
Rep 10	89.03%	98%	88.72%	94.90%	590	144	0.2%
Rep 11	89.20%	97.97%	88.77%	94.57%	593	148	0.2%
Rep 12	89.20%	98.02%	88.85%	94.81%	579	144	0.2%

As shown in Table 2, the libraries produced with the TruSeq Nano method are highly consistent with one another in terms of percent alignments to the reference genome and quality scores. Median fragment lengths (593 bp) are also highly reproducible between replicates. Percent PCR Duplicates—a key quality metric that measures the amount of artifacts produced via PCR—was quite low for the libraries built using the BFX.[P] On balance, these metrics show that the libraries produced are high-quality and sequenceable.

Conclusion

Reliable and efficient automation solutions for NGS library construction are essential in order 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 is capable of creating up to 96 TruSeq Nano DNA libraries ready for PCR thermocycling in just over 8 hours from low-input samples.

Author

Mary Blair
Beckman Coulter Life Sciences, Indianapolis, IN 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
FC-121-4003	Illumina	TruSeq Nano DNA HT kit
FC-121-4002	Illumina	TruSeq Nano DNA LT kit
AM6050	Life Technologies	Human Reference Brain RNA
740000	Agilent Technology	Universal Human Reference RNA
KK4835	Kapa Biosystems	Library Quantification Kit—Illumina/ABI
14380	USB® Corporation	Stock *E. coli* gDNA

Consumables Used			
Part Number	**Manufacturer**	**Description**	**Qty for 96-Sample Run**
717253	Beckman Coulter	Biomek AP96 P250 Tips, Pre-Sterile with Barrier (case of 10 racks)	2 Tip Boxes
379503	Beckman Coulter	Biomek Span-8 P250 Tips, Pre-Sterile with Barrier (case of 10 racks)	2 Tip Boxes
A21586	Beckman Coulter	Biomek P50 Tips, Pre-Sterile with Barrier (case of 10 racks)	8 Tip Boxes
717256	Beckman Coulter	Biomek AP96 P20 Tips, Pre-Sterile with Barrier (case of 10 racks)	2 Tip Boxes
B01124	Beckman Coulter	Biomek Span-8 P1000 Tips, Pre-Sterile with Barrier, 1025 µl (case of 5 racks)	1 Tip Box
372790	Beckman Coulter	Quarter Reservoir (case of 48)	3 Reservoirs
372788	Beckman Coulter	Quarter Reservoir, Divided by Length (case of 48)	2 Reservoirs
372786	Beckman Coulter	Half Modular Reservoir	1 Reservoir
372795	Beckman Coulter	Reservoir Frame* (1 each)	1 Frame
A32782	Beckman Coulter	Agencourt SPRIPlate 96R—Ring Super Magnet Plate*	1 Magnet
A83054	Beckman Coulter	Blue Heater/Chiller 24-Well 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 1.5 mL Microfuge Tubes** (case of 25)	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	4 Plates
HSP-9641	Bio-Rad	Hard-Shell® Thin-Wall 96-Well Skirted PCR Plate	11 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.

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

Biomek FX^{P} Dual Arm Multi-96 and Span-8 Workstation Configuration Used[2]		
Part Number	**Manufacturer**	**Description**
719654	Beckman Coulter	ALP, Tip Wash, 8-Channel
719363	Beckman Coulter	96-Well Wash Station
379448	Beckman Coulter	ALP, Shaking, Orbital, Single-Position, Biomek
719590	Beckman Coulter	Waste, Span-8, ALP
719357	Beckman Coulter	ALP, Standard Single-Position
719361	Beckman Coulter	ALP, Cooling/Heating, Single-Position, Biomek
719948	Beckman Coulter	ALP, High-Density, 12-Position, 4x3
719366	Beckman Coulter	Biomek 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. Contact a Beckman Coulter sales consultant for a system quotation at www.beckmancoulter.com.

BECKMAN COULTER

Life Sciences

For Beckman Coulter's worldwide office locations and phone numbers, please visit "Contact Us" at www.beckmancoulter.com

AAG-344TCH07.14-A

High Throughput TruSeq Stranded mRNA Library Construction on the Biomek FX^P

Zach Smith and Scott D. Michaels
The Center for Genomics and Bioinformatics
Indiana University
Bloomington, IN USA

Mary Blair
Life Science Division
Beckman Coulter, Inc.
Indianapolis, IN USA

Abstract

A collaboration between Beckman Coulter, the Center for Genomics and Bioinformatics at Indiana University, and Illumina, Inc. has resulted in the automation of the TruSeq Stranded mRNA sample preparation kit from Illumina on the Biomek FX^P. This automation method generates high quality stranded mRNA sequencing libraries compatible with Illumina sequencers. Data has been generated on the Illumina HiSeq 2500 and MiSeq platforms confirming that libraries produced with this automation method yield target-specific, highly uniform, strand-specific sequence data, with no detectable cross contamination of template samples, while reducing the risk of use error.

Introduction

The continuing development of next generation sequencing (NGS) platforms is providing unparalleled levels of sequencing capacity at relatively low cost. This advance makes possible larger scale genomic studies to understand the genetic and epigenetic underpinnings of a wide range of biological processes, including development, behavior and disease.

A significant bottleneck to fully realizing the capacity of Illumina's sequencing systems is the manual preparation of large numbers of sequencing libraries, which can be labor-intensive and time-consuming. 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 precious reagents and samples, and heating, 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 power, flexibility and efficiency to consistently provide high quality template libraries at a throughput needed to take advantage of the high capacity of NGS, at a reasonable cost.

TruSeq Stranded mRNA Sample Preparation kits provide the clearest, most complete view of the mRNA transcriptome by providing precise assessment of strand orientation, uniform coverage, high-confidence mapping of alternate transcripts and gene fusion events as well as the detection of allele-specific expression. In addition to enabling the discovery of these and other features without prior knowledge, it is a highly accurate and cost-efficient gene expression quantification tool with multiplexing enabled by 96 available unique index pairs.

This application note describes the automation of the Illumina TruSeq Stranded mRNA kit on the Biomek FX^P for transcriptome library construction. One to 96 samples can be run simultaneously, and setup requires only a few clicks of the mouse and one-time pipetting of the required kit components into the reservoirs. The pipetting tools and software improve the ease and speed of reaction setup. The method then runs automatically, providing purification and fragmentation of mRNA from total RNA, first strand cDNA synthesis, second strand cDNA synthesis, AMPure XP purification, 3′ adenylation, and adaptor ligation, double AMPure XP purification and PCR enrichment. The end result is as many as 96 sequence-ready libraries in just two days, from as little as 50[1] ng of mRNA. The resulting high quality libraries display consistent insert sizes, minimal adapter dimers, low numbers of ribosomal sequences, and high fidelity to the reference genome, enabling a detailed survey of the transcriptome under investigation. All of this was accomplished with minimal hands-on time (see Table 1) and no detectable sample cross-contamination.

IB-18319A

Table 1. Processing Time

MAJOR PROCESS DESCRIPTION	AUTOMATED/ HANDS ON TIME**		
	24 SAMPLES	48 SAMPLES	96 SAMPLES
cDNA Synthesis: Day 1			
Prepare Reagents/Set up Inst	25 min	27 min	30 min
Method Run	4 hrs	4 hrs 20 min	4 hrs 50 min
Total	**4 hrs 25 min**	**4 hrs 47 min**	**5 hrs 20 min**
Library Construction: Day 2			
Prepare Reagents/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 35 min**
Total	**7 hrs 20 min**	**7 hrs 54 min**	**8 hrs 55 min**

**Timing does not include thawing of reagents and PCR thermocycling

Materials

REAGENTS	SOURCE
TruSeq Stranded mRNA Sample Preparation Kit	Illumina, San Diego, CA
AMPure XP	Beckman Coulter, Brea, CA
RiboGreen	Life Technologies, Carlsbad, CA
PicoGreen	Life Technologies
R6K ScreenTape	Agilent Technologies, Santa Clara, CA
MiSeq Reagent Kit v2	Illumina
TruSeq Rapid SBS 200 Cycle Kit	Illumina
Tomato RNA *Drosophila* RNA *Daphnia pulex* RNA *Tribolium* RNA *Enchenopa binotata* and *Umbonia crassicornis* RNA	RNA samples provided by various researchers in the Department of Biology at Indiana University
Universal Human Reference RNA	Agilent Technologies
TapeStation 2200 D1K High Sensitivity Screentape with the associated reagents and consumables	Agilent Technologies

LABWARE	SOURCE
Biomek Span-8 P1000 Tips, Pre-sterile with Barrier	Beckman Coulter
Biomek Span-8 P250 Tips, Pre-sterile with Barrier	Beckman Coulter
Biomek P50 Tips, Pre-sterile with Barrier	Beckman Coulter
Biomek AP96 P20 Tips, Pre-sterile with Barrier	Beckman Coulter
Biomek AP96 P250 Tips, Pre-sterile with Barrier	Beckman Coulter
Quarter Reservoir	Beckman Coulter
Reservoir, Half	Beckman Coulter
Quarter Reservoir, Divided by Length	Beckman Coulter
Frame for Reservoirs	Beckman Coulter
Agencourt® SPRIPlate® 96R - Ring Super Magnet Plate	Beckman Coulter
Abgene 96-Well Storage Plate, Square Well, 1.2 mL	Fisher Scientific
2mL SuperClear™ Screw Cap Microcentrifuge Tubes-Conical Bottom	VWR
Hard-Shell® Thin-Wall 96-Well Skirted PCR Plates	Bio-Rad Laboratories

Instruments

Biomek® FX^{P} Laboratory Automation Workstation (P/N B05450)	Illumina MiSeq
Agilent 2200 TapeStation Instrument	Illumina HiSeq 2500
Eppendorf MasterCycler ep or equivalent thermocycler with heated lid	

Results and Discussion

Setting up Library Construction

Automated library construction using 96 samples was set up by making a few selections in the Biomek FX^P user interface (Figures 1 and 2). The user selects either the TruSeq Stranded mRNA method or the TruSeq RNA v2 method; this application note is focused on the TruSeq Stranded mRNA method. The automation method is flexible, allowing the user to choose which steps to perform. Users also have the option to use custom plates for the adaptor labware, and to choose which adaptors to use with which samples, by selecting their own transfer files. Setting up the PCR reaction to enrich for fragments with the proper adaptors is also optional (Figure 1).

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 2).

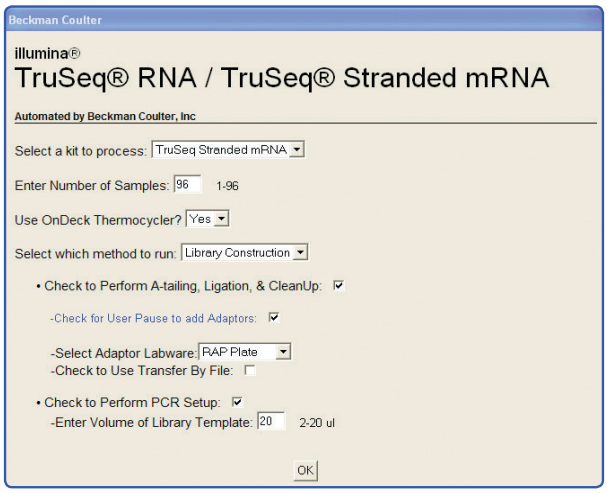


Figure 1. Intuitive user interface enables quick and flexible setup of runtime parameters

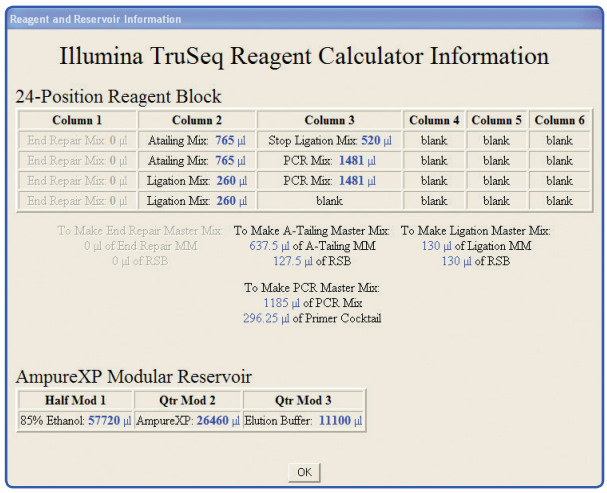


Figure 2. Required reagent volumes are automatically calculated and displayed to help users avoid errors.

Sample Layout

The RNA samples to be sequenced were placed in a 96 well plate configured as shown in Table 2 to assess cross contamination between wells. The library sequences from various species can be differentiated after sequencing using the individual index sequences added during the adaptor ligation step of library preparation. If cross contamination existed, we should be able to detect its presence by mapping the reads of a particular library against the reference genomes of libraries prepared in adjacent wells. Except for the tomato libraries in columns 11 and 12 of the library construction plate, all libraries contained 1ug of total RNA. Tomato libraries in columns 11 and 12 began library construction with 3ug of total RNA.

Table 2. RNA Template Sample Setup on a 96 Well Plate

SPECIES TYPE												
Row/ Column	1	2	3	4	5	6	7	8	9	10	11	12
A	Tomato	Tomato	Tomato	Tomato	Tomato	Human UHR	D. melanogaster	D. melanogaster	D. pulex	Tribolium	Tribolium	Tomato
B	Tomato	Tomato	Tomato	Tomato	Tomato	D. melanogaster	D. melanogaster	Human UHR	D. pulex	Tribolium	Human UHR	Tomato
C	Tomato	Tomato	Tomato	Tomato	Tomato	D. melanogaster	D. melanogaster	Enchenopa binotata	D. pulex	Tribolium	D. melanogaster	Tomato
D	Tomato	Tomato	Tomato	Tomato	Tomato	D. melanogaster	D. melanogaster	Umbonia crassicornis	Human UHR	Tribolium	D. melanogaster	Tomato
E	Tomato	Tomato	Tomato	Tomato	Tomato	D. melanogaster	D. melanogaster	Human UHR	D. pulex	Tribolium	Tomato	Tomato
F	Tomato	Tomato	Tomato	Tomato	Tomato	D. melanogaster	D. melanogaster	D. pulex	Tribolium	Human UHR	Tomato	Tomato
G	Tomato	Tomato	Tomato	Tomato	Tomato	D. melanogaster	D. melanogaster	D. pulex	Tribolium	Human UHR	Tomato	Tomato
H	Tomato	Tomato	Tomato	Tomato	Human UHR	D. melanogaster	Human UHR	D. pulex	Tribolium	Tribolium	Tomato	Tomato

Consistent Library Results

Total RNA from each sample shown in Table 2 was re-suspended in a final volume of 50 µL prior to starting the method. RNA fragmentation time was 7 minutes at 94°C. Illumina RAP plate adaptors were used during the course of library construction. The pre-enrichment library was eluted in a final volume of 20 µL, and 10 µL was used in the enrichment PCR, which was a 12 cycle amplification. Post-enrichment libraries were eluted in 25 µL of 10mM Tris, pH 8.5.

The adaptor-enriched libraries were analyzed for uniformity of size using the Agilent 2200 TapeStation. The results shown in Figure 3 illustrate very consistent size distribution across all 96 samples.

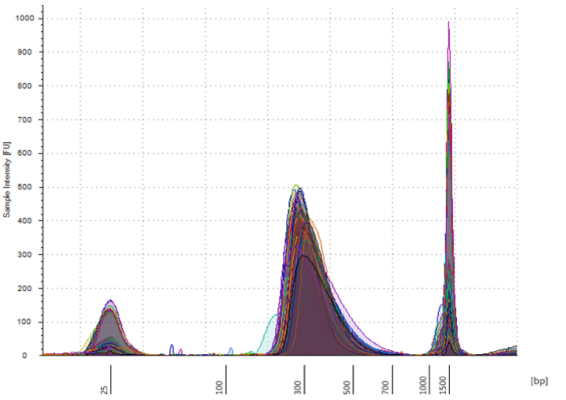


Figure 3. Agilent 2200 results for 96 stranded mRNA TruSeq libraries created on the Biomek FX^P

High Quality, Consistent Sequencing Reads

After generating the 96 libraries using the TruSeq Stranded mRNA Kit, 24 of them were sequenced on two lanes of an Illumina HiSeq 2500 instrument using a TruSeq Rapid SBS 200-cycle kit. The data was then analyzed by scientists at Illumina and the Center for Genomics and Bioinformatics. The HiSeq 2500 run was successful, generating 572,468,106 reads following trimming.

The base read counts (total number of reads assigned to a sample), base counts (total number of bases assigned to a sample), and the Q30 base counts (high quality base assignments) were then determined. As shown in Figure 4, the 24 sequenced libraries constructed using the Biomek FX^P provided highly consistent base read counts. Figure 5 illustrates that the base counts were also highly reproducible for the stranded mRNA libraries, as the majority of the reads consist of bases with Q scores higher than 30. Figure 6 outlines the total sequence yield for each library.

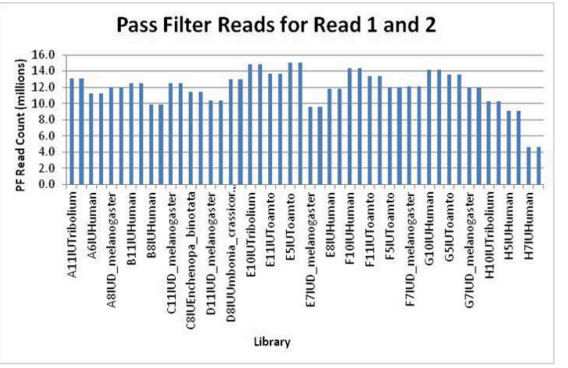


Figure 4. Pass Filter Reads for Read 1 and 2 of the Illumina HiSeq 2500 Run.

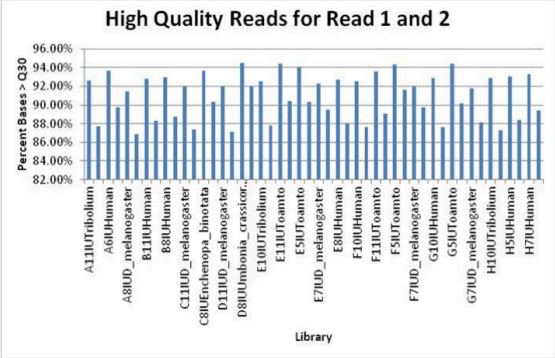


Figure 5. High quality reads for Read 1 and 2 of the Illumina HiSeq 2500 Run.

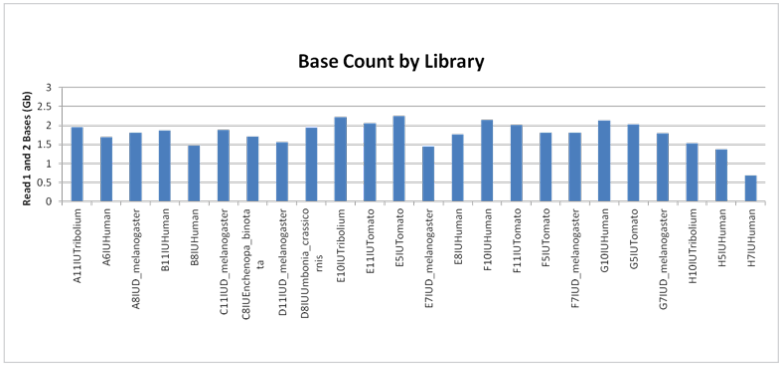


Figure 6. Total base counts for each library, combining Read 1 and Read 2 results

Highly Efficient Interrogation of the Transcriptome

The goal of every transcriptome sequencing project is to obtain results that provide the most information on all RNA transcripts, with a minimum of extraneous sequence information. The results from the human libraries constructed using the Biomek FX^P and the TruSeq Stranded mRNA Kit are highly targeted to the polyA transcriptome, with very low percentages of adapter dimers and sequence that do not align to the polyA transcriptome, or that align with ribosomal, 5s RNA, or mitochondrial sequences (Table 3).

Table 3. Key Sequencing Results Percentage (%) of PF Reads*

UHR LIBRARY	READ	% ALIGNED	% UNALIGNED	% HUMRIBOSOMAL	% ADAPTER DIMER
UHR2-1 ssRNA TruSeq Library	1	95.6%	4.4%	0.9%	0.2%
UHR2-2 ssRNA TruSeq Library	1	95.5%	4.5%	1.2%	0.3%
UHR2-3 ssRNA TruSeq Library	1	95.9%	4.1%	0.9%	0.1%
UHR2-4 ssRNA TruSeq Library	1	95.1%	4.9%	0.9%	0.3%
UHR2-5 ssRNA TruSeq Library	1	95.2%	4.8%	1.1%	0.2%
UHR2-7 ssRNA TruSeq Library	1	95.5%	4.5%	13.0%	0.2%
UHR2-8 ssRNA TruSeq Library	1	95.5%	4.5%	2.4%	0.2%
UHR2-9 ssRNA TruSeq Library	1	94.4%	5.6%	1.1%	0.5%
UHR2-1 ssRNA TruSeq Library	2	93.3%	6.7%	0.9%	0.2%
UHR2-2 ssRNA TruSeq Library	2	93.4%	6.6%	1.2%	0.3%
UHR2-3 ssRNA TruSeq Library	2	94.0%	6.0%	0.9%	0.1%
UHR2-4 ssRNA TruSeq Library	2	92.8%	7.2%	0.9%	0.3%
UHR2-5 ssRNA TruSeq Library	2	92.7%	7.3%	1.1%	0.2%
UHR2-7 ssRNA TruSeq Library	2	93.5%	6.6%	13.0%	0.2%
UHR2-8 ssRNA TruSeq Library	2	93.2%	6.9%	2.4%	0.2%
UHR2-9 ssRNA TruSeq Library	2	92.1%	7.9%	1.1%	0.6%

High Coverage Uniformity

On-target sequencing must also be efficient, with uniform coverage across the transcriptome, few duplicates and polyA only fragments, and a high percentage of correct strand reads. Uniform coverage is assessed by analyzing the coverage for 1000 genes, 5′ to 3′, and averaging the base counts per position. The median value and CV for each position across the length of each of the 1000 genes is calculated, and then all 1000 genes are plotted together and the median coefficient of variation (median CV in Table 4) is reported. The lower the median CV, the more even the coverage, and the less probability of 5′ or 3′ bias. Table 4 illustrates the coverage uniformity of the sequencing results for the human libraries, as well as low percentages of poly A-only fragments and duplicates. Duplicates in mRNA sequencing must be interpreted carefully, as one gene may have multiple transcripts or duplicates may be created during PCR at the adaptor enrichment step, and these can be resolved by paired end sequencing. The correct strand percentages are very high for the stranded mRNA libraries.

Table 4. Additional Sequencing Results

UHR LIBRARY	READ	POLYA (% OF PF READS)	DUPLICATES (%)	MEDIAN CV COVERAGE	CORRECT STRAND (% OF ALIGNED READS)	PF READ COUNT	PF BASES (GB)	PF BASES > Q30 (% OF PF BASES)
UHR2-1 ssRNA TruSeq Library	1	0.15%	1.53%	0.5242	99.28%	9,108,330	0.6831	93.06%
UHR2-2 ssRNA TruSeq Library	1	0.14%	1.35%	0.5105	99.43%	11,269,877	0.8452	93.60%
UHR2-3 ssRNA TruSeq Library	1	0.16%	1.46%	0.5347	99.39%	4,570,360	0.3428	93.32%
UHR2-4 ssRNA TruSeq Library	1	0.15%	1.45%	0.5195	99.36%	9,870,498	0.7403	92.95%
UHR2-5 ssRNA TruSeq Library	1	0.14%	1.62%	0.5217	99.38%	11,788,148	0.8841	92.72%
UHR2-7 ssRNA TruSeq Library	1	0.14%	1.79%	0.5364	99.52%	14,299,293	1.0724	92.51%
UHR2-8 ssRNA TruSeq Library	1	0.14%	1.56%	0.5174	99.45%	14,186,244	1.064	92.89%
UHR2-9 ssRNA TruSeq Library	1	0.14%	1.53%	0.5197	99.39%	12,493,267	0.937	92.80%
UHR2-1 ssRNA TruSeq Library	2	0.27%	1.53%	0.5242	99.28%	9,108,330	0.6831	88.38%
UHR2-2 ssRNA TruSeq Library	2	0.27%	1.35%	0.5105	99.43%	11,269,877	0.8452	89.71%
UHR2-3 ssRNA TruSeq Library	2	0.27%	1.46%	0.5347	99.39%	4,570,360	0.3428	89.44%
UHR2-4 ssRNA TruSeq Library	2	0.28%	1.45%	0.5195	99.36%	9,870,498	0.7403	88.75%
UHR2-5 ssRNA TruSeq Library	2	0.29%	1.62%	0.5217	99.38%	11,788,148	0.8841	88.09%
UHR2-7 ssRNA TruSeq Library	2	0.28%	1.79%	0.5364	99.52%	14,299,293	1.0724	87.62%
UHR2-8 ssRNA TruSeq Library	2	0.25%	1.56%	0.5174	99.45%	14,186,244	1.064	87.65%
UHR2-9 ssRNA TruSeq Library	2	0.30%	1.53%	0.5197	99.39%	12,493,267	0.937	88.30%

Minimal Cross Contamination

In order to assess cross contamination of the libraries that might have occurred during construction, the scientists at the Center for Genomics and Bioinformatics used Blastn to search the reads against all three genomes, keeping the best hit from each genome and only considering matches that covered at least 35bp. Any read that mapped to the expected reference from the list of contaminants was rejected, as well as any read that mapped to both potential contaminants.

The Biomek FX^{P} added reagents starting in Column 1 and moving across the plate from left to right until it reached the end of the samples (Table 2). If sample carry-over during library construction occurred, we would expect to observe tomato sequences in the human UHR2-1 library prepared in well H5 of the library construction plate, but no cross contamination in the samples in column 1. Following sequencing, reads from the sequenced libraries were mapped against the correct reference genome and the reference genomes of possible contaminant species using BowTie with an edit distance of 3. Reads were only assigned to a particular species if the reads mapped only to a single genome.

As can be seen in Table 5, the number of non-human reads in any of the human UHR libraries is quite low. The same holds true for the *Drosophila* libraries, in which we detect very few non-*Drosophila* reads. The high number of unassigned reads in the tomato, *Tribolium, Enchenopa,* and *Umbonia* libraries reflects the current state of reference genomes for these species, which are not as complete as those for human and *Drosophila*. Taken together, these results show that the libraries derived from the automated stranded mRNA method on the Biomek FXP show no discernible evidence of cross contamination during library construction.

Table 5. Mapping to Reference Genomes

LIBRARY PLATE WELL	SPECIES	TOTAL READS (ALL LANES, ALL READS)	REFERENCE GENOME MAPPING PERCENTAGES					
			Daphnia	Drosophila	H. sapiens	Tomato	Tribolium	unassigned
E7	Drosophila	19,214,090	0.000%	86.811%	0.026%	0.030%	0.016%	13.117%
F7	Drosophila	24,124,996	0.000%	84.878%	0.021%	0.022%	0.000%	15.078%
G7	Drosophila	23,859,338	0.000%	84.326%	0.020%	0.015%	0.000%	15.639%
A8	Drosophila	24,089,160	0.000%	83.621%	0.088%	0.002%	0.041%	16.248%
C11	Drosophila	25,074,808	0.000%	84.404%	0.014%	0.003%	0.033%	15.546%
D11	Drosophila	20,772,508	0.000%	84.682%	0.012%	0.003%	0.027%	15.276%
C8	Enchenopa	22,757,524	0.015%	0.109%	0.030%	0.008%	0.043%	99.796%
H5	Human	18,216,660	0.008%	0.001%	81.131%	0.005%	0.012%	18.844%
A6	Human	22,539,754	0.011%	0.006%	81.103%	0.003%	0.003%	18.875%
H7	Human	9,140,720	0.008%	0.005%	81.833%	0.002%	0.020%	18.132%
B8	Human	19,740,996	0.007%	0.002%	81.000%	0.002%	0.000%	18.989%
E8	Human	23,576,296	0.010%	0.022%	80.714%	0.049%	0.023%	19.183%
F10	Human	28,598,586	0.148%	0.009%	75.432%	0.052%	0.008%	24.351%
G10	Human	28,372,488	0.024%	0.008%	80.398%	0.015%	0.003%	19.553%
B11	Human	24,986,534	0.008%	0.003%	80.068%	0.004%	0.001%	19.915%
E5	Tomato	29,987,210	0.090%	0.006%	0.019%	73.578%	0.011%	26.296%
F5	Tomato	24,065,104	0.071%	0.008%	0.024%	76.216%	0.000%	23.680%
G5	Tomato	27,038,110	0.068%	0.007%	0.019%	76.141%	0.000%	23.764%
E11	Tomato	27,386,464	0.003%	0.019%	0.043%	48.448%	0.025%	51.463%
F11	Tomato	26,794,430	0.281%	0.019%	0.031%	60.697%	0.004%	38.968%
E10	Tribolium	29,664,318	0.025%	0.006%	0.062%	0.035%	74.705%	25.166%
H10	Tribolium	20,463,272	0.056%	0.002%	0.028%	0.011%	69.281%	30.622%
A11	Tribolium	26,114,234	0.009%	0.036%	0.021%	0.008%	73.260%	26.666%
D8	Umbonia	25,890,506	0.027%	0.156%	0.011%	0.006%	0.045%	99.756%

Conclusion

Reliable and efficient automation solutions for NGS library construction are essential in order 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 money and gives researchers valuable, worry-free walk-away time. The Biomek FX^{P} suite of automated methods creates TruSeq Stranded mRNA libraries that are highly consistent and that produce high quality sequencing data on Illumina sequencers, with no detectable cross contamination of template samples.

Notes

1. 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.

For Beckman Coulter's worldwide office locations and phone numbers, please visit www.beckmancoulter.com/contact

B2013-14332 IB-18319A www.beckmancoulter.com © 2013 Beckman Coulter, Inc. PRINTED IN U.S.A.

Automation of the Illumina® TruSeq® DNA PCR-Free Sample Preparation Kit on the Biomek FX^P Automated Liquid Handler

Abstract

In this technical note, we describe the automation of the Illumina TruSeq DNA PCR-Free Sample Preparation Kit on the Beckman Coulter BiomekFX^P Dual-Arm System, Multichannel Pipettor and Span-8 Pipettor automated liquid handler (Biomek FX^P). The Illumina PCR-Free automated method is designed to work with the Illumina TruSeq DNA PCR-Free Sample Preparation Kit (FC-121-3001, FC-121-3002 or FC-121-3003), and allows the user to create up to 96 individually barcoded DNA libraries per method run—using either the LT or HT versions of the Illumina TruSeq DNA PCR-Free Kit. As the name of the kit implies, the libraries generated with this method do not undergo PCR amplification, which reduces nucleic acid composition bias and eliminates the risk of artifacts introduced via PCR.

The method provides users with the option to utilize either off-deck incubations using an external thermocycler, or to perform incubations on-deck with a Biometra T-Robot thermocycler integrated to the Biomek FX^P liquid handler. The method employs individual Span-8 probes to deliver enzyme and reagent transfers while DNA cleanup, wash, and elution transfers are performed using the multichannel 96 pipetting head. A static Peltier unit ensures that enzyme master mixes are kept cool during the course of the method. The Illumina PCR-Free automated method can generate up to 96 Illumina TruSeq DNA PCR-Free libraries in approximately 5 hours. The method also incorporates all recommended stop points described in the Illumina TruSeq DNA PCR-Free protocol to allow users the maximum amount of flexibility in planning their experiments.

Protocol

This automated method utilizes the Biomek FX^P automated liquid handler in conjunction with the Illumina TruSeq DNA PCR-Free Sample Preparation Kit protocol (15036187 Rev. B). Automation consumables, instrument configuration, and other details can be found at the end of this document. An overview of the method workflow can be seen in Figure 1.

Biomek FX^P Workstation

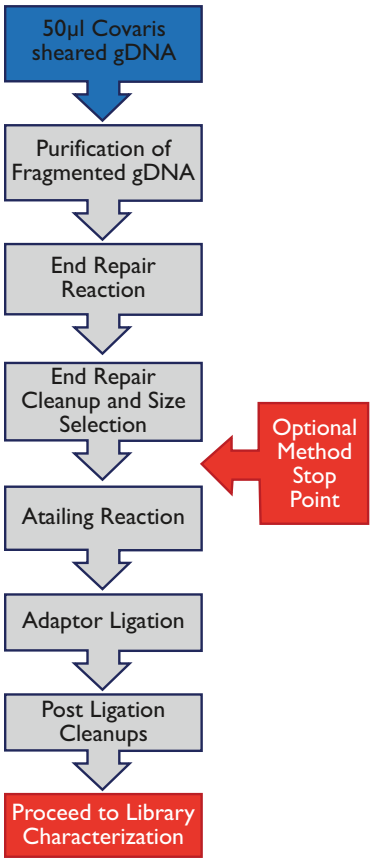


Fig. 1. Automated Illumina TruSeq DNA PCR-Free workflow. Blue indicates sample inputs; red indicates stop points; and grey indicates method steps.

BECKMAN COULTER
Life Sciences

The automated Illumina TruSeq DNA PCR-Free method utilizes an HTML-driven User Interface (UI) that allows the user to customize his workflow by offering a number of different options. Among the major options offered by the UI, the user may select: (1) any number of samples between 1 and 96 to process; (2) whether to use on-deck or off-deck incubations; (3) which size selection option (350 bp inserts or 550 bp inserts) to be used in the library construction procedure; and (4) how much of the library construction workflow to run. Several options are provided around the addition of the TruSeq adapters to the deck, including an optional pause step in the method to add the TruSeq adapter labware to the deck and the ability to perform custom TruSeq adapter transfers based on a user-supplied CSV-formatted file. Additionally, the user can specify the type of labware containing the TruSeq adapters including tubes, any number of 96-well PCR plate types, or the Illumina DAP plate supplied with the HT version of the Illumina TruSeq DNA PCR-Free Sample Preparation Kit. Finally, the UI allows the user to enable event logging—which records major events such as master mix additions—in a LIMS accessible file. An image of the UI is presented in Figure 2.

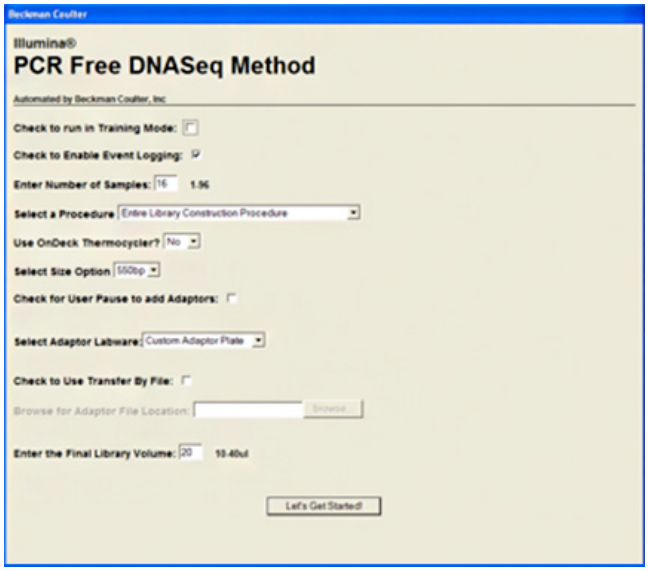


Fig. 2. Automated Illumina PCR-Free user interface.

In addition to the user interface, the automated Illumina PCR-Free method provides the user with an HTML-driven reagent calculator that provides the user with the final volumes of all of the reagents and master mixes required on the deck. It also gives instructions on how to generate the various master mixes based upon the number of samples to be processed and the amount of the workflow that the user wishes to pursue. An image of the reagent calculator is shown in Figure 3.

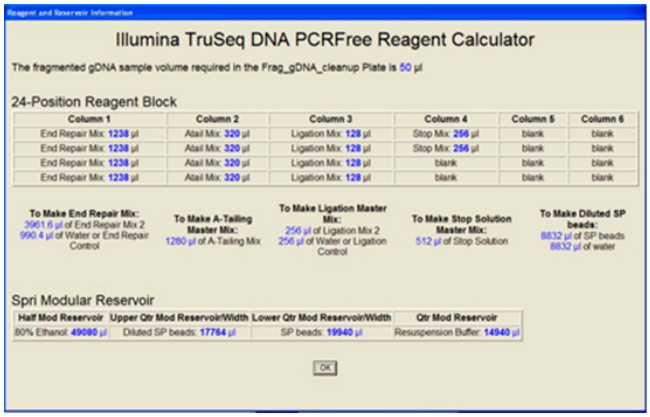


Fig. 3. Automated Illumina PCR-Free reagent calculator.

Experimental Design and Results

Eight 1 µg aliquots of Human Female Reference Genomic DNA (Promega P/N G1521) were sheared using a Covaris® S220 (Covaris) utilizing the settings outlined in the Illumina TruSeq DNA PCR-Free Library Preparation Kit protocol (15036187 Rev. B) for a 350 bp insert size. Following shearing, the input DNA was pooled into a single tube, vortexed, and assayed on the Agilent 2200 TapeStation (Agilent Technologies) using D1000 High-Sensitivity ScreenTape (P/N 5067-5584) to ensure the targeted insert size of 350 bp was reached (Figure 4).

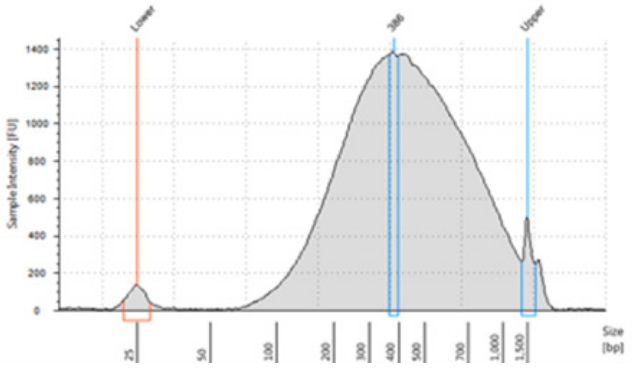


Fig. 4. Human Reference gDNA following shearing on the Covaris S220.

Following library construction, the quality and size distribution of the libraries was analyzed using the Agilent DNA High Sensitivity kit (5067-4626) and Agilent 2100 Bioanalyzer. Representative Bioanalyzer 2100 results are shown in Figure 5. The library concentrations were measured using KAPA SYBR Fast Universal 2X qPCR Master Mix library quantification kits (P/N KK4824, Kapa Biosystems), and are summarized in Figure 6.

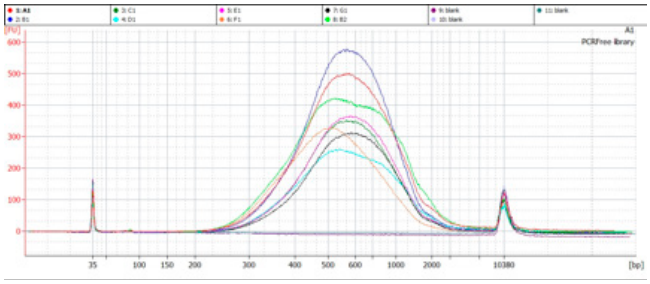


Fig. 5. Illumina TruSeq DNA PCR-Free libraries constructed with the Biomek FX[P]

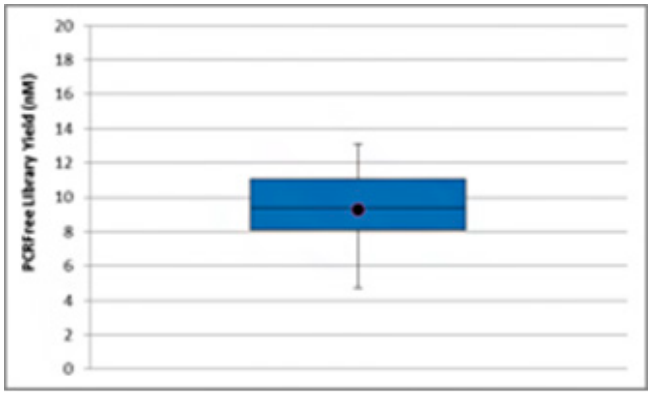


Fig. 6. Sequence-ready library yields as determined by qPCR. Mean library yield was 9.3 nM.

Seven Illumina TruSeq DNA PCR-Free libraries were sequenced on an Illumina MiSeq® 2x101 cycle paired end run, which generated over 9 million pass filter reads from the Illumina TruSeq DNA PCR-Free libraries. The reads were then mapped to the human reference genome (hg19) using Bowtie 2 mapping software implemented on the public instance of Galaxy (usegalaxy.org) using default parameters. Mapping metrics are presented in Figure 7. Following mapping, insert size distributions were calculated for each of the 7 libraries using the Picard Insertion Size Metrics tool, also installed on the public instance of Galaxy. Median size insert values for the 7 libraries were 386±6 bp. Standard deviations for the 7 libraries were 86±2 bp. A representative size insert distribution is presented in Figure 8.

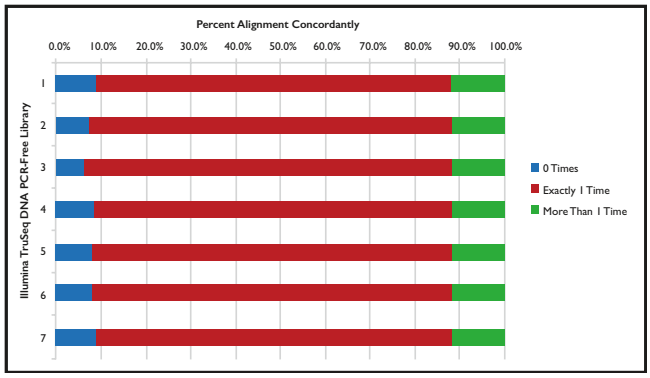


Fig. 7. Bowtie 2 mapping results for Illumina TruSeq DNA PCR-Free libraries.

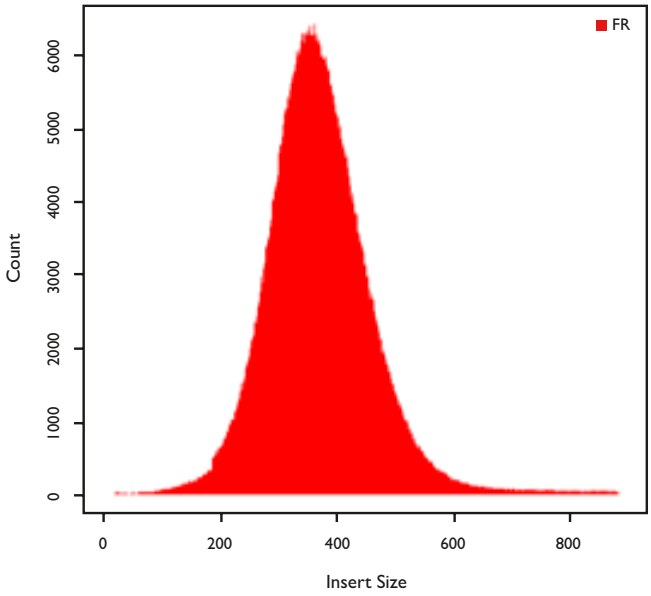


Fig. 8. Size insert distribution calculated using Picard Insertion Size Metrics tool for Illumina TruSeq DNA PCR-Free library[I]

Conclusion

Illumina TruSeq PCR-Free libraries migrate later in automated electrophoresis platforms, such as the Agilent 2100 Bioanalyzer, due to the structure of the Illumina TruSeq adapters. Our results, shown in Figure 3, reflect this fact and are consistent with similar Agilent 2100 Bioanalyzer data presented in the Illumina TruSeq DNA PCR-Free protocol. qPCR validation of Illumina TruSeq DNA PCR-Free libraries created using the Biomek FX^P, presented in Figure 4, shows that the yield of sequence-ready libraries was more than double the 2 nM minimum amount required by the Illumina TruSeq DNA PCR-Free protocol.

In summation, using the Beckman Coulter Biomek FX^P, in conjunction with the Illumina TruSeq DNA PCR-Free Library Preparation Kit, results in sequence-ready, amplification-free DNASeq libraries appropriate for sequencing on all Illumina sequencing platforms.

Deck configuration of the Biomek FXP Dual-Arm Multi-96 and Span 8 Liquid Handler.

ALPs/Devices Required			
Part Number	**Qty.**	**Manufacturer**	**Description**
719948	1	Beckman Coulter, Inc.	ALP, High-Density, 12-Position, 4 x 3
379448	1	Beckman Coulter, Inc.	ALP, Shaking, Orbital, Single-Position
719357	2	Beckman Coulter, Inc.	ALP, Standard Single-Position
719361	1	Beckman Coulter, Inc.	ALP, Cooling/Heating, Single-Position
A93942	1	Beckman Coulter, Inc.	Shaking Peltier ALP
719590	1	Beckman Coulter, Inc.	Waste, Span-8, ALP
719356	1	Beckman Coulter, Inc.	Disposable Tip Loader ALP
719654	1	Beckman Coulter, Inc.	ALP, Tip Wash, 8-Channel
719363	1	Beckman Coulter, Inc.	Wash Station including Pump and Tubes
719366	1	Beckman Coulter, Inc.	Biomek FX Device Controller
Request a Quote	1	Beckman Coulter, Inc.	(*Optional*) Biometra T-Robot for On-Deck Incubations

Auxiliary Equipment for QC Testing		
Part Number	**Manufacturer**	**Description**
G2940CA	Agilent Technology	Agilent 2100 Bioanalyzer
5067-4626	Agilent Technology	High-Sensitivity DNA Kit
4351405	Life Technologies	7900HT Fast Real-Time PCR System with Fast 96-Well Block Module
KK4824	Kapa Biosystems	Library Quantification Kit—Illumina/Universal
SY-410-1003	Illumina	MiSeq System

Labware Required			
Part Number	**Qty.**	**Manufacturer**	**Description**
B01124	1	Beckman Coulter, Inc.	Biomek Span-8 P1000 Tips, Pre-Sterile with Barrier
379503	1	Beckman Coulter, Inc.	Biomek Span-8 P250 Tips, Pre-Sterile with Barrier
A21586	3	Beckman Coulter, Inc.	Biomek P50 Tips, Pre-Sterile with Barrier
717253	1	Beckman Coulter, Inc.	Biomek AP96 P250 Tips, Pre-Sterile with Barrier
372790	1	Beckman Coulter, Inc.	Quarter Reservoir
534681	1	Beckman Coulter, Inc.	Half Reservoir, Nonpyrogenic
372792	1	Beckman Coulter, Inc.	Quarter Reservoir, Divided by Width
372795	1	Beckman Coulter, Inc.	Frame for Reservoirs
A32782	1	Beckman Coulter, Inc.	Agencourt SPRIPlate 96R—Ring Super Magnet Plate
A83054	1	Beckman Coulter, Inc.	Blue Heater/Chiller 24-Well Block
AB-1127	5	Thermo Scientific	ABgene 96-Well Storage Plate, Square Well, 1.2 mL
16466-042	14	VWR	2 mL SuperClear® Screw Cap Microcentrifuge Tubes—Conical Bottom
HSP-9641	4	Bio-Rad	Hard-Shell® Thin-Wall 96-Well Skirted PCR Plates
MSL-2022	1	Bio-Rad	Arched Auto-Sealing Lids*

* Optional: For on-deck thermocycling only.

User-Supplied Consumables		
Part Number	**Manufacturer**	**Description**
AB00138-01000	American Bioanalytical	Ethanol
FC-121-3001 or FC-121-3002 or FC-121-3003	Illumina	Illumina TruSeq DNA PCR-Free Sample Prep LT Set A (24 samples); Illumina TruSeq DNA PCR-Free Sample Prep LT Set B (24 samples); or Illumina TruSeq DNA PCR-Free Sample Prep HT (96 samples)

Software Used in QC Testing

Bowtie 2 (version 0.2) installed on Galaxy Main (usegalaxy.org)

Picard Insertion Size Metrics tool (version 1.56) installed on Galaxy Main (usegalaxy.org)

Author

Zach Smith, M.S.
Beckman Coulter Life Sciences, Indianapolis, IN USA

References

1. The PCR process is covered by patents owned by Roche Molecular Systems, Inc. and F. Hoffman La Roche, Ltd.

Galaxy Citations

2. Goecks J, Nekrutenko A, Taylor J and The Galaxy Team. Galaxy: a comprehensive approach for supporting accessible, reproducible, and transparent computational research in the life sciences. *Genome Biol*. 11(8); R86: (Aug 25 2010).

3. Blankenberg D, Von Kuster G, Coraor N, Ananda G, Lazarus R, Mangan M, Nekrutenko A, Taylor J. Galaxy: a web-based genome analysis tool for experimentalists. *Current Protocols in Molecular Biology.* Chapter 19: Unit 19.10.1–21: (Jan 2010).

4. Giardine B, Riemer C, Hardison RC, Burhans R, Elnitski L, Shah P, Zhang Y, Blankenberg D, Albert I, Taylor J, Miller W, Kent WJ, Nekrutenko A. Galaxy: a platform for interactive large-scale genome analysis. *Genome Research.* 15(10); 1451–5: (Oct 2005).

BECKMAN COULTER
Life Sciences

For Beckman Coulter's worldwide office locations and phone numbers, please visit "Contact Us" at www.beckmancoulter.com

AAG-313TCH07.14-A

Automated Directional RNASeq Library Preparation using the Biomek FX^P Liquid Handler.

Zach Smith, M.S. Beckman Coulter, Inc. | Daniela Munafo, Ph.D. New England Biolabs, Inc. Ipswich, MA USA

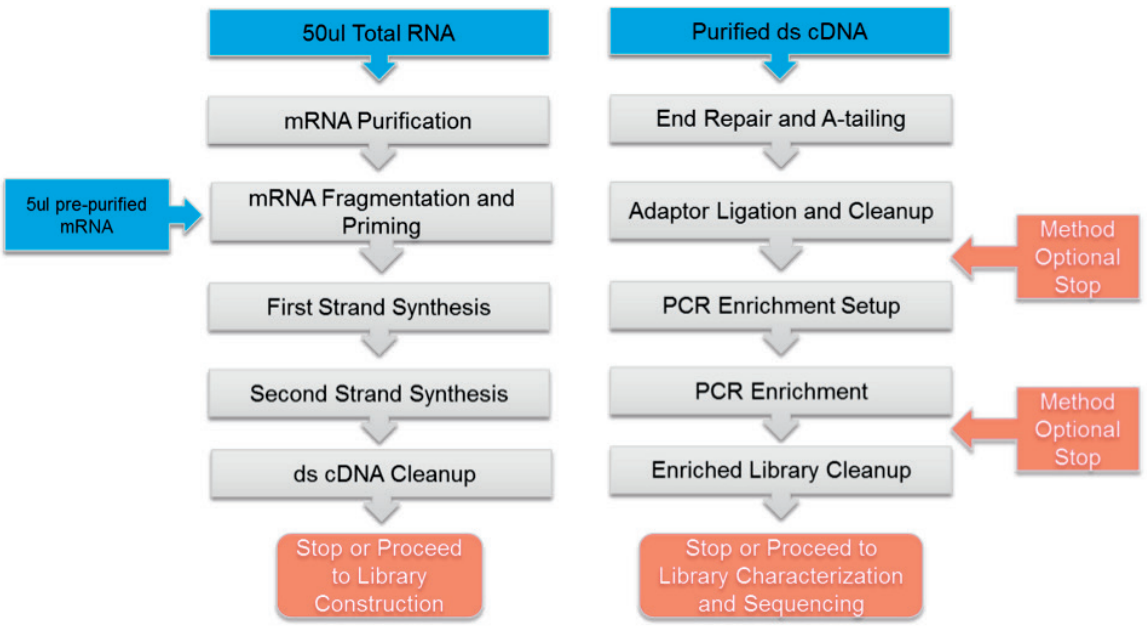
Introduction

The purpose of this tech-note is to describe the automation of the New England Biolabs NEBNext (Registered trademark) Ultra Directional RNA Library Prep Kit for Illumina on the Beckman Coulter Biomek FXP automated liquid handler. The Biomek NEB Ultra Directional RNA Method was designed to work with NEBNext Ultra Directional RNA Library Prep Kit for Illumina protocol (Catalog Number E7420S/L), and provides a solution for simultaneously constructing up to 96 individually bar-coded directional RNASeq libraries per method run.

The method provides users with the option to utilize either off-deck incubations using an external thermocycler, or to perform incubations on-deck with a Biometra TRobot thermocycler integrated to the Biomek liquid handler. The method employs individual Span-8 probes to deliver enzyme and reagent transfers while DNA cleanup, wash, and elution transfers are performed using the multi-channel 96 pipetting head. A static Peltier unit ensures that enzyme master mixes are kept cool during the course of the method.

The data presented below includes a comparison of library construction using the Biomek FX^P method at 25ng and 100ng total RNA inputs. Automated sample preparation allows for the simultaneous construction of up to 96 directional RNASeq libraries in about 9.5 hours. For convenience, this method can be run with in a single nine and one-half hour run, or as a two day run, with day 1 consisting of sample preparation through cDNA synthesis and day 2 consisting of the sample preparation through the end of library construction. Figure 1 shows the workflow of the NEBNext Ultra Directional RNA Library Prep Kit for Illumina protocol.

Figure 1. Automation Workflow. Blue indicates method inputs, red indicates potential stop points, and grey indicates method steps



BECKMAN COULTER Life Sciences

NEW ENGLAND BioLabs Inc.

AAG-161TCH07.14-A

Materials and Methods

The data was generated using the materials and methods described in the field installation guide.

Results and Discussion

25ng and 100ng Universal Human Reference (UHR) RNA (Agilent Technologies) was used as input for the New England Biolabs NEBNext Ultra Directional RNA Library Prep Kit for Illumina on the Beckman Coulter Biomek FX^{P} automated liquid handler. UHR RNA was assayed on the Agilent Bioanalyzer 2100 using an RNA6000 Pico kit (PN# 5067-1513) prior to the run to ensure high sample quality (data not shown). The purified adapter ligated library was amplified to 15 cycles per instruction of the New England Biolabs NEBNext Ultra Directional RNA Library Prep Kit for Illumina protocol using the Bio-Rad S1000 Thermal Cycler (Bio-Rad., Inc).

The quality and size distribution of the library was analyzed using the Agilent DNA High Sensitivity kit (PN# 5067-4626) and Agilent Bioanalyzer 2100. Representative Bioanalyzer 2100 results are shown in Figure 2. The library concentrations were measured using KAPA SYBR Fast Universal 2X qPCR Master Mix library quantification kits (PN# KK4824, KAPA Biosystems), and are summarized in Figure 3.

Figure 2. 25ng UHR RNA (left) and 100ng UHR RNA (right) NEBNext Ultra Directional RNA libraries for Illumina sequencing.

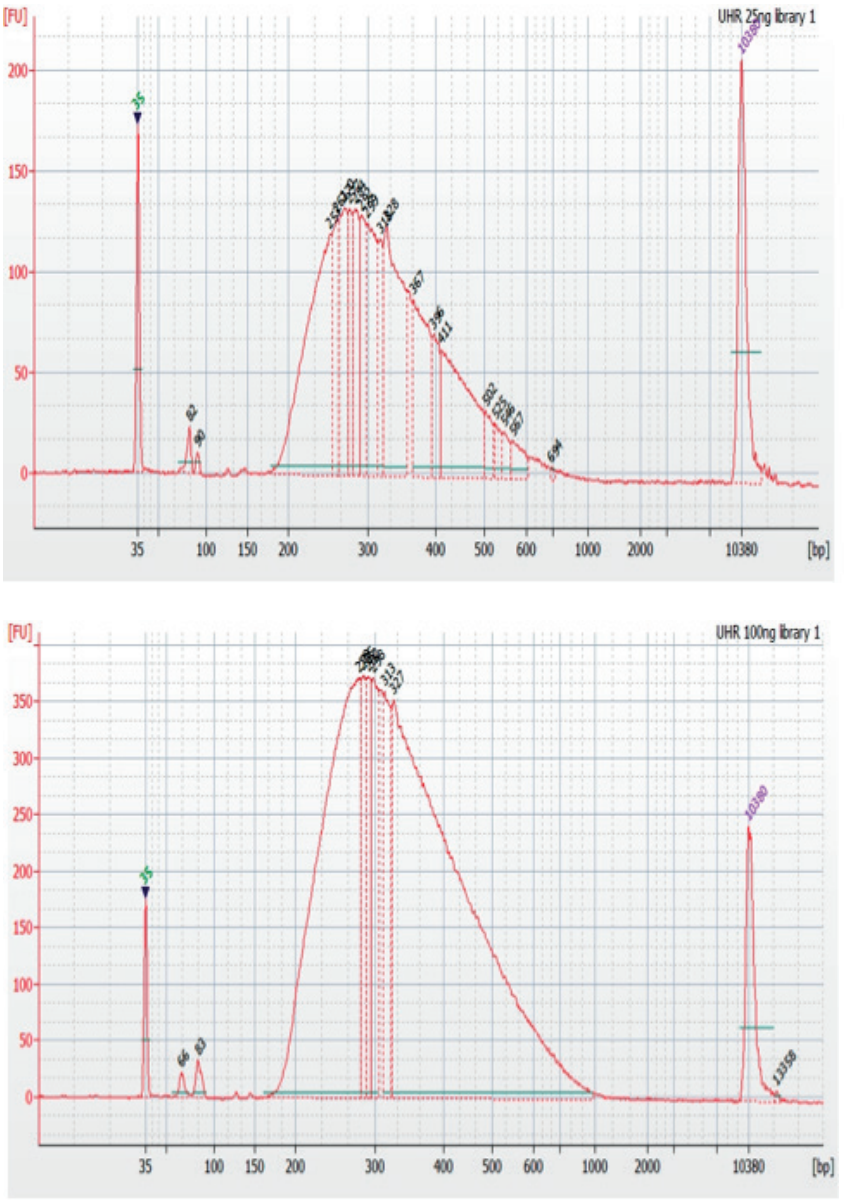
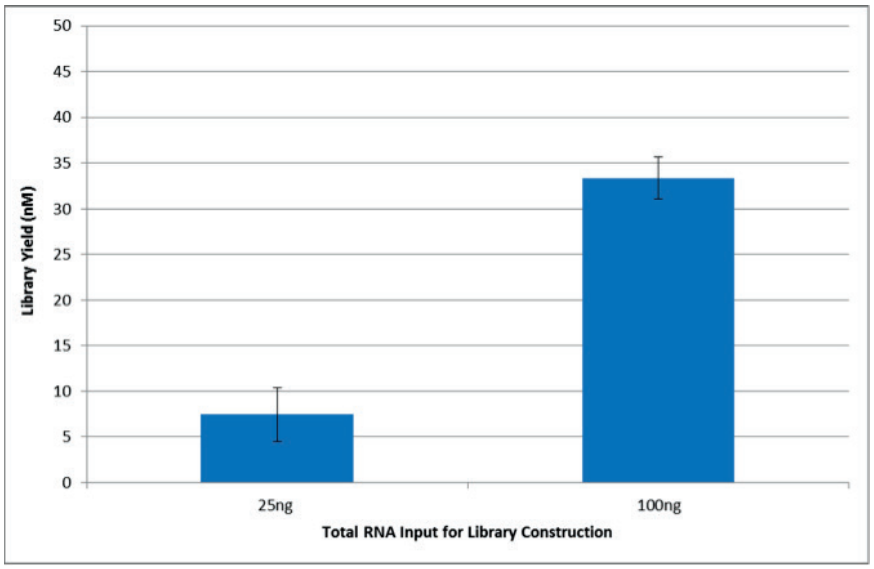
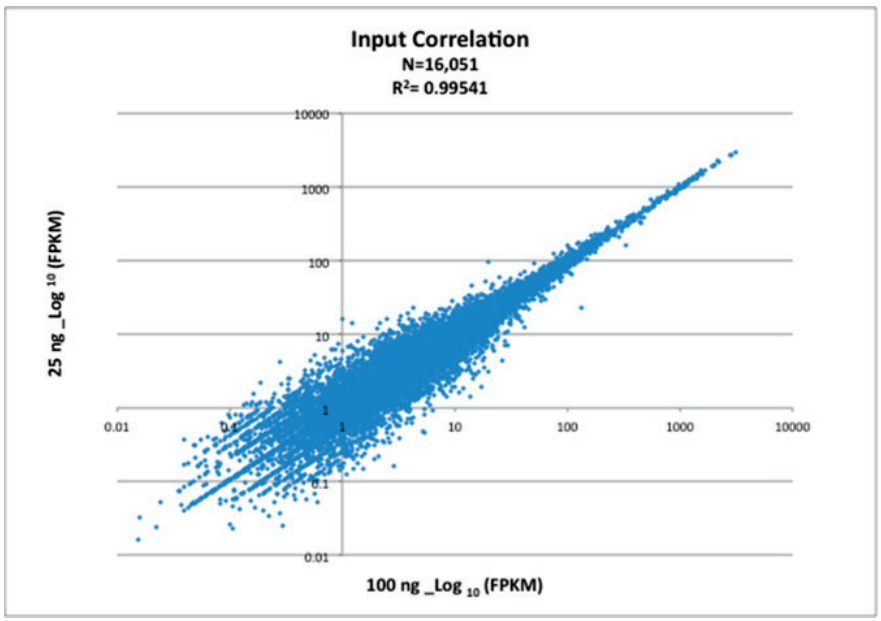


Figure 3. qPCR quantification of 25ng UHR RNA (left) and 100ng UHR RNA (right) NEBNext Ultra Directional RNA libraries for Illumina sequencing. Error bars indicate standard deviation.



Libraries were sequenced at New England Biolabs using a MiSeq 2x100 cycle paired end sequencing run. Reads were assayed using FastQC and aligned to the reference genome (HG19 build) using Tophat2. Following mapping, Cufflinks was used to measure transcript abundances in terms of fragments per kilobase of exon per million fragments mapped (FPKM). Bioinformatics analysis was performed at New England Biolabs using a local instance of Galaxy (https://usegalaxy.org). As shown in Figure 4, there is a high degree of correlation between 25ng and 100ng total RNA input libraries.



Conclusion

For optimal sequencing results, high quality RNA should be used for any RNASeq experiment.

The use of Beckman Coulter automation solutions in conjunction with the New England Biolabs NEBNext Ultra Directional RNA Library Prep Kit for Illumina allows for the construction of high quality, reproducible directional RNASeq libraries from limiting sample inputs.

Automation Labware

PART NUMBER	MANUFACTURER	DESCRIPTION	QUANTITY REQUIRED FOR 96 SAMPLES
B01124	Beckman Coulter, Inc.	Biomek Span-8 P1000 Tips, Pre-sterile with Barrier	2
379503	Beckman Coulter, Inc.	Biomek Span-8 P250 Tips, Pre-sterile with Barrier	3
A21586	Beckman Coulter, Inc.	Biomek P50 Tips, Pre-sterile with Barrier	5
717256	Beckman Coulter, Inc.	Biomek AP96 P20 Tips, Pre-sterile with Barrier	2
717253	Beckman Coulter, Inc.	Biomek AP96 P250 Tips, Pre-sterile with Barrier	4
372790	Beckman Coulter, Inc.	Quarter Reservoir	6
534681	Beckman Coulter, Inc.	Reservoir, Half	3
372795	Beckman Coulter, Inc.	Frame for Reservoirs‡	2
A32782	Beckman Coulter, Inc.	Agencourt® SPRIPlate® 96R - Ring Super Magnet Plate‡	1
A83054	Beckman Coulter, Inc.	BCI Tube Block‡	1
C5064	Acme Automation	ReactorAdaptor96Flat‡**	1
AB-1127	Fisher Scientific	Abgene 96-Well Storage Plate, Square Well, 1.2 mL	5
16466-042	VWR	2mL SuperClear™ Screw Cap Microcentrifuge Tubes- Conical Bottom	16 tubes
HSP-9641	Bio-Rad	Hard-Shell® Thin-Wall 96-Well Skirted PCR Plates	8
MSL-2022	Bio-Rad	Arched Auto-Sealing Lids*	1

‡One Time purchase
* For on-deck thermocycling only
** For on-deck Adaptor_Plate only

Biomek ALPs

PART NUMBER	MANUFACTURER	DESCRIPTION
719654	Beckman Coulter, Inc.	Span-8 Wash ALP
719363	Beckman Coulter, Inc.	Multichannel Wash Station including pump and tubing
379448	Beckman Coulter, Inc.	Orbital Shaker ALP, Single Position
719590	Beckman Coulter, Inc.	Span-8 Disposal ALP
719357	Beckman Coulter, Inc.	Static 1x1 ALP Platform
719361	Beckman Coulter, Inc.	Static Peltier ALP
719948	Beckman Coulter, Inc.	4x3 ALP kit
719366	Beckman Coulter, Inc.	Biomek FX Device Controller

Auxiliary Equipment

PART NUMBER	MANUFACTURER	DESCRIPTION
G2940CA	Agilent Technology	Agilent 2100 Bioanalyzer
5067-4626	Agilent Technology	High Sensitivity DNA kit

User-Supplied Reagents

PART NUMBER	MANUFACTURER	DESCRIPTION
Not available	User Preferred	Elution Buffer – Nuclease free water, TE, 10mM Tris, pH 8.5
A63881	Beckman Coulter, Inc.	AmpureXP
AB00138-01000	American Bioanalytical	Ethanol
E7420S (24 rxns) or E7420L (96 rxns)	New England Biolabs	NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina®
E7335L (Set 1, 96 rxns) or E7500L (Set2, 96 rxns)	New England Biolabs	NEBNext® Multiplex Oligos for Illumina® (Index Primers Set 1 or 2
E7490L (96 rxns)	New England Biolabs	NEBNext Poly(A) mRNA Magnetic Isolation Module
A1410	Sigma	Actinomycin D (dissolved in dimethylsulfoxide [DMSO] to 5 µg/µl)

***Note:** unless otherwise indicated, the recommended manufacturer is Beckman Coulter.

BECKMAN COULTER
Life Sciences

For Beckman Coulter's worldwide office locations and phone numbers, please visit "Contact Us" at www.beckmancoulter.com

AAG-161TCH07.14-A

NEW ENGLAND BioLabs Inc.

Illumina TruSight® HLA Sequencing Panel Automated on the Biomek FX^P HLA SP Liquid Handler

Zach Smith, MS, Senior Applications Scientist, Beckman Coulter, Inc.

Nate Baird, PhD, Scientist, Illumina

Brad Baas, PhD, Senior Scientist, Illumina

Introduction

The Human Leukocyte Antigen (HLA) system consists of a collection of 21 genes located on chromosome 6 that display high levels of polymorphism, resulting in over 12,500 HLA alleles characterized in the IPD-IMGT/HLA database as of January 2015.[1] The variability observed in the HLA system allows the immune system to discriminate between host cells, non-host cells, infected cells, and malfunctioning cells. In addition, certain HLA alleles have been shown to be associated with a number of auto-immune disorders including Type 1 Diabetes, rheumatoid arthritis, and inflammatory bowel diseases[2] as well as certain cancers.[3] Therefore, HLA typing has a number of valuable applications, including reducing the risk of transplant rejection by matching transplant recipients with donors that are close HLA matches in addition to providing a potential diagnostic tool for assessing the risk of certain HLA-associated disorders.

TruSight® HLA Sequencing Panel

In this technical note, we describe the automation of the Illumina TruSight® HLA Sequencing Panel on the Beckman Coulter Biomek FX^P HLA SP. An application specific configuration of the Biomek FX^P Dual Mutli-96 Span-8 automated liquid handler (Figure 8). DNA libraries prepared for next-generation sequencing (NGS) using the TruSight® HLA Sequencing Panel enable high-resolution, unambiguous HLA typing with a single assay. Briefly, three Class I HLA loci (HLA-A, -B, and -C) as well as eight Class II HLA loci (HLA-DPA1, -DPB1, -DQA1, -DQB1, and -DRB1/3/4/5) are amplified using locus specific primers. These amplicons, which range from 2.6kb to 10.3kb in size, are normalized, tagmented, and amplified into uniquely indexed libraries for each locus in each genomic DNA sample. Finished libraries are then pooled and loaded onto the Illumina MiSeq® system for sequencing, with the option of using either a single MiSeq® v2 standard flow cell or MiSeq® v2 Nano flow cell, depending on the number of samples per run.

The Biomek FX^P TruSight® HLA automation method standardizes library preparation and significantly reduces manual labor. Up to 192 Illumina TruSight® HLA libraries can be generated from 24 genomic DNA samples in approximately 11 hours. By limiting the need for user interactions, the automated method reduces the incidence of costly pipetting errors and provides for more uniform sample processing. The resulting library pool is ready to load directly onto the MiSeq® Reagent Cartridge while advanced data handling features speed the preparation of the required Sample Sheet by reporting out the indexing i5 and i7 primers associated with each library.

Biomek FXP Dual- Arm Multi-96 and Span-8 Liquid Handler

BECKMAN COULTER Life Sciences | illumina®

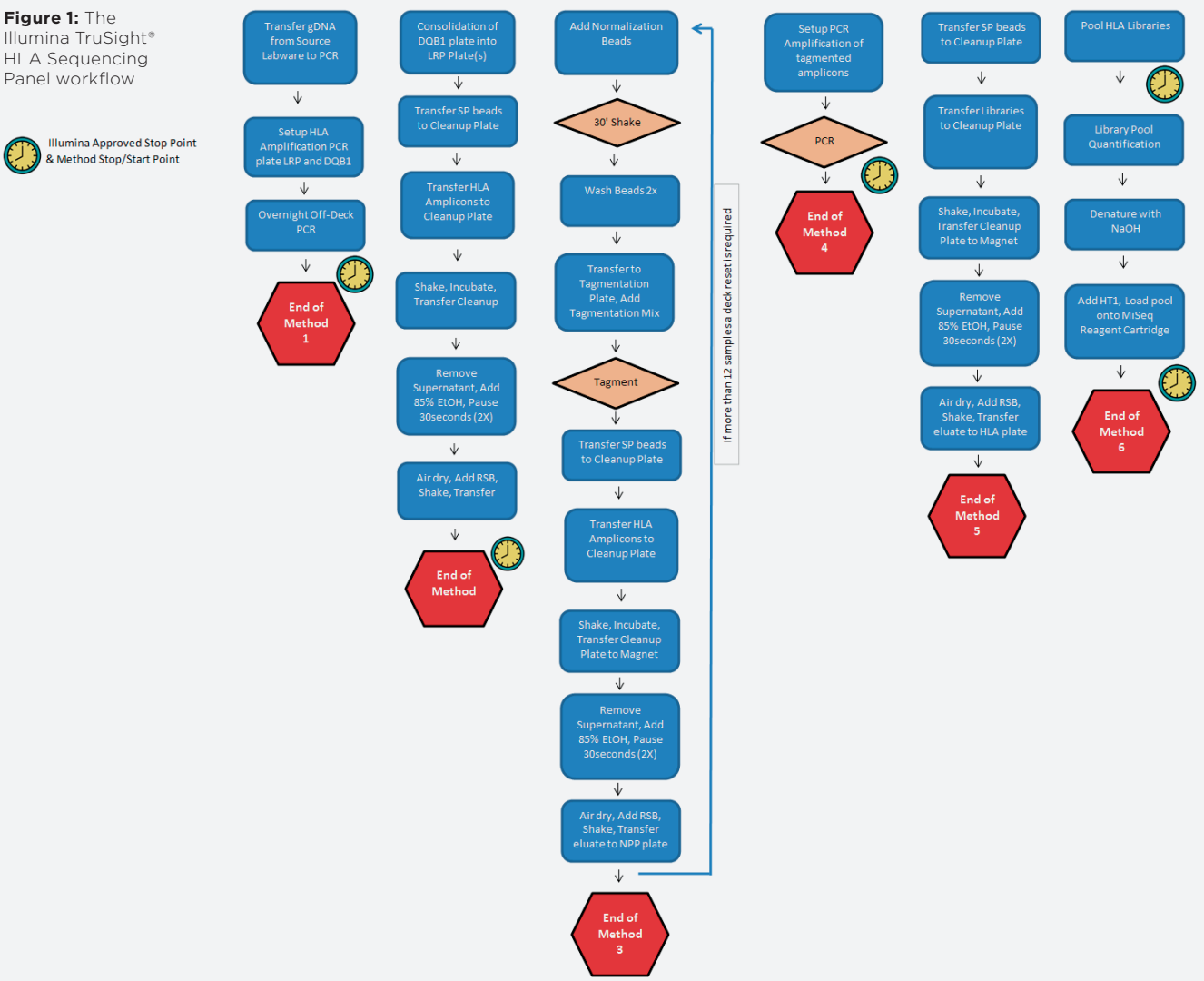
AAG-814TCH02.15-A

For added convenience, the Biomek FX^P TruSight® HLA automation method is preconfigured to employ fully skirted PCR plates or non-skirted PCR plates to accommodate a range of off-deck thermocyclers. For greater hands-off time, the method also includes the option of utilizing an integrated Biometra TRobot thermocycler for on-deck incubations and thermocycling. The method employs individual Span-8 probes to deliver enzyme and reagent transfers while steps involved in sample cleanups using Sample Preparation Beads, are performed using the multi-channel 96 pipetting head. This provides significant time and consumables savings. On-deck peltier units or chill-blocks ensure automation plates are kept cool during master mix transfers. The method also incorporates all recommended stop points described in the Illumina TruSight® HLA Sequencing Panel Library Preparation Guide to allow users the maximum amount of flexibility in planning their experiments.

Protocol

The Biomek FX^P TruSight® HLA automation method utilizes the Beckman Coulter Biomek FX^P HLA SP (hereafter Biomek FX^P) in conjunction with the Illumina TruSight® HLA Sequencing Panel Library Reference Guide (15056536 Rev. A). Automation consumables, instrument configuration, and other details can be found at the end of this document. The automation method consists of six sub-methods operated by a single HTML-driven User Interface (UI). The Illumina TruSight® HLA Sequencing Panel workflow can be seen in Figure 1. The automation workflow can be seen in Figure 2.

Figure 1: The Illumina TruSight® HLA Sequencing Panel workflow



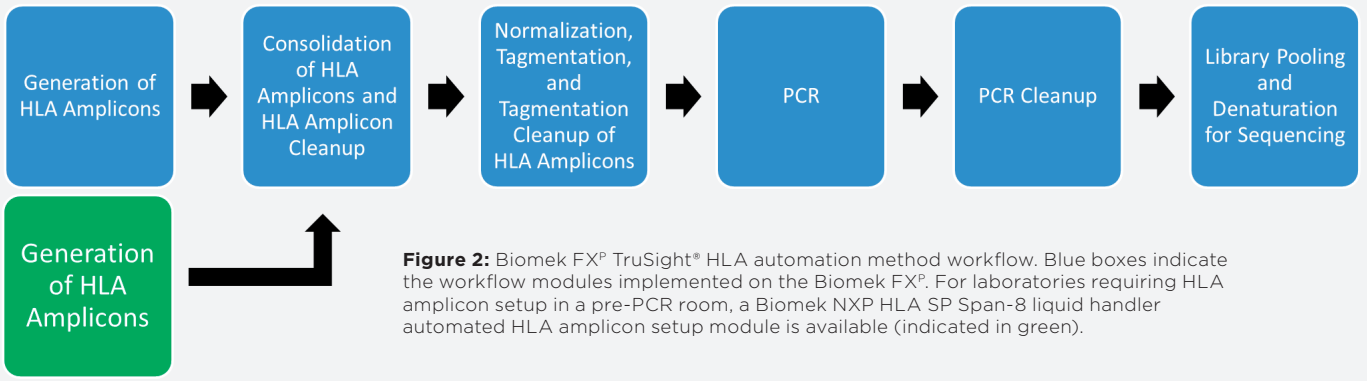


Figure 2: Biomek FX^P TruSight® HLA automation method workflow. Blue boxes indicate the workflow modules implemented on the Biomek FX^P. For laboratories requiring HLA amplicon setup in a pre-PCR room, a Biomek NXP HLA SP Span-8 liquid handler automated HLA amplicon setup module is available (indicated in green).

The automation method utilizes an HTML-driven UI that offers users a number of different options for customizing their workflow (Figure 3). For scheduling flexibility, the UI also allows the user to select between any one of the six sub-methods that make up the automation method. In each sub-method, the user can select to process any number of genomic DNA samples between 1 and 24. Depending on the sub-method selected and the number of genomic DNA samples being processed, the UI will update to include features specific to the sub-method automatically. For example, when the PCR sub-method is selected, the user is prompted to select which Nextera® XT v2 primer sets (A, B, C, or D) will be used for each Nextera® PCR Plate (NPP). This information is then used not only to populate the HTML-driven reagent calculator, but also to generate two .csv files that record which i5/i7 primer combinations were delivered to each well of the NPP plates. These files can then be used to generate the Sample Sheet for the sequencing run in Illumina Experiment Manager. The PCR sub-method also gives the user the option to perform the library amplification using an on-deck TRobot thermocycler or to use an off-deck thermocycler. Another sub-method with multiple options in the UI can be found in the Normalization, Tagmentation, and Tagmentation Cleanup sub-method, where the tagmentation incubation can be performed either on an on-deck TRobot thermocycler or an off-deck thermocycler. Finally, the Library Pooling sub-method offers the user the choice of whether to pool the libraries or to pool, dilute, and denature the pooled libraries. The number of library pools is defined by the type of MiSeq® flow cell being used (either Version 2 Standard or Version 2 Nano) as outlined in the Illumina TruSight® HLA Sequencing Panel Library Reference Guide. If the user chooses to dilute and denature the resulting library pool(s), the resulting denatured library pool(s) can be loaded directly onto the MiSeq® Reagent Cartridge.

In addition to the UI, the automation method provides an HTML-driven Reagent Calculator (Figure 4) that provides the user with the final volumes of all reagents and master mixes required on the deck as well as instructions on how to generate the various master mixes based upon the number of samples to be processed and which sub-method is selected.

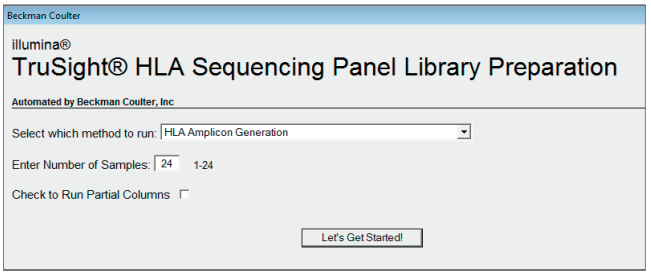


Figure 3: Biomek FX^P TruSight® HLA automation method UI.

Experimental Design and Results

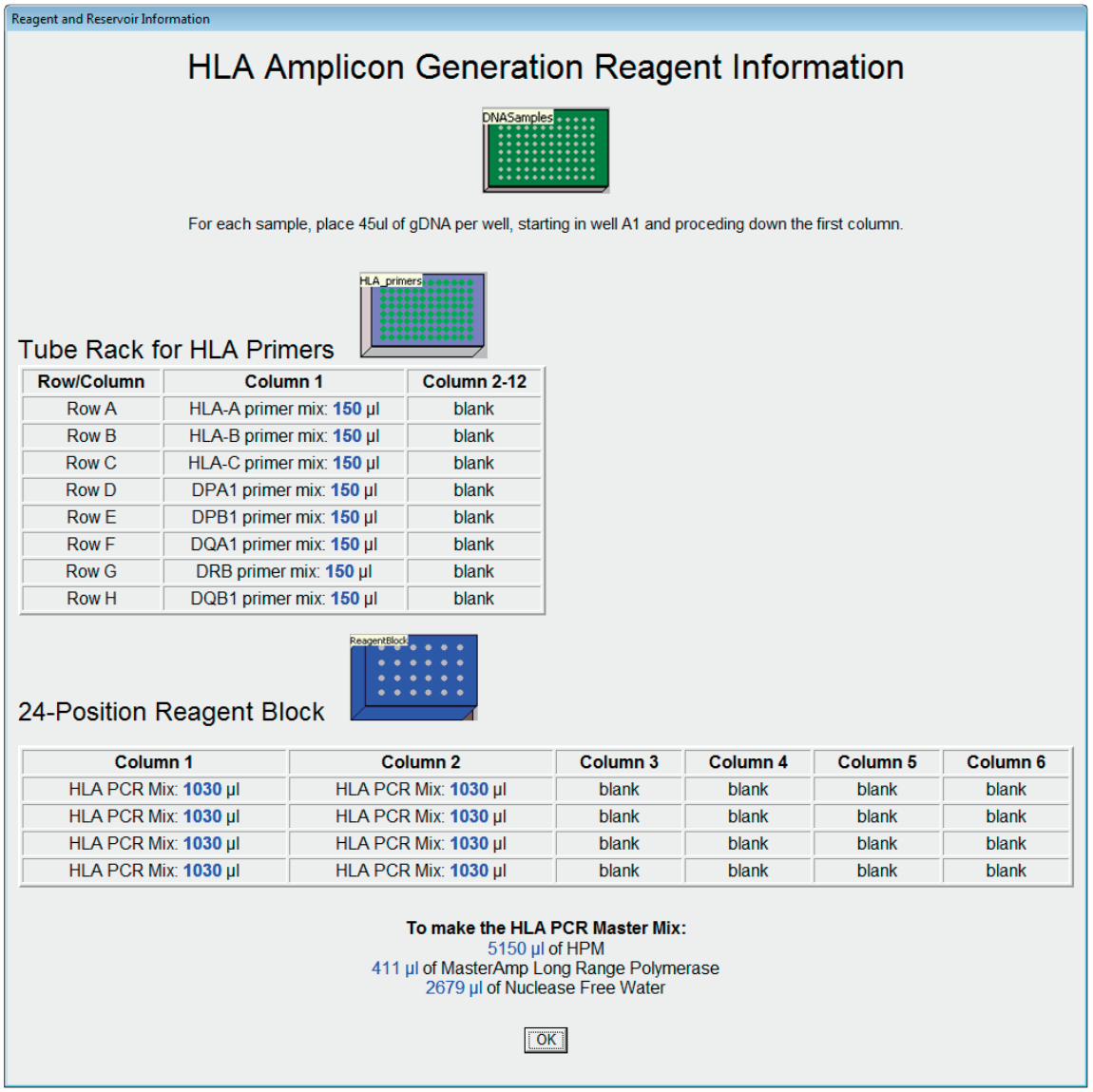
Twenty-three genomic DNA samples obtained from the International Histocompatibility Working Group (IHWG)[4] were quantified using Quant-iT™ Picogreen (Life Technologies), normalized to a final concentration of 10ng/µl, and arrayed in a 96 well BioRad Hard-Shell PCR plate (BioRad). The HLA amplification reactions were set up with the Biomek FX^P using the HLA Amplification sub-method and the HLA amplification reactions were performed in BioRad S1000 thermocyclers. A negative

control sample, ultrapure water, was also included. Following amplification cleanup using the using the HLA Cleanup sub-method, selected samples were assayed using the Bioanalyzer 2100 with a DNA 12000 chip (Agilent), the results of which are presented in Figure 5. The purified HLA amplicons were stored overnight at 4˚C.

Using the Normalization, Tagmentation, and Tagmentation Cleanup sub-method in conjunction with the PCR sub-method, the HLA amplicons were normalized, tagmented, cleaned, and used as template for the library amplification reactions. Thermocycling was performed off-deck in BioRad S1000 thermocyclers and reactions were allowed to hold at 10˚C overnight. The following day, the PCR Cleanup sub-method was used to purify the HLA amplicon libraries. Selected samples were diluted 1:10 in ultrapure water and assayed on the Bioanalyzer 2100 using a DNA High Sensitivity chip (Agilent), the results of which are presented in Figure 6. 192 libraries were pooled, diluted, and denatured using the Library Pooling sub-method and a 2 x 251 paired end sequencing run was performed using a 500-cycle MiSeq® v2 Standard Reagent Kit.

Three 23-sample runs with a negative control sample were performed using the automation method and the workflow described above. FASTQ reads for each run were transmitted to Illumina for analysis using Conexio Assign v1.0.0.719 (Beta 2) (Conexio Genomics) software with default parameters. The sample data

Figure 4: Biomek FX^P TruSight® HLA automation method reagent calculator for the HLA Amplification sub-method.



Row/Column	Column 1	Column 2-12
Row A	HLA-A primer mix: 150 µl	blank
Row B	HLA-B primer mix: 150 µl	blank
Row C	HLA-C primer mix: 150 µl	blank
Row D	DPA1 primer mix: 150 µl	blank
Row E	DPB1 primer mix: 150 µl	blank
Row F	DQA1 primer mix: 150 µl	blank
Row G	DRB primer mix: 150 µl	blank
Row H	DQB1 primer mix: 150 µl	blank

Column 1	Column 2	Column 3	Column 4	Column 5	Column 6
HLA PCR Mix: 1030 µl	HLA PCR Mix: 1030 µl	blank	blank	blank	blank
HLA PCR Mix: 1030 µl	HLA PCR Mix: 1030 µl	blank	blank	blank	blank
HLA PCR Mix: 1030 µl	HLA PCR Mix: 1030 µl	blank	blank	blank	blank
HLA PCR Mix: 1030 µl	HLA PCR Mix: 1030 µl	blank	blank	blank	blank

generated across the three automated method runs was highly reproducible when compared with the manual sample data generated internally at Illumina as well as with the known HLA types of the IHWG genomic DNA samples. As shown in Figure 7, 99% of the HLA allele typing results generated with the automated method matched HLA typing results generated with the manual preparation of TruSight® HLA libraries as well as with known reference alleles in the 410 alleles that were analyzed. Examination of negative controls showed that all three runs were devoid of contaminating sequences from neighboring wells, indicating that the automation method does not produce sample cross-contamination.

Figure 5: Agilent DNA1200 gel image following HLA Amplicon purification.

Figure 6: Agilent High Sensitivity gel image following HLA library purification.

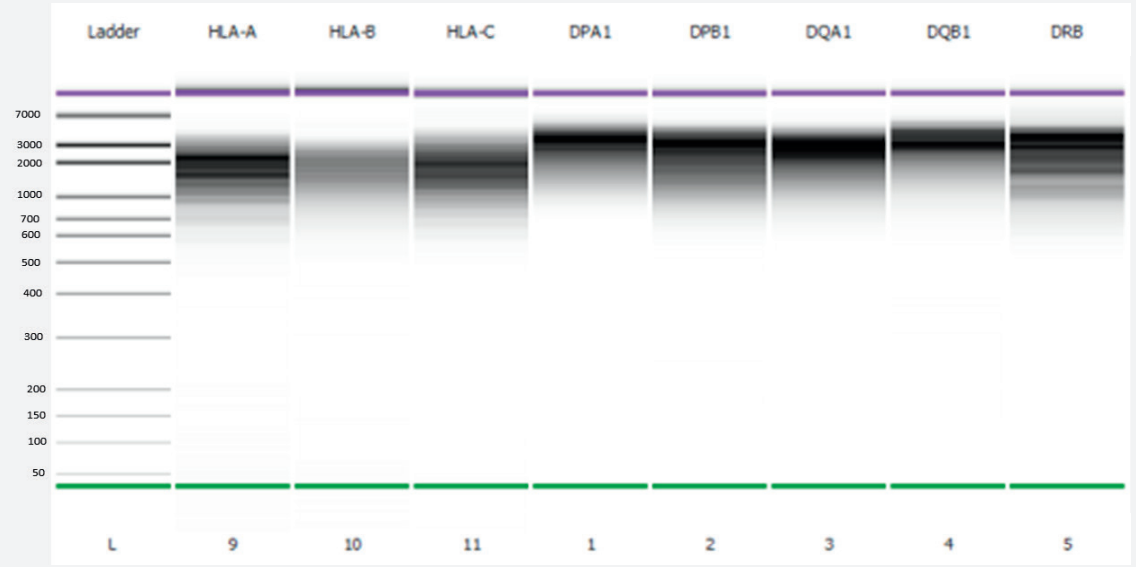


Figure 7: Comparison between automated TruSight® HLA for Biomek FX^P automation method and manual TruSight® HLA sequencing data.

	Total	HLA-A	HLA-B	HLA-C	HLA-DPA1	HLA-DPB1	HLA-DQA1	HLA-DQB1	HLA-DRB1	HLA-DRB3	HLA-DRB4	HLA-DRB5
Allele Reproducibility	99%	99%	98%	100%	100%	100%	100%	100%	99%	97%	96%	92%
Alleles Analyzed	410	46	46	46	46	46	46	42	46	22	16	8
Bases Analyzed	6,822,330	486,312	369,012	463,128	1,349,088	961,584	904,452	955,962	736,368	275,352	222,096	98,976
Bases Mismatched	18	4	8	0	0	0	0	0	2	2	0	2
Samples Analyzed	23											

Conclusion

The Biomek FX^{P} TruSight® HLA automation method streamlines the intensive workflow of the Illumina TruSight® HLA Sequencing Panel, while providing highly reproducible and accurate HLA typing results with a complete sample to sequence workflow.

References

1. Robinson J, Halliwell JA, Hayhurst JH, Flicek P, Parham P, Marsh SGE. The IPD and IMGT/HLA database: allele variant databases Nucleic Acids Research (2015) 43:D423-431

2. Fernando MMA, Stevens CR, Walsh EC, De Jager PL, Goyette P, et al. (2008) Defining the Role of the MHC in Autoimmunity: A Review and Pooled Analysis.PLoS Genet 4(4): e1000024. doi: 10.1371/journal.pgen.1000024

3. Madeleine MM, Johnson LG, Smith AG, Hansen JA, Nisperos BB, Li S, et al. Comprehensive analysis of HLA-A, HLA-B, HLA-C, HLA-DRB1, and HLA-DQB1 loci and squamous cell cervical cancer risk. Cancer Res (2008) 68(9):3532–910.1158/0008-5472.CAN-07-6471

4. www.ihwg.org

Software

Conexio Assign v1.0.0.719 (Beta 2) www.conexio-genomics.com

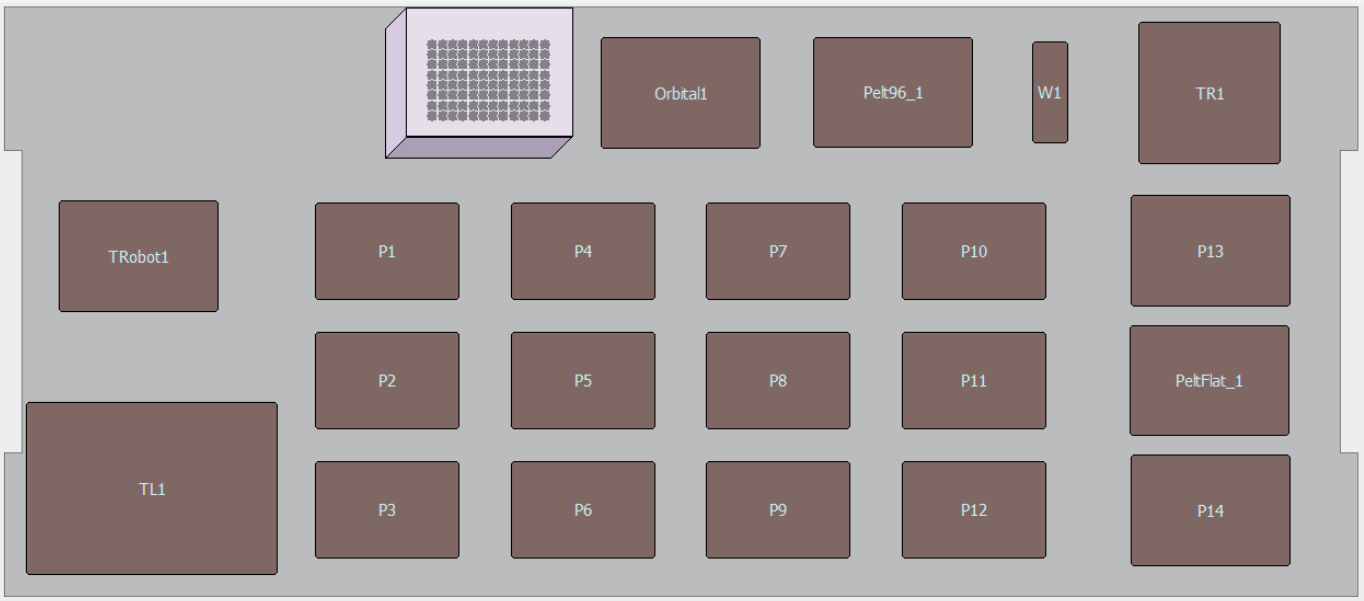
AAG-814TCH02.15-A

Reagent Details (if applicable)

ALPs/ Devices Required

PART NUMBER	QTY	MANUFACTURER	DESCRIPTION
719948	1	Beckman Coulter, Inc.	ALP, High-Density, 12-Position, 4 x 3
379448	1	Beckman Coulter, Inc.	ALP, Shaking, Orbital, Single-Position
719357	2	Beckman Coulter, Inc.	ALP, Standard Single-Position
A93938*	2	Beckman Coulter, Inc.	Static Peltier ALP
719590	1	Beckman Coulter, Inc.	Waste, Span-8, ALP
719356	1	Beckman Coulter, Inc.	Disposable Tip Loader ALP
719654	1	Beckman Coulter, Inc.	ALP, Tip Wash, 8-Channel
719363	1	Beckman Coulter, Inc.	Wash Station including pump and tubes
719366	1	Beckman Coulter, Inc.	Biomek FX Device Controller
Contact Beckman Coulter, Inc.	1	Biometra	(Optional) Biometra T-Robot for On-Deck incubations

*Method can be configured to use a chill-block on a 1X1 in place of one Static Peltier ALP.



Deck configuration of the Biomek FX^{P} Dual Arm Multi -96 and Span 8 Liquid Handler.

Labware Required

MANUFACTURER PART #	PER 24 SAMPLE RUN	MANUFACTURER	MANUFACTURERS NAME
B01124	5	Beckman	Biomek Span-8 P1000 Tips, Pre-sterile with Barrier
379503	15	Beckman	Biomek Span-8 P250 Tips, Pre-sterile with Barrier
A21586	15	Beckman	Biomek P50 Tips, Pre-sterile with Barrier
717256	6	Beckman	Biomek AP96 P20 Tips, Pre-sterile with Barrier
717253	8	Beckman	Biomek AP96 P250 Tips, Pre-sterile with Barrier
372790	4	Beckman	Quarter Reservoir
534681	3	Beckman	Reservoir, Half
372788	2	Beckman	Quarter Reservoir, Divided by Length
372795‡	1	Beckman	Frame for Reservoirs
4900‡	1	ThermoScientific	Empty Latch Racks for 500 µl ScrewTop Tubes
A32782‡	1	Beckman	Agencourt® SPRIPlate® 96R - Ring Super Magnet Plate
A83054‡	1	Beckman	BCI Tube Block
AB-1127	8	Fisher Scientific	Abgene 96-Well Storage Plate, Square Well, 1.2 mL
16466-042	14	VWR	2mL SuperClear™ Screw Cap Microcentrifuge Tubes - Conical Bottom
HSP-9641	12	Bio-Rad	Hard-Shell® Thin-Wall 96-Well Skirted PCR Plates
MSL-2022*	4	Bio-Rad	Arched Auto-Sealing Lids
4312063**‡	4	Bio-Rad	MicroAmp® Splash-Free 96-Well Base
HSS-9641**	12	Bio-Rad	Hard-Shell® Thin-Wall 96-Well Non Skirted PCR Plates
C5064‡	1	Acme Automation	ReactorAdaptor96Flat

* Optional: for On-Deck Thermo cycling only **Non-skirted plate option only, not compatible with TRobot ‡ One time purchase

User-Supplied Consumables

PART NUMBER	MANUFACTURER	DESCRIPTION
AB00138-01000	American Bioanalytical	Ethanol
FC-142-1001	Illumina	Illumina TruSight® HLA Sequencing Panel
FC-131-200(1,2,3,4)	Illumina	Nextera® XT Index Kit v2 (Set A, B, C, or D)
MS-102-2003 or MS-103-1003	Illumina	MiSeq® Reagent Kit v2 (500 cycles) or MiSeq® Reagent Nano Kit v2 (500 cycles)

Auxiliary Equipment for QC Testing

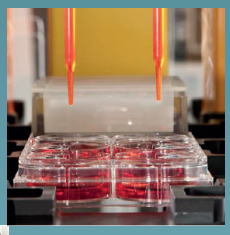
PART NUMBER	MANUFACTURER	DESCRIPTION
G2940CA	Agilent Technologies	Agilent 2100 Bioanalyzer
5067-1508	Agilent Technologies	Agilent DNA 12000 Kit
5067-4626	Agilent Technologies	Agilent High Sensitivity DNA Kit
SY-410-1003	Illumina	MiSeq® System

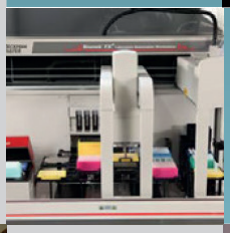
For Research Use Only. Not for use in diagnostic procedures.

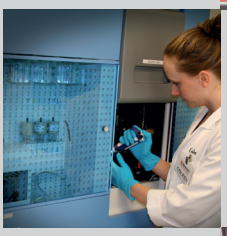
For Beckman Coulter's worldwide office locations and phone numbers, please visit www.beckmancoulter.com/contact

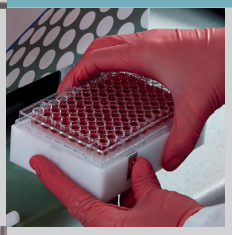
NUCLEIC ACID EXTRACTION AND PURIFICATION KITS

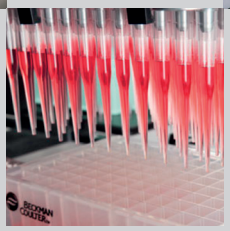
HIGH RECOVERY OF NUCLEIC ACIDS WITH SUPERIOR YIELD AND PURITY







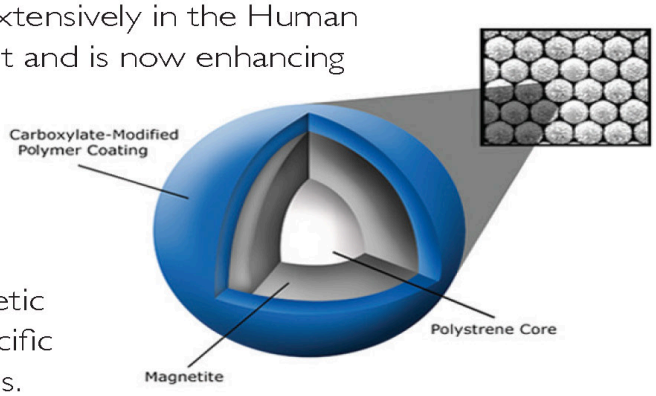




BECKMAN COULTER
Life Sciences

BECKMAN COULTER'S SPRI CHEMISTRIES

The SPRI technology is a patented purification technology developed at MIT's Whitehead Institute (Hawkins, et al., Nucleic Acids Res. 1995 (23):4742-4743). It is a simple and highly efficient means of microplate-based nucleic acid isolation used extensively in the Human Genome Project and is now enhancing NGS sample prep in the same way. Nucleic acids are immobilized onto paramagnetic beads using specific buffer conditions.



Flexibility is added to the system through modification of binding buffers, which alters selectivity for the type and size of immobilized nucleic acid. The targeted nucleic acid is easily isolated from the sample without the need for centrifugation or filtration, thus creating an automation-friendly format.

Key Features	Benefits
Paramagnetic beads	Easily automated with no centrifugation or filtration
Uniform bead size	Highly reproducible results
Negatively charged surface	Elution with aqueous solution without the use of chaotropic salts
Non-styrene polymer surface	Low non-specific binding
High surface area to mass ratio	Increased binding capacity
Fast magnetic response time	Rapid purifications
Size optimized	Low settling rates for automation application

AAG-1128FLY09.15-A

DNA EXTRACTION KITS

DNAdvance

- gDNA from Mammalian tissue and cells, saliva, Genotek Orgagene, and mouse tail
- Process 96 well plate in 75 mins

GenFind v2

- gDNA from whole blood, serum, mammalian tissue, cell culture, saliva, Buccal swab, FTS card, Bacterial
- Whole Blood processing from 50 – 400 ul
- 96 samples in approximately 2.5 hours

CosMC Prep

- Plasmid DNA from E. Coli
- High quality purification of BACs, cosmids, fosmid, low copy plasmids, and high copy plasmids
- 24 plates in 8 hrs
- Requires Centrifugation step

RNA EXTRACTION KITS

RNAdvance Blood

- RNA from blood (PAXgene tubes), cells, and tissue
- Process 96 well plate in approximately 3.5 hrs
- No detectable gDNA
- Yield Approximately 700 ng of RNA per 400 ul of blood

RNAdvance Cell v2

- RNA from mammalian cell culture, <50 K cells
- Two 96-well plates in approximately 2 hr
- Supports miRNA extraction

RNAdvance Tissue

- RNA from tissue, Cells (50 k to 1 M cells)
- Efficient removal of genomic DNA and other contaminants

FormaPure

- FFPE sample types
- About 4 hrs to process 96-well plate
- Consistent RNA purity
- Superior recovery

CLEAN UP KITS

CleanSeq

- Efficient removal of Big Dye terminator and sequencing reaction contaminants
- Long Phred 20 read length averaging over 700 bps
- Pass rates 85% or higher

AMPure XP

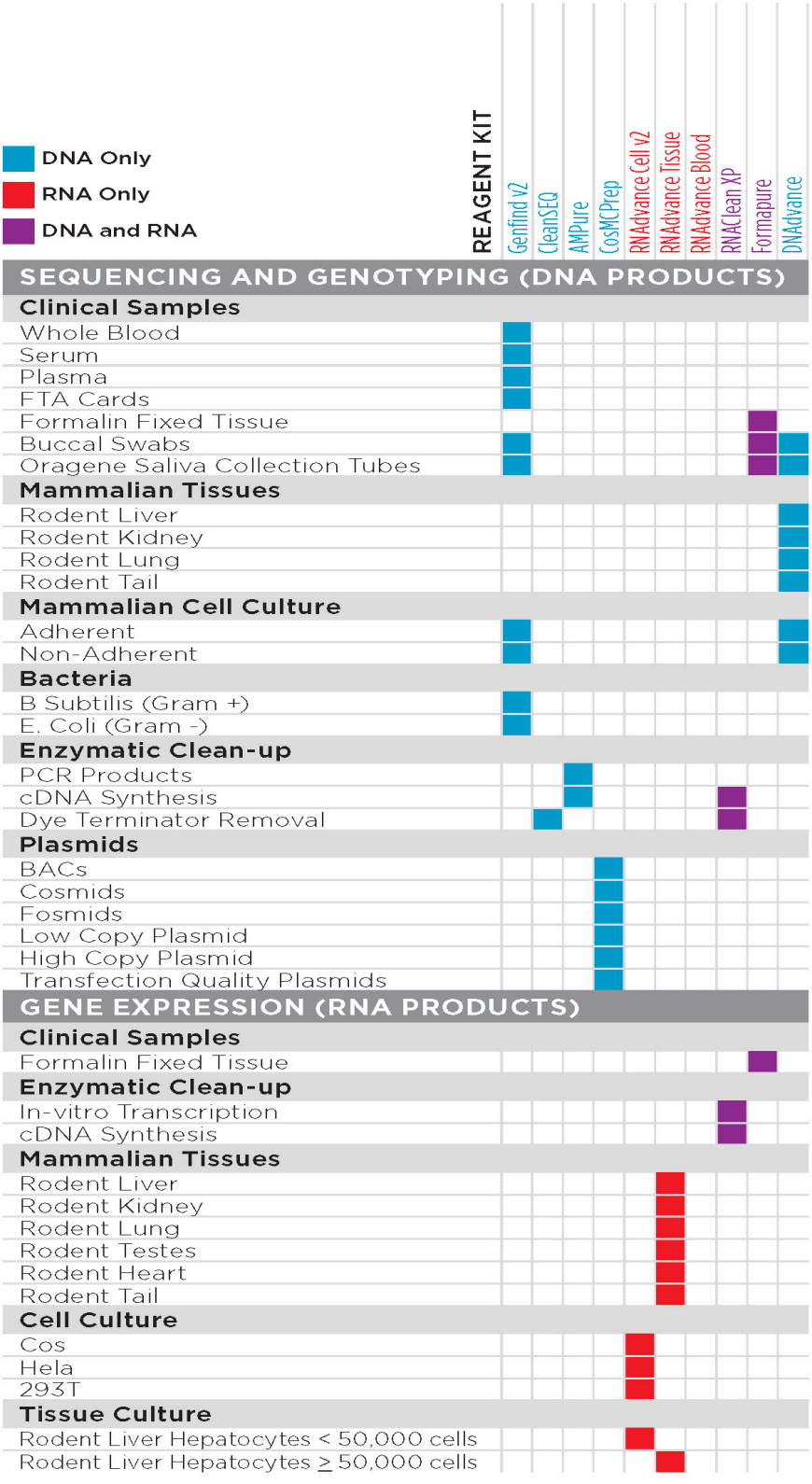
- Removes primers, unincorporated dNTPs, primer dimers, salts and other contaminants
- Eliminate fragments <50 bp.
- Recovers both double and single stranded DNA templates
- No PCR degradation after storage at 4 C for 7 days
- Captures DNA > 100 bp

RNAClean XP

- For Post-reaction cDNA and cRNA cleanup
- High recovery of small and large nucleic acid products
- Efficient removal of salts, unicorporated primers and dNTPs

SPRIselect

- Used for fragment size selection for library construction in NGS process
- Manufactured to a tight specification to provide lot to lot consistency
- Guideline to aide in optimizing desired size range



BECKMAN COULTER
Life Sciences

For Beckman Coulter's worldwide office locations and phone numbers, please visit "Contact Us" at beckmancoulter.com

09.15

Solid Phase Reverse Immobilization (SPRI) Bead Technology for Micro RNA Clean Up using the Agencourt RNAClean XP Kit

Bee Na Lee, Ph.D. Staff Application Scientist, Beckman Coulter Life Sciences

Introduction

Micro RNAs are small non-coding ribonucleic acids with sizes between 18 and 40 nucleotides (nts). The majority of miRNAs are composed of approximately 22 nts. Purification of these small miRNA fragments after enzymatic reactions can be challenging because most commonly-used column methods were designed to recover fragment sizes that are greater than 50 nts. Therefore, these column methods often produce lower miRNA yields.

This application note describes the use of Beckman Coulter's Agencourt RNAClean XP kit for miRNA clean up and concentration of miRNA and total RNA from low yield samples. The RNAClean XP kit is a Solid Phase Reverse Immobilization (SPRI) paramagnetic bead-based method for RNA and cDNA purification. The magnetic bead procedure does not require centrifugation or filtration steps. It is a simple, rapid, automation-friendly protocol that produces high quality samples.

miRNA purification is achieved in three steps.

Step 1: Mix miRNA with binding buffer containing beads and isopropanol.

Step 2: Wash the miRNA bound beads with fresh 85% ethanol.

Step 3: Elute the miRNA from the beads with nuclease free water.

The results show that the SPRI bead purification protocol gives two times higher miRNA recovery compared to a column purification method.

Materials and Methods

Different amounts of the RNAClean XP reagent, 100% isopropanol and input sample volume were tested to achieve optimal binding buffer conditions for miRNA purification. Table 1 shows the ratio of the binding buffer to sample volume for miRNA purification. For example, 50 µL of sample containing 1 µg RNA (Life Technologies, AM7950) was mixed thoroughly with 90 µL of RNAClean XP (Beckman Coulter, A63987) plus 270 µL of 100% isopropanol (American Bioanalytical, AB07015) in a 96 well plate format (ABGene, T5050G), and incubated at room temperature for 5 minutes. The sample plate was placed on an Agencourt SPRIPlate 96-Ring Super Magnet Plate (Beckman Coulter, A32782) for 15 minutes or until the solution turns completely clear to separate the magnetic beads. The RNA bound beads were washed two times with 300 µL of freshly-made 85% ethanol, prepared from 100% ethanol (American Bioanalytical, AB000138). The beads were air dried for 5 minutes and RNA was eluted with 50 µL of nuclease free water.

For miRNA clean up and purification using the MinElute Column modified protocol (Qiagen, 74204): 100 µL of sample containing 1 µg RNA was mixed thoroughly with 350 µL RLT +250 µL 100% ethanol. The sample mixture was applied to the first column and centrifuged for 15 sec at 10,000 rpm. 50 µL of RLT and 500 µL 100% ethanol were then added into the collected flow through. The flow through was mixed well with RLT+100% ethanol and was transferred to the second column. After the second centrifugation for 15 sec at 10,000 rpm, the column was washed with RB buffer and then with 100% ethanol, the purified miRNA was eluted with 50 µL of nuclease free water.

The concentration and purity of the miRNA and RNA purified from both RNAClean XP and MinElute Column was measured with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). 1 µL of. purified miRNA was analyzed by an Agilent Small RNA Kit (Agilent Technologies, 5067-1548) using the 2100 Bioanalyzer (Agilent Technologies).

MicroRNA let-7c gene expression was determined by Taqman microRNA assay (Life Technologies 4427975, assay ID000379). 50 ng and 100 ng of total RNA was used for the reverse transcription reaction using the TaqMan micro RNA Reverse Transcription kit (Life Technologies, 4366596) and 1.33 µL of cDNA was used per PCR reaction in triplicate using Taqman Universal Master Mix II (Life Technologies, 4440038). For the RT and qPCR reaction set up, see details in reference IB-17265A (www.Beckman.com).

AAG-245APP06.14-A

SAMPLE VOLUME (µL)	RNAClean XP (µL)	100% ISOPROPANOL (µL)
50	90	270
100	180	270
150	270	270
200	360	270

Table 1: Binding buffer formulation for miRNA clean up

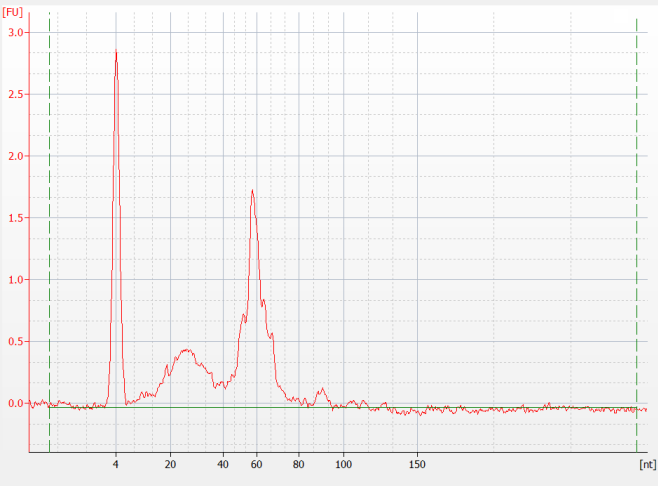
Results and Discussion

RNAClean XP resulted in highly efficient miRNA and small RNA recovery.

A 50 µL RNA sample containing miRNAs with sizes between 18-60 nts was purified using the RNAClean XP reagent and eluted with 50 µL of nuclease free water. 1 µL of the pre-purified control miRNA and post-purified, eluted miRNA were analyzed on the Agilent Small RNA Chip to determine miRNA recovery. Figure 1 shows that the estimated recovered miRNA amount was not significantly different from pre- and post-purified samples. Figure 1 shows an example of the miRNA profiling. The estimated miRNA concentration of pre-purified control sample was at 554 pg/µL with 67% miRNA (left panel), and the estimated purified miRNA concentration was at 541 pg/µL with 63% miRNA (right panel). The average estimated miRNA concentration purified from a total of four samples was at 663 pg/µL+/-93 with 62%+/-2.9 of miRNA (data not shown).

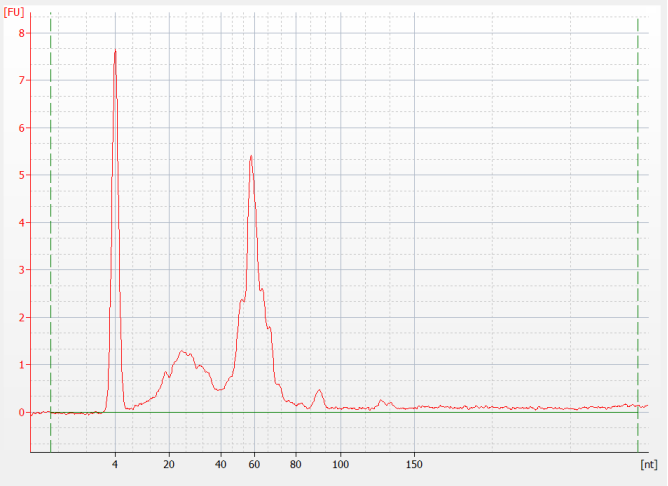
Figure 1. RNAClean XP gave very high miRNA recovery

Pre-purified Sample



Small RNA Concentration (pg/µL)	828.1
miRNA Concentration (pg/µL)	554.2
miRNA / Small RNA Ratio (%)	67

Post-purified Sample



Small RNA Concentration (pg/µL)	862.0
miRNA Concentration (pg/µL)	541.4
miRNA / Small RNA Ratio (%)	63

RNAClean XP protocol showed higher miRNA recovery than the column method.

100 µL of RNA containing 20 ng/µL of total RNA plus miRNA (Life Technologies, AM7950) was used to determine miRNA plus RNA recovery. Eight samples were purified using the RNAClean XP protocol (Beckman Coulter, AAG-244APP06.14-A) and eight samples were purified using the MinElute Column method. The results showed that the RNAClean XP method gave higher miRNA plus RNA yields compared to the column method (Table 2). In an independent experiment, 100 µL of samples containing 5.5 ng/µL of total RNA plus miRNA (Life Technologies, AM7950) were tested for RNA recovery, eight samples were purified using each method. The data consistently showed that RNAClean XP gave 80 - 94% recovery, whereas the column method only had 39 - 49% recovery (Table 3). It is important to note that majority of the larger RNA fragments were removed in the column method.

METHOD	AVERAGE RNA RECOVERY +/- STD DEV	AVERAGE % RECOVERY
RNAClean XP	16 ng/µL +/- 1.34	80%
MinElute Column	7.76 ng/µL +/- 3.16	39%

Table 2: Average RNA and miRNA recovery from RNAClean XP and MinElute column.

METHOD	AVERAGE RNA RECOVERY +/- STD DEV	AVERAGE % RECOVERY
RNAClean XP	5.2 ng/µL +/- 0.55	94%
MinElute Column	2.7 ng/µL +/- 0.64	49%

Table 3: Average RNA and miRNA recovery from RNAClean XP and MinElute column.

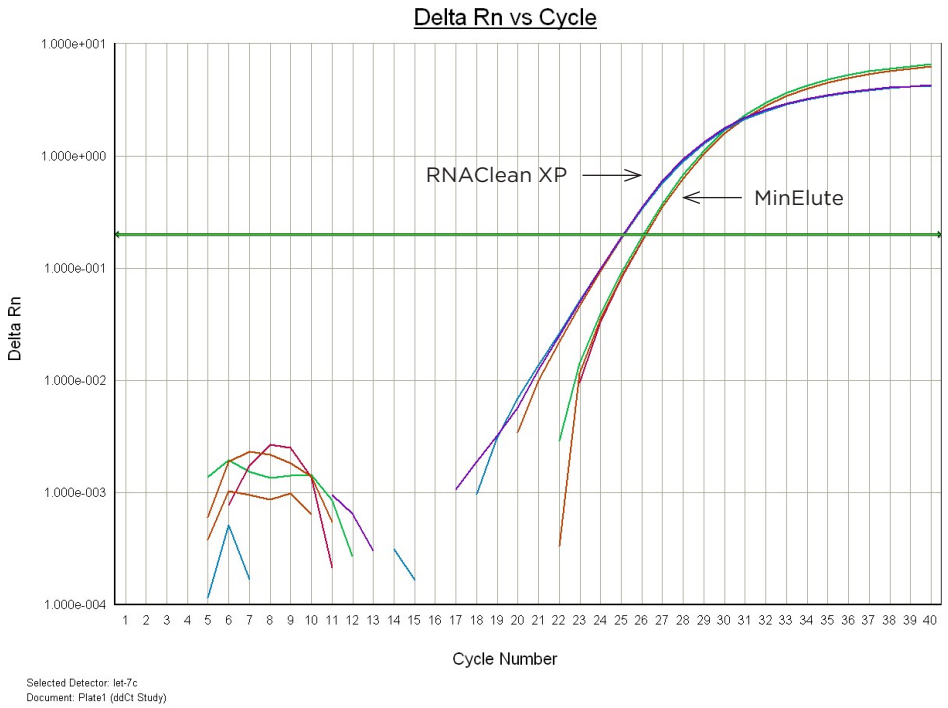
RNAClean XP purified samples showed higher miRNA let7c gene expression.

To determine the miRNA recovery efficiency, 50 ng of the purified RNA samples from the RNA Clean XP and MinElute column were used to determine miRNA let-7c gene expression using the Taqman microRNA assay (Life Technologies 4427975, assay ID000379). The results show that the average cycle threshold (Ct) for RNAClean XP purified RNA samples was 25 cycles, whereas the MinEluted purified RNA samples gave an average Ct at 26 cycles in 50 ng per reaction (Table 4 and Figure 2), suggeting that RNAClean XP has higher let 7c gene expression as compared to the column method. In consistent with this finding, 2 times more miRNA input amount was needed from the MinElute purified sample in order to archieve the same cycle threshold for let 7c gene expression (data not shown).

METHOD	AVERAGE RNA RECOVERY +/- STD DEV
RNAClean XP	24.98 +/- 0.145
MinElute Column	26.30 +/- 0.03

Table 4: Average Ct value for the let-7c gene expression in a 50 ng reaction, a total of three independent samples were tested for each method.

Figure 2. Overlaid amplification plots from RNACleanXP and MinEluted purified RNA samples.



Conclusion

The data from this study shows that RNAClean XP has higher miRNA recovery when compared to the column miRNA purification method. The RNAClean XP protocol enables recovery of both miRNA and total RNA in one procedure. It does not require centrifugation, filtration or precipitation steps. It provides scalable throughput as well as being automation-friendly.

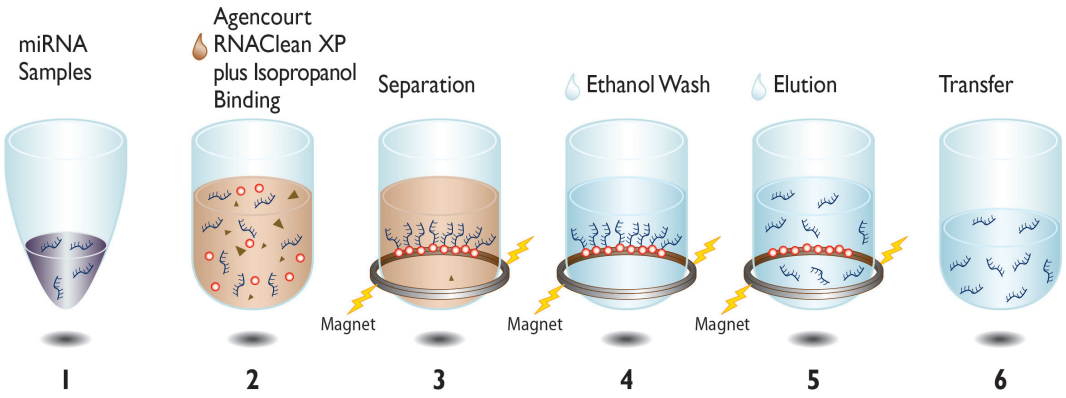
Reference

For miRNA size selection and enrichment, use the SPRIselect reagent (Beckman Coulter, B23318) and the miRNA enrichment supplemental protocol (IB-18479A, www. Beckman.com).

Agencourt® RNAClean™ XP

SUPPLEMENTAL PROTOCOL FOR Micro RNA CLEAN UP USING AGENCOURT® RNAClean® XP

PROCESS OVERVIEW



Introduction

The Agencourt RNAClean XP system utilizes Beckman Coulter's solid-phase paramagnetic bead technology for high-throughput purification of RNA or cDNA for in vitro applications such as transcription, antisense RNA (aRNA) amplification as well as RNA and cDNA probe synthesis. This supplemental protocol enables recovery of micro RNA (miRNA), small RNA and total RNA from enzymatic reactions, concentrating miRNA and total RNA from a diluted sample.

Materials Supplied by the User

Consumables and Hardware:

Magnetic Separator: Agencourt SPRIPlate 96R-Ring Super Magnet Plate: Beckman Coulter Life Sciences, A32782) or Agencourt SPRIStand-Magnetic 6-tube Stand (for 1.7 mL tubes) (Beckman Coulter Life Sciences, A29182)

Prep Plate: ABgene 1.2 mL 96-Well Storage Plate, Square Well, UBottom (ABGene # 1127).

Reagents:

- 100% Isopropanol: American Bioanalytical, AB07015
- 100% ethanol: American Bioanalytical, AB00138
- 85% ethanol made with nuclease free water (Note: 85% ethanol is hygroscopic. *Fresh 85% ethanol should be prepared for optimal results).*
- Nuclease-Free Water (not DEPC-Treated) (Ambion, AM9932)

BECKMAN COULTER Life Sciences

Procedure

Note: Shake the Agencourt RNAClean XP bottle to re-suspend any magnetic particles that may have settled.

1. Add the appropriate volume of RNAClean XP and 100% Isopropanol (American Bioanalytical, part # AB07015) into the well containing 50-200 µL of miRNA sample according to the instructions in the table below.

SAMPLE VOLUME (µL)	RNAClean XP (µL)	100% ISOPROPANOL (µL)
50	90	270
100	180	270
150	270	270
200	360	270

2. Mix the total reaction volume by pipetting up and down 10 times and incubate at room temperature for 5 minutes. Place the reaction vessel on an Agencourt SPRIPlate 96R-Ring Super Magnet Plate or Agencourt SPRIStand-Magnetic 6-tube and allow the RNAClean XP beads to settle on the magnet for 15 minutes or until the supernatant turns completely clear.
3. Remove and discard the entire clear supernatant.
   NOTE: *Care should be taken not to aspirate a significant amount of beads during this step as the desired miRNA and total RNA is associated with the beads. Significant bead loss will result in reduced yield.*
4. With the reaction vessel still on the magnet, add 600 µL of 85% nondenatured ethanol (freshly made from 100% ethanol) and incubate at room temperature for 30 seconds.
5. Remove and discard the ethanol supernatant.
   NOTE: *Care should be taken not to aspirate a significant amount of beads during this step as the desired miRNA and total RNA is associated with the beads. Significant bead loss will result in reduced yield.*
6. Repeat steps 4-5 with the ethanol wash for a total of 2 ethanol washes. Air dry the beads for 5 minutes.
7. To elute the sample, remove the reaction vessel from the magnet and add 20-50 µL of molecular biology grade RNase/DNase free water or 10 mM Tris-HCl, pH8.5.
   NOTE: *Elution volume should be large enough so that the liquid level is high enough on the magnet for the beads to settle.*
8. Mix the total elution volume by pipetting up and down 10 times to resuspend the beads and incubate at room temperature for 2 minutes.
9. Place the reaction vessel on an appropriate magnetic stand or plate and allow the RNAClean XP beads to settle to the magnet for 2 minutes.
10. Transfer the eluted samples to an appropriate storage vessel and keep at -80˚C for long term storage.

For more information, visit www.Beckman.com or contact 1-800-369-0333

BECKMAN COULTER
Life Sciences

Micro RNA and Total RNA Purification from Tissues using the AgencourtRNAdvance Tissue Kit

Bee Na Lee, Ph.D. Staff Application Scientist, Beckman Coulter Life Sciences

Introduction

Micro RNAs are small naturally occurring non-coding ribonucleic acids with sizes between 18 and 40 nucleotides (nts) that have been demonstrated to play a significant role in the regulation of gene expression. As a result, interest in smaller RNA species, such as miRNA, has increased. Here we describe the purification of total RNA, including miRNA and other small RNA molecules from fresh frozen tissue samples using the Agencourt SPRI (Solid Phase Reverse Immobilization) magnetic bead based chemistry. Extracting RNA from tissues often involves phenol and chloroform as well as multiple centrifugation steps. The SPRI method is an easy, rapid, high yielding, robust and automation-friendly nucleic acid purification procedure that does not require vortexing, centrifugation or filtration steps. The RNAdvance Tissue protocol has additional steps for lysing and purifying the sample. This technical note demonstrates that the SPRI extraction method produces high quality miRNA and RNA using two homogenization methods. The RNA yield and quality is comparable to a commonly used column method.

Materials and Methods

Rat liver, brain and heart tissue were homogenized using either a Precellys 24 (Bertin) or Ultra Turrax (IKA) homogenizer. For the Precellys homogenization, 100 mg of tissue was homogenized in a CK28_7 mL tube (Bertin Technologies, KT03961-1-302.7) containing 1 mL of RNAdvance Tissue lysis buffer (Beckman Coulter Life Sciences, A32646) and antifoaming Dx Reagent (Qiagen, 19088). The homogenization conditions are as follows: 6500 rpm for 20 sec at the 1st cycle and 6000 rpm for 20 sec at the 2nd cycle. For the Ultra Turrax tissue dispersing device, 100 mg of tissue in 4 mL of lysis buffer was homogenized at the highest speed for 60 seconds or until the tissue was homogenized completely. Lysate was adjusted to 10 mg per 400 µL lysis buffer and then digested with proteinase K at 37˚C for 25 minutes. RNA was extracted according to the instructions for the RNAdvance Tissue miRNA protocol (www.beckman.com, AAG-230APP07.14-A) using the tube format method. For the miRNeasy Micro kit (Qiagen, 217084) samples, 50-100 mg of rat liver tissue was homogenized using either Precellys 24 (Bertin Technologies) or the Ultra Turrax dispersing element as described above. Lysate was adjusted to 5mg per 700 µL Qiazol buffer and then RNA was extracted according to the miRNeasy Micro Kit protocol (Qiagen, 217084).

The RNA concentration and purity were measured with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). The RNA purity was determined by the OD260/OD280 and OD260/OD230 ratios. 1 µL of the diluted RNA sample was analyzed by an Agilent RNA 6000 Pico chip (Agilent Technologies, 5067-1513) using the 2100 Bioanalyzer (Agilent Technologies) to determine RNA integrity. Let-7c miRNA gene expression was determined by Taqman microRNA assay (Life Technologies 4427975, assay ID000379). 50 ng of total RNA was used for the reverse transcription reaction using the TaqMan microRNA Reverse Transcription kit (Life Technologies, 4366596) and 1.33 µL of cDNA was used per PCR reaction in triplicate using Taqman Universal Master Mix II (Life Technologies, 4440038).

BECKMAN COULTER
Life Sciences

Results and Discussion

Summary of RNA yields from three different tissue types using the RNAdvance Tissue Kits.

An average of 10 mg of three tissue types was used to evaluate RNA yield. Liver (soft), heart (fibrous) and brain (lipid rich) tissue was prepared for RNA extraction. Eight samples from each type of tissue were extracted for total RNA and miRNA. The RNA was eluted in 40 µL of nuclease free water. The results show that liver and heart tissue gave at least three times higher RNA yields as compared to more difficult brain tissue (lipid rich) (Table 1). All samples show high RNA quality as indicated by RIN (RNA Integrity Number) scores (Table 2). The lower RIN scores observed in brain samples that could have resulted from the higher amounts of smaller RNA in the samples as indicated by the relatively larger area of small RNA present in the electropherograms. The software has no way to detect that the fluorescence in this area is due to enrichment for small RNA and calculates it as degraded RNA (Figure 1). Figure 2 shows the examples of RNA profiling from the liver, heart and brain. The OD260/OD280 ratio for all 24 samples was above 1.9, and the OD260/OD230 ratio was above 1.2 (data not shown).

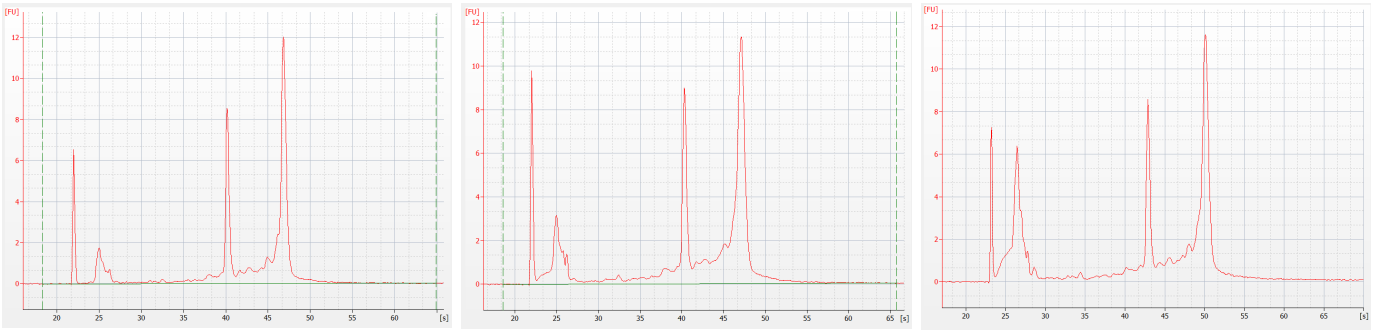
TISSUE	LIVER	HEART	BRAIN
Average Yield (µg) per 10 mg	53.70 +/- 4.0	46.6 +/- 2.8	14.72 +/- 4.5

Table 1: The average yield was calculated from a total of 8 samples for each tissue type.

TISSUE	LIVER	HEART	BRAIN
RIN	9.0-9.8	9.0-9.5	8.0-8.70

Table 2: The average RIN was calculated from a total of 8 samples for each tissue type.

Figure 1. Example of total RNA Profiling:

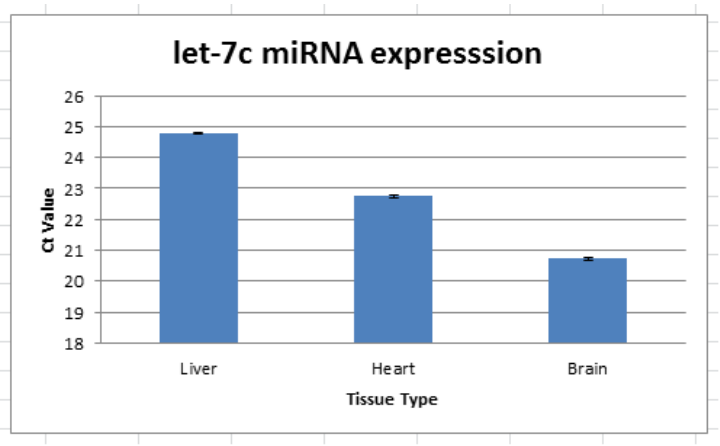


RNA Pico Chip data: 1:1000 dilution for liver (left) and heart samples (middle) and 1:300 for brain sample (right).

Micro RNA let-7c gene expression in liver, heart and brain tissues.

50 ng of total RNA was used for let-7c gene expression to determine miRNA gene expression. The results show that brain tissue expressed let-7c miRNA more abundantly than either liver or heart tissue (Figure 2). The average cycle threshold (Ct) was calculated from three samples in each tissue type. The average Ct value for let-7c gene expression in the brain was 20.72+/-0.038, heart was 22.75+/-0.038 and liver was 24.78+/-0.022. The minus RT and controls with no template showed negative amplification, indicating that the amplification resulted from miRNA alone.

Figure 2. Average Ct value for the let-7c gene expression in a 50 ng reaction



The average Ct value was calculated from three samples extracted from different experiments.

The RNAdvance Tissue purification kit gave comparable RNA yield as well as miRNA extraction efficiency as compared to column purification.

A total of 16 different liver tissue replicates were used to evaluate miRNA extraction efficiency between the SPRI reagents and a common column method (miRNeasy column extraction). Two homogenization methods were used for this study (IKA Ultra Turrax tissue dispersing element and Precellys' homogenizer) in order to have an impartial comparison. RNA was extracted either using the RNAdvance Tissue Kits or miRNeasy Micro Kits. The results showed that RNAdvance Tissue Kits and miRNeasy Micro Kits gave comparable yield from both homogenization methods. The calculated average yield prepared from the RNAdvance Tissue Kit was between 24-30 µg per 5 mg liver tissue whereas the calculated average yield from the miRNeasy Micro Kit was between 19-27 µg per 5 mg liver tissue (Table 3 and 4). The average cycle threshold (Ct) value for the let -7c miRNA expression in RNA samples prepared from both methods was at 25 cycles, indicating that both extraction methods gave similar miRNA extraction efficiency (Figure 3).

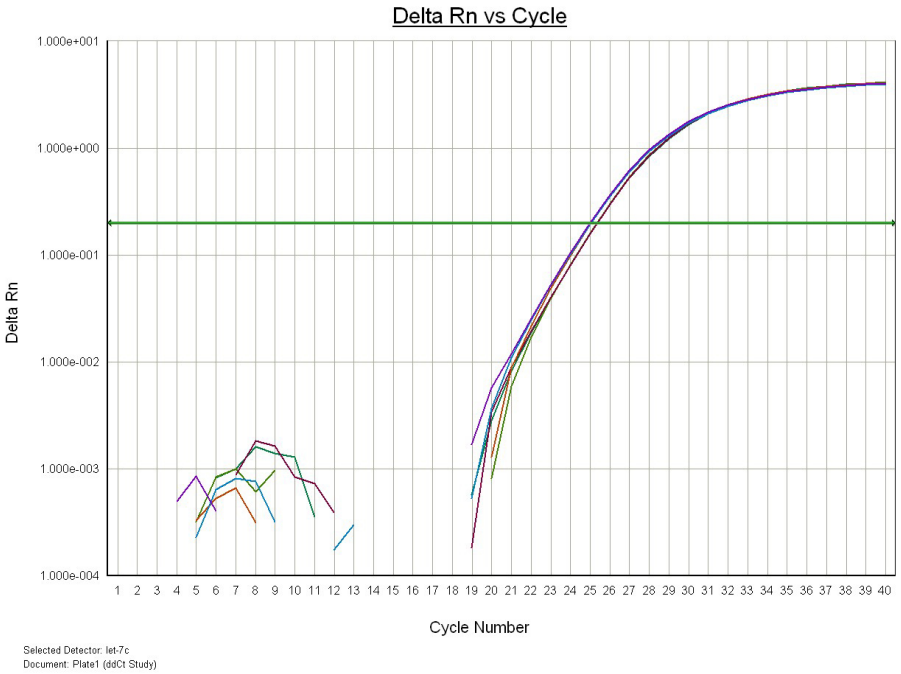
METHOD	YIELD PER 5 mg LIVER TISSUE (µg)	AVERAGE YIELD PER 5 mg LIVER TISSUE (µg)
RNAdvance Tissue	24-30	27.04 +/-2.0
miRNeasy	19-23	21.17+/-2.0

Table 3: The average RNA yield and quality prepared from tissue dispersing homogenizer

METHOD	YIELD PER 5 mg LIVER TISSUE (µg)	AVERAGE YIELD PER 5 mg LIVER TISSUE (µg)
RNAdvance Tissue	25-30	27.80+/-2.2
miRNeasy	21-27	23.26+/-2.5

Table 4: The average RNA yield and quality prepared from Precellys homogenizer.

Figure 3. Overlay of let-7c gene expression from the RNAdvance Tissue Kits and miRNeasy Micro Kits.



Taqman qPCR amplication plots show that both extraction methods gave a Ct at 25 cycles.

Conclusion

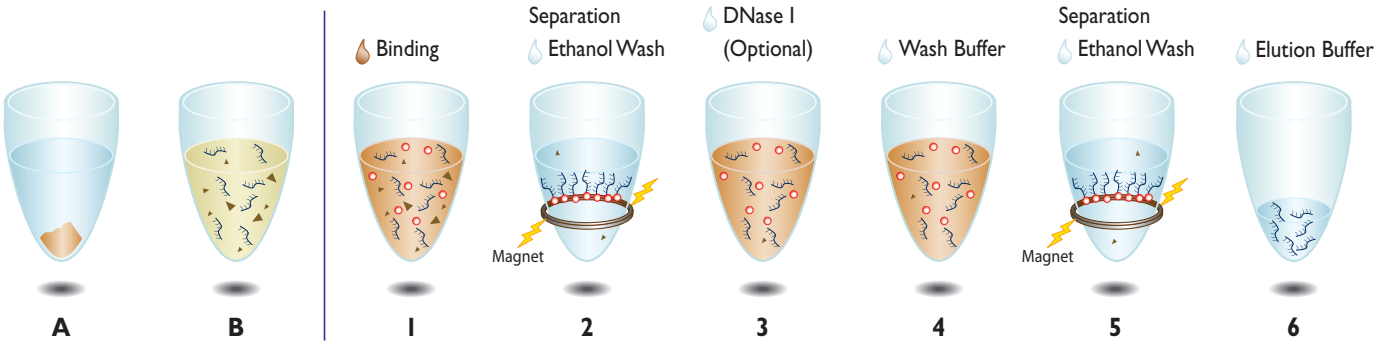
The data from this study shows that the RNAdvance Tissue Kit provides high quality RNA and miRNA. The RNAdvance Tissue Kit can be used for up to 10 mg per extraction, whereas the miRNeasy Micro Kit is optimized only up to 5 mg tissue per extraction (see miRNeasy protocol). The magnetic bead based extraction protocol does not require centrifugation, filtration or precipitation steps and the use of phenol chloroform. It provides scalable throughput and it is automation-friendly.

Agencourt® RNAdvance™ Tissue Kit

SUPPLEMENTAL PROTOCOL FOR Micro RNA AND TOTAL RNA ISOLATION FROM TISSUE

PROCESS OVERVIEW

Agencourt RNAdvance Tissue



Introduction

The Agencourt RNAdvance Tissue total RNA purification kit utilizes Beckman Coulter's patented Agencourt SPRI paramagnetic bead-based technology to isolate micro RNA (miRNA) and total RNA. The protocol can be performed in both 96-well and single tube formats. Purification begins with the homogenization and lysis of tissue. Following lysis, there is an immobilization of RNA onto the magnetic beads allowing for the RNA to be separated away from contaminants using a magnetic field. The RNA is then treated with DNase and the contaminants rinsed away using a simple wash procedure. The Agencourt RNAdvance Tissue kit is amenable to automation as it utilizes magnetic separation, thus eliminating the need for vacuum filtration or centrifugation.

Note for miRNA and Total RNA extraction

If the RNAdvance Tissue Kit is to be used for miRNA isolation:

- **100% Isopropanol should not be added directly to the Wash buffer bottle.** See page 3 for Wash buffer preparation
- 600 µL of Isopropanol is added in the Bind Buffer at step 9 instead of 400 µL
- 85% ethanol should be used instead of 70% ethanol in all ethanol washes

Materials Supplied by the User

Consumables and Hardware:

- Agencourt SPRIPlate 96R – Ring Super Magnet Plate (Beckman Coulter Life Sciences, A32782) or Agencourt SPRIStand – Magentic 6-tube Stand (for 1.7 mL tubes) (Beckman Coulter Life Sciences, A29182)
- Tissue homogenizer
- 1.7 mL microcentrifuge tubes (Fisher Scientific, NC9448938) for tube format
- 96-Well Riplate-2.2 mL (World Wide Medical Products, 99181000) for plate format
- 37°C water bath or heat block for proteinase K digestion and DNase treatment

BECKMAN COULTER
Life Sciences

Reagents:

- 100% Isopropanol; American Bioanalytical AB07015 or equivalent
- 85% ethanol; freshly prepared/diluted from 100% ethanol (American Bioanalytical, AB00138 or equivalent)
- DNase I (RNase-free); Ambion AM2222 or AM2224
- Reagent grade water, nuclease-free; Ambion AM9932

Materials Supplied in the Kit

Reagent	Refered to in the Protocol As	Storage Conditions on Arrival	Storage Conditions once In Use (isopropanol, PK or buffer added)
Lysis Buffer	Lysis Buffer	Room Temperature	Room Temperature
Binding Buffer	Bind Buffer	4°C	4°C
Wash Buffer	Wash Buffer	Room Temperature	Room Temperature
Proteinase K	PK	-20°C	-20°C
Proteinase K Storage Buffer	PK Buffer	Room Temperature	-20°C

Kit Specifications

The Agencourt RNAdvance Tissue kit is manufactured under RNase-free conditions and has been tested and certified not to contain contaminating nucleases. The Agencourt RNAdvance Tissue kit can be used in 96 well and single tube formats.

General Remarks on Handling RNA

RNases are ubiquitous and general precautions should be followed in order to avoid the introduction of contaminating nucleases during the Agencourt RNAdvance Tissue procedure. The most common sources of RNase contamination are hands, dust particles, and contaminated laboratory instruments, solutions and glassware. When working with RNA, the following procedures should be followed to limit RNase contamination:

- Always work with gloved hands and change gloves frequently.
- Use RNase free, filtered pipette tips for pipetting whenever possible.
- Use dedicated RNase free equipment, e.g. pipettes, pipette tips, gels boxes, etc.
- Avoid using reagents, consumables and equipment that are in common use for other general lab processes.
- When available, work in a separate room, fume hood or lab space.
- Use plastic, disposable consumables that are certified RNase free.
- Purchase reagents, such as commonly used buffers and water, that are certified RNase free.
- Prepare small individual aliquots of such buffers to avoid repeated transfer out of stock buffers. This lowers the risk of contaminating the stock solution.
- Wipe down work surfaces with commercial RNase inhibiting surfactant solutions or 85% ethanol before starting work.

Before starting

- Preheat water baths or heat blocks to 37°C
- Ensure that Agencourt RNAdvance Tissue Proteinase K and Wash Buffer have been assembled according to the following instructions:

Agencourt RNAdvance Tissue 96 Well and Tube Procedure

Assembly Steps 1 and 2 are only performed once for each new Agencourt RNAdvance Tissue kit. If these have already been completed during a previous experiment, please skip ahead to Step 3.

1. **Add PK Buffer to PK tube per the chart below:**

	50 Prep Kit Part # A32645	96 Prep Kit Part # A32649	384 Prep Kit Part # A32646
Volume of PK Buffer to Add	1.2 mL	2.3 mL	8.4 mL

Mix components by inverting the tube/ bottle several times. Do not vortex. Store this solution at -20°C when not in use.

2. **Add 100% isopropanol to Wash Buffer according to the instruction below.**

For miRNA and total RNA isolation: Add 100% Isopropanol to the Wash Buffer in a proportion of 2:1 (Isopropanol: Wash Buffer). To make 10 mL of wash buffer solution, add 6.67 mL of 100% Isopropanol with 3.33 mL of Wash Buffer in a 15 mL conical tube.

For total RNA isolation only: Add 100% Isopropanol to the Wash Buffer in a proportion of 1: 1.5 (Isopropanol: Wash Buffer). To make 10 mL of wash buffer solution, add 4 mL of 100% Isopropanol to 6 mL of Wash Buffer in a 15 mL conical tube.

3. **Prepare Lysis Buffer**

Prepare this solution and use within 10 minutes – discard any unused solution.

For each sample combine 20 µL PK with 400 µL of Lysis Buffer. Example: for ten isolations mix 4.0 mL of Lysis with 200 µL of proteinase K. (It is generally recommended to prepare an additional 10% to account for dead volume.

Note: Pipette enzyme directly into the liquid and pipette mix up and down to remove any residual enzyme from the inside of the tip. A light vortex can be done to ensure homogeneity, but avoid foaming.

4. **Prepare Bind Buffer**

Prepare this solution fresh and per isolation – discard any unused solution. For each sample combine

80 µL Bind Buffer with 600 µL of isopropanol for a total of 680 µL.

5. **To perform the optional DNase step: Prepare DNase solution.**

Prepare this solution fresh and per isolation – discard any unused solution. Combine 80 µL nuclease

free water, 10 µL 10X DNase buffer, and 10 µL of DNase I.

6. **Homogenize up to 10 mg of tissue per 400 µL of Lysis Buffer.**

Note: Please refer to Appendix 1 of the RNAdvance Tissue Kit protocol for recommended homogenization methods and equipment (Agencourt RNAdvance Tissue protocol 000473v003, page 8-10).

Homogenization may be scaled up to any volume using 400 µL per 10mg tissue ratio. A larger lysate volume can be prepared and then split 400 µL lysate into each well or tube for extraction.
Complete homogenization and lysis of the tissue is a highly critical step in the isolation of high quality total RNA.

7. **Transfer 400 µL of homogenized lysate to processing plate (96-Well Riplate-2.2 mL), and seal with plate seal, or to a 1.7 mL microcentrifuge tube.**

8. **Incubate plate/tube in water bath for 25 minutes at 37°C.**

Following incubation lysate may be frozen indefinitely at -80°C.

9. **Shake Bind Buffer vigorously to resuspend magnetic particles before using. Add 680 µL of Bind Buffer and slowly pipette mix 10 times. Incubate at room temperature for 10 minutes.**

10. **Place on magnet for 10-20 minutes or wait until the solution turns completely clear (6-10 minutes for tube format).**

96 well plate format, use Agencourt SPRIPlate 96R Super Magnet Plate

Microcentrifuge tube format – use Agencourt SPRIStand.

11. **Fully remove supernatant from the plate/ tube and discard.**

    This step must be performed while the plate/ tube is situated on the magnet. Avoid disturbing the separated magnetic beads by aiming the pipette tip for the center of the well/ tube bottom. If beads are drawn out, leave a few microliters of supernatant behind. The liquid may be colored due to tissue homogenization.

12. **REMOVE plate/ tube from the magnet and wash by adding 1000 µL of Wash Buffer. Pipette mix 10 times.**

    Try to avoid bubbles while tip mixing.

13. **Return plate/ tube to the magnet for 5 minutes or wait for the solution to turn completely clear.**

14. **Fully remove supernatant from the plate/ tube and discard.**

    This step must be performed while the plate/ tube is situated on the magnet. Avoid disturbing the separated magnetic beads by aiming the pipette tip for the center of the well/ tube bottom. If beads are drawn out, leave a few microliters of supernatant behind. Colored liquid can be due to tissue homogenization.

15. **REMOVE plate/ tube from the magnet and wash by adding 800 µL of 85% ethanol per well and gently pipette mix 5 times.**

16. **Return plate/ tube to the magnet for 5 minutes or wait until the solution to turn completely clear.**

17. **Remove as much ethanol as possible, then, REMOVE the plate/ tube from the magnet.**

    Pipette slowly to avoid aspirating beads.

18. **Optional: Add 100 µL of DNase solution with the plate OFF the magnet. Incubate at room temperature for 1 minute without mixing to hydrate the beads.**

    If the DNase step is not required, skip to step 20. Perform only a total of 3 ethanol washes.

    Pipette mix 5 times to resuspend the beads in the DNase solution.

    Seal and incubate plate/ tube in a 37°C water bath for 15 minutes to facilitate digestion of DNA. DO NOT REMOVE THE DNase SOLUTION.

19. **Add 550 µL of Wash Buffer and pipette mix 5 times. Incubate at room temperature for 4 minutes.**

    Place plate/ tube onto the magnet and separate for 7 minutes or wait until the solution to turn completely clear

20. **Aspirate supernatant and wash by adding 600 µL of 85% Ethanol. Pipette mix 5 times.**

21. **Return plate/ tube to the magnet for 5 minutes or wait for the solution to turn completely clear.**

    Remove ethanol and discard.

22. **Repeat steps #20-21 for a total of 3 ethanol washes (including wash from #15 if omitting DNase step).**

    Remove final ethanol wash completely and allow beads to dry for 5 minutes at room temperature. (3 minutes at room temperature for tube format).

    Beads do not need to be completely dry, but all traces of liquid should be gone (i.e. no remaining droplets or puddles)

23. **Remove plate/ tube from the magnet and elute by adding a minimum of 40 µL of nuclease free water. Pipette mix 10 times and incubate at room temperature for 2 minute.**

24. **Return plate/ tube to the magnet for 2 minutes and carefully transfer eluted RNA away from the beads into fresh plate/ tube for storage.**

BECKMAN COULTER

Life Sciences

Micro RNA and Total RNA Purification from Blood Stabilized in PAXgene Blood RNA Tubes using the Agencourt RNAdvance Blood Kit

Introduction

The RNA content and gene expression profiles of blood samples provide useful information to study and understand both systemic and tissue-specific pathological changes. Here we describe an RNAdvance Blood kit supplemental protocol, that can be used to extract miRNA and total RNA from blood stabilized in PAXgene Blood RNA Tubes. This method uses Beckman Coulter's SPRI (Solid Phase Reverse Immobilization) magnetic bead-based chemistry—which provides an easy, rapid, high yielding, robust and automation-friendly nucleic acid purification procedure that does not require vortexing, centrifugation or filtration steps. This data shows that the RNAdvance Blood method produces high-quality miRNA and RNA in one eluate from PAXgene stabilized blood samples.

Materials and Methods

Blood was collected in PAXgene Blood RNA Tubes (PreAnalytiX) from consenting healthy adults, stored for 20–24 hours at room temperature and frozen at -20°C. The tubes were thawed for 2 hours at room temperature before processing. 400 µL of PAXgene blood was digested with 300 µL lysis buffer containing 20 µL of proteinase K at 37°C for 15 minutes. RNA was extracted following the instructions for the RNAdvance Blood miRNA supplemental protocol (Beckman Coulter document #AAG-567SP10.14-A). The RNA concentration and purity were measured with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). The RNA purity was determined by the OD260/OD280 and OD260/OD230 ratios. 1 µL of the 1:10 diluted RNA sample was analyzed by an Agilent RNA 6000 Pico chip (Agilent Technologies, 5067-1513) using the 2100 Bioanalyzer (Agilent Technologies) to determine RNA integrity. miR16, let7c and RNU44 miRNA gene expression was determined by TaqMan® microRNA assay (Life Technologies 4427975, assay ID002171, assay ID000379 and assay ID001094 respectively). For messenger RNA gene expression, cDNA was synthesized using a B2M, ACTB or HPRT1 gene specific reverse primer (TCTGCT-CCCCACCTCTAAGT, CACCTTCACCGTTCCAGTTT and AACAATCCGCCC AAAGGGAA respectively). PCR products were amplified using a primer probe mix cocktail. B2M (forward primer, GGACTGGTCTTTCTA-TCTCTTGTAC; reverse primer, ACCTCCATGATGC-TGCTT AC; probe CTGCC TGTGAACCATGTGACTTTG). ACTB (forward primer ACAGAGCCTCGCCTTTG, reverse primer CCTTGCACATGCCGGAG, probe TCATCCATGGTGAGCTGGCGG). HPRT1 (forward primer AGA TGGTCAAGGTCGCAAG, reverse primer GTATTCATTATAGTCAAGGGCATATCC, probe TGGTGAAAAGGACCC CACGAAGT). 50 ng of total RNA was used for the reverse transcription reaction using the TaqMan® microRNA Reverse Transcription kit (Life Technologies, 4366596) and 1.33 µL of cDNA was used per PCR reaction in triplicate using TaqMan® Universal Master Mix II (Life Technologies, 4440038).

BECKMAN COULTER
Life Sciences

Results and Discussion

Summary of RNA Yields from PAXgene Blood Using the RNAdvance Blood Kits

Four PAXgene Blood samples from 4 different donors were used to evaluate RNA yield, purity and quality. A total of 12 samples were extracted for total RNA and miRNA. The RNA was eluted in 40 µL of nuclease free water. The results show that the average RNA concentration was at 29.18+/-3.56 ng/µL (range between 21.3 µg/µL to 33.6 µg/µL) with an average yield at 1.2 µg (range between 852 ng to 1344 ng). The average OD260/OD280 ratio for all 12 samples was at 2.1 (range between 2.05–2.2), and the average OD260/OD230 ratio was at 1.8 (range between 1.42–1.98). The RIN (RNA Integrity Number) scores for 12 samples were from 7.0–8.5 (Table 1).

Table 1. The Average Yield and Purity. Calculated from a total of 12 samples from 4 different donors.

Average Concentration (ng/µL)	Average Yield (µg)	Average OD260/ OD280	Average OD260/ OD230	RIN Scores
29.18+/-3.56	1.2+/-0.16	2.1+/-0.05	1.8+/-0.14	7.0-8.5

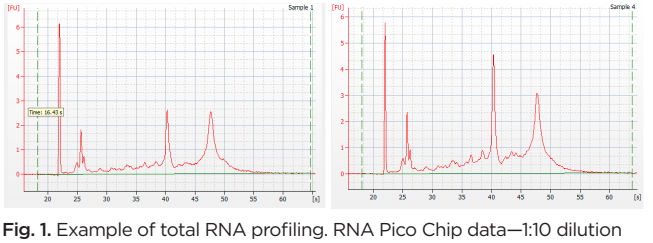


Fig. 1. Example of total RNA profiling. RNA Pico Chip data—1:10 dilution PAXgene Blood RNA samples.

Micro RNA and Messenger RNA Gene Expression Indicating miRNA and Total RNA Were Successfully Extracted from PAXgene Blood Samples

50 ng of total RNA was used to determine miRNA and messenger RNA gene expression. For miRNA gene expression, let-7c, miR16 and RNU44 were used for evaluation. The average cycle threshold (Ct) was calculated from triplicates in each donor. The average Ct value for let-7c gene expression was 22.13+/-0.024, miR16 gene expression was 16.77+/-0.004 and RNU44 gene expression was 22.47+/-0.004 (Figure 2). The minus RT and controls with no template showed no amplification, indicating that the amplification resulted from miRNA alone (data not shown). For messenger RNA gene expression, B2M, ACTB and HPRT1 genes were used for evaluation, with the results shown in Figure 3. The average Ct value for B2M gene expression was 22.98+/-0.015, ACTB gene expression was 24.69+/-0.042 and HPRT1 gene expression was 31.85+/-0.045. The minus RT and controls with no template showed no amplification, indicating that the amplification resulted from messenger RNA alone (data not shown).

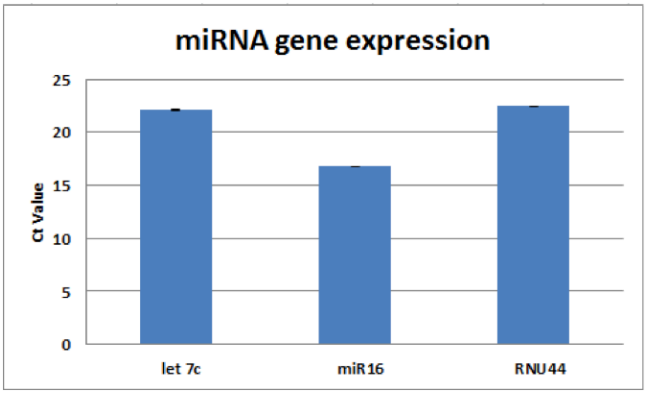


Fig. 2. Average Ct value for the let-7c, miR16 and RNU44 gene expression. The average Ct value was calculated from two different samples from the same donor.

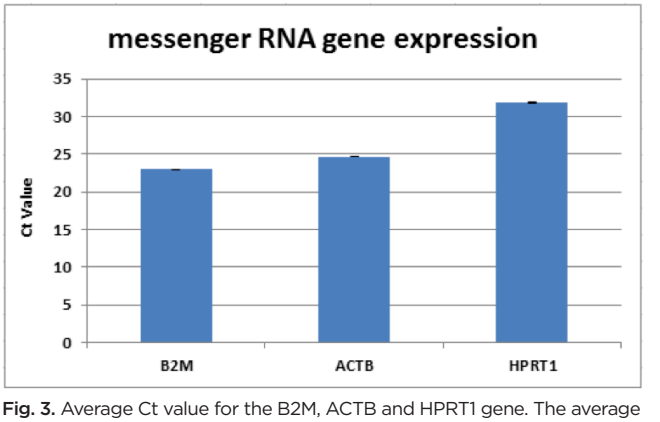


Fig. 3. Average Ct value for the B2M, ACTB and HPRT1 gene. The average Ct value was calculated from two different samples from the same donor.

Conclusions

The data from this study demonstrates that the RNAdvance Blood Kit provides high-quality RNA and miRNA from PAXgene stabilized blood samples. The magnetic bead-based extraction protocol provides automation-friendly, scalable throughput and does not require vortexing, centrifugation, or filtration steps.

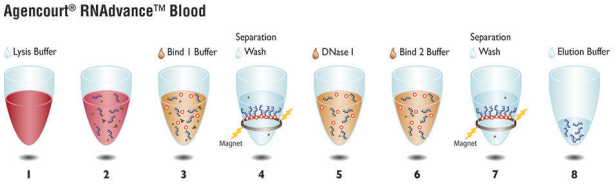
Author

Bee Na Lee, PhD, *Staff Application Scientist*
Beckman Coulter Life Sciences

Agencourt RNAdvance Blood Kit

Supplemental Protocol for Micro RNA and Total RNA Isolation from PAXgene Preserved Blood

Process Overview



Introduction

The Agencourt RNAdvance Blood RNA purification kit utilizes Beckman Coulter's patented Agencourt SPRI paramagnetic bead-based technology to isolate total RNA from PAXgene preserved blood. The protocol is modified to isolate micro RNA (miRNA) and total RNA from 400 µL of PAXgene preserved blood per well in 96-well or 2 mL tube formats.

Note for miRNA and Total RNA Extraction

If the RNAdvance Blood kit is to be used for miRNA and total RNA isolation:

- **100% Isopropanol should not be added directly to the Wash Buffer bottle.** See *Reagent Preparation* for Wash Buffer preparation.
- 500 µL of Isopropanol is added in the Bind 1 at Step 4 instead of 400 µL (also see Step 3 of *Reagent Preparation*).
- Add 400 µL of Isopropanol in the Bind 2 Buffer at Step 16.
- 85% ethanol should be used instead of 70% ethanol in all ethanol washes.

General Remarks on Handling RNA

RNases are ubiquitous and general precautions should be followed in order to avoid their introduction to your sample during the Agencourt RNAdvance Blood procedure. The most common sources of RNase contamination are hands, dust particles, and contaminated laboratory instruments, solutions and glassware. When working with RNA, the following procedures should be followed to limit RNase contamination:

- Always work with gloved hands and change gloves frequently.
- Use RNase-free, filtered pipette tips for pipetting whenever possible.
- Use dedicated RNase-free equipment, e.g. pipettes, pipette tips, gels boxes, etc.
- Avoid using reagents, consumables and equipment that are in common use for other general lab processes.
- When available, work in a separate room, fume hood or lab space.
- Use plastic, disposable consumables that are certified RNase-free.
- Purchase reagents, such as commonly used buffers and water, that are certified RNase-free.
- Prepare small individual aliquots of such buffers to avoid repeated transfer out of stock buffers. This lowers the risk of contaminating the stock solution.
- Wipe down work surfaces with commercial RNase-inhibiting surfactant solutions or 85% ethanol before starting work.

BECKMAN COULTER
Life Sciences

Materials Supplied by the User

Consumables and Hardware:

- Agencourt SPRIPlate 96R-Ring Super Magnet Plate (Beckman Coulter Life Sciences A32782) or Agencourt SPRIStand—Magnetic 6-Tube Stand for 1.7 mL Tubes (Beckman Coulter Life Sciences A29182)
- 1.7 mL microcentrifuge tubes (Fisher Scientific, NC9448938) for tube format
- 96-Well Riplate—2.2 mL (Ritter Medical, 43001-0200) for plate format
- 1.2 mL 96-well plate (ABGene #AB-1127; http://www.abgene.com)
- 37°C and 55°C water bath or heat block for proteinase K digestion and DNase treatment
- Plate Seals (ABGene #0580; http://www.abgene.com)

Reagents:

- 100% Isopropanol (American Bioanalytical AB07015 or equivalent)
- 85% ethanol; freshly prepared/diluted from 100% ethanol (American Bioanalytical, AB00138 or equivalent)
- DNase I—RNase-free (Ambion® AM2222 or AM2224)
- Reagent-grade water, nuclease-free (Ambion® AM9932)

Calculation of Yield

To determine yield of RNA, Beckman Coulter recommends using an OD260 measurement. To determine RNA quality, dilute samples to 1–2 ng/µL for analysis using the Agilent 2100 Bioanalyzer PicoChip assay.

Reagent Preparation

Prepare the following reagents in advance for both the 96-well and 2 mL tube protocols:

1. **Add PK Buffer to the Proteinase K tube/bottle. (Final concentration is 50 mg/mL.)**

   For the 50 prep kit, add 1.2 mL of PK Buffer per tube of Proteinase K.

   For the 384 prep kit, add 10 mL of PK Buffer to the bottle of Proteinase K.

   Mix components by inverting the tube/bottle several times. To avoid foaming, do not vortex. The solution will appear cloudy immediately after mixing—let the solution sit for 5 minutes to clear prior to using. *Store the Proteinase K solution at -20°C when not in use.*

2. **Wash Buffer Preparation.**

   For miRNA and Total RNA Isolation—Add 100% Isopropanol to the Wash Buffer in a proportion of 1:1 (Isopropanol: Wash Buffer). Example: To make 10 mL of Wash Buffer solution, add 5 mL of 100% Isopropanol with 5 mL of Wash Buffer in a 15 mL conical tube, and vortex thoroughly for 10 seconds.

   For Total RNA Isolation Only—Add 100% Isopropanol to the Wash Buffer in a proportion of 1:1.5 (Isopropanol: Wash Buffer). To make 10 mL of Wash Buffer solution, add 4 mL of 100% Isopropanol with 6 mL of Wash Buffer in a 15 mL conical tube, and vortex thoroughly for 10 seconds.

3. **Prepare Bind 1/Isopropanol Solution.**

   Prepare this Solution Fresh and Per Isolation—Discard Any Unused Solution. 510 µL of Bind 1/Isopropanol Solution are required per sample. Vortex the tube containing Bind 1 Buffer for at least 30 seconds to fully resuspend the beads. Combine 10 µL Bind 1 Buffer with 500 µL 100% Isopropanol and mix thoroughly.

4. **Prepare DNase Solution.**

   100 µL of 1X DNase solution are required per sample. Combine 80 µL nuclease-free water, 10 µL 10X DNase buffer, and 10 µL of DNase I. Make this solution fresh for each set of samples.

RNAdvance Blood miRNA Isolation Procedure

Thaw frozen tubes of PAXgene blood at room temperature. Cap the tubes tightly, then mix by inverting each tube several times or by vortexing.

1. **Aliquot 400 µL of PAXgene preserved blood into each well of a 2.2 mL processing plate or 1.7 mL tube.**

2. **Add Proteinase K and Lysis Buffer.**
   - Add 20 µL of Proteinase K (50 mg/mL, See *Reagent Preparation*, Step 1)
   - Add 300 µL of Lysis Buffer

   Mix thoroughly by pipetting up and down 10 times.

3. **Lysis and Protein Digestion.**

   Seal plate with a plate seal. Incubate samples in water bath at 55°C for 15 minutes. Before proceeding to the next step, let the samples cool for 2 minutes to room temperature.

   Note: *When using this plate in conjunction with a water bath, make sure the plate does not tip over and the seal does not get wet. Should the seal get wet or condensation form on the seal, spin the liquid down and very carefully remove the seal.*

4. **Shake Bind 1 Bottle vigorously to resuspend magnetic particles before using.**

   Prepare Bind 1/Isopropanol Solution as described in Reagent Preparation, *Step 3. Add 510 µL of Bind 1/ Isopropanol Solution to the samples and pipette mix 10 times. Incubate samples at room temperature for 5 minutes.*

5. **Place 2.2 mL processing plate on Agencourt SPRIPlate 96R-Ring Super Magnet Plate (or 1.7 mL tubes on SPRIStand) and separate for 15 minutes or wait for the solution to turn completely clear.**

6. **Fully remove supernatant from the 2.2 mL processing plate (or 1.7 mL tube) and discard.**

   This step must be performed while the 2.2 mL processing plate is situated on the magnet. *The following technique is recommended when working with opaque supernatant:* Place the pipette tip on the side of the well and carefully aspirate the liquid by following the liquid level down until approximately 200–250 µL remains in the well. Next, carefully place the pipette tip at the center of the bottom of the well, and slowly aspirate the remaining liquid, revealing the ring of beads.

7. **Remove the 2.2 mL processing plate (or 1.7 mL tube) from the magnet and wash the beads by adding 800 µL of Wash Buffer.**

   Pipette mix for 10 times. (Isopropanol must be added to Wash Buffer—See Reagent Preparation, *Step 2).* Pipette mix 10 times to resuspend the magnetic beads.

8. **Transfer the suspension to a 1.2 mL processing plate.**

   Be sure to transfer all of the sample solution and magnetic beads to the new plate (Skip this step if using tube format). Transferring the samples to the smaller plate allows for easier pipetting in subsequent steps.

9. **Place 1.2 mL processing plate (or 1.7 mL tube) on the magnet and separate for 7 minutes or wait for the solution to turn completely clear.**

10. **Completely remove supernatant from the 1.2 mL processing plate (or 1.7 mL tube) and discard.**

    This step must be performed while the plate is situated on the magnet. Do not to disturb the ring of separated magnetic beads.

11. **Remove the 1.2 mL processing plate (or 1.7 mL tube) from the magnet and add 800 µL of 85% ethanol.**

    Pipette mix 10 times to resuspend the magnetic beads.

12. **Return 1.2 mL processing plate (or 1.7 mL tube) to the magnet for 3 minutes or wait for the solution to turn completely clear.**

13. **Remove as much ethanol as possible and allow magnetic beads to dry for 3 minutes at room temperature.**

    Pipette slowly to avoid disturbing the beads. If too much ethanol is present (more than 5 µL), the DNase digestion will be inhibited, thereby affecting downstream applications.

14. **Remove the 1.2 mL processing plate from the magnet and add 100 µL of DNase solution**

    (See *Reagent Preparation*, Step 4).

    Pipette mix 5 times carefully—avoid bubbles and foaming.

15. **Seal plate with a plate seal and incubate 1.2 mL processing plate (or 1.7 mL tube) in water bath for 15 minutes at 37°C.**

16. **DO NOT REMOVE THE DNase SOLUTION.**

    Add 200 µL of Bind 2 Buffer plus 400 µL of 100% Isopropanol and pipette mix 10 times. Incubate at room temperature for 5 minutes.

    Note: *Do not mistake Bind 2 for Bind 1.*

17. **Place 1.2 mL processing plate (or 1.7 mL tube) onto the magnet for 5 minutes or wait for the solution to turn completely clear.**

18. **Remove supernatant and discard.**

    Wash by adding 800 µL of 85% ethanol. Pipette mix 10 times. Let sit for approximately 3 minutes and then remove ethanol while processing plate remains situated on the magnet plate.

19. **Repeat Step 18 one more time for a total of 2 ethanol washes.**

20. **Allow magnetic beads to dry for 3 to 5 minutes at room temperature.**

    Beads do not need to be completely dry, but the traces of liquid should be gone (i.e., droplets or puddles).

21. **Remove 1.2 mL processing plate (or 1.7 mL tube) from the magnet and elute RNA by adding 20 µL to 40 µL of nuclease-free water.**

    Pipette mix 10 times and incubate at room temperature for 2 minutes. On average, a 20 µL elution will produce a 20–50 ng/µL solution of RNA.

22. **Return the plate to the magnet for 2 minutes and carefully transfer eluted RNA away from the beads and into a fresh plate for storage.**

BECKMAN COULTER
Life Sciences

MicroRNA Extraction from Exosomes using Beckman's Agencourt RNAdvance Cell v2 Kits

Randy P. Carney[1], Bee Na Lee, Sidhartha Hazari[1], Alisha Knudson[1], and Kit S. Lam[1]
[1]University of California, Davis and Beckman Coulter Life Sciences

Summary

Exosomes are cell-derived vesicles that are present in different types of biological fluids, such as blood, urine, and cultured medium of cell cultures. Exosomes are typically defined as those nano-sized vesicles (bewteen ~30 nm-150 nm diameter) released from the cell when multi-vesicular bodies fuse with the plasma membrane, although some similarly sized vesicles may be released directly from the plasma membrane and co-purified along with exosomes. These circulating extracellular vesicles carry a variety of functional components including DNA, proteins, lipids, RNA, non-coding RNA and microRNA. The delivery of miRNA and other molecules can function as intercellular communication vehicles by transferring these signaling molecules between specific cells. Therefore, exosomes have the capacity to influence a larger diversity of genes' expressions and regulate biological pathways such as tumorigenesis and immune response. Studying exosome gene expression profiling becomes important and could shed light on the cancer-associated RNA biomarkers for cancer diagnostics and therapeutics.

This application note describes the purification of miRNA and RNA from exosome samples isolated from cell culture medium, using Beckman's Agencourt RNAdvance Cell v2 kit. The method uses Beckman Coulter's SPRI (Solid Phase Reverse Immobilization) paramagnetic bead-based chemistry, which provides an easy, rapid, high yielding, robust and automation-friendly nucleic acid purification procedure that does not require vortexing, centrifugation or filtration steps. The data shows that miRNA and coding RNA was successfully extracted from exosomes.

Materials and Methods

Exosomes from IMR-90 and Jurkat cells were isolated via differential ultracentrifugation procedure. Cells were grown to at least 10% confluency before adding exosome collection media (EMEM for IMR-90, RPMI 1640 for Jurkat cells) containing 10% FBS. Endogenous bovine exosomes from the FBS were pelleted and removed prior to cell incubation by ultracentrifugation at 100,000 x g for 18 hours (Beckman Coulter Optima L-80 XP Preparative Ultracentrifuge, SW 32 Ti rotor). Cells were incubated for about 24-48 hrs until the cell growth reached a plateau. The cultured media was removed and subjected to a series of centrifugation steps (Sorvall Legend X1R centrifuge, Fiberlite F15-8x50cy fixed-angle rotor) as described: low speed spins at 300 x g for 10 min, 2000 x g for10 min, and 10,000 x g for 30 min. Supernatant from each step of centrifugation was discarded to remove cells, dead cells, and cell debris respectively. Cleared supernatant was concentrated by a factor of 10 and ultracentrifuged at 100,000 x g for 70 mins (Beckman Coulter Optima L-80 XP Preparative Ultracentrifuge, SW 32 Ti rotor). Pellet was re-suspended in ~24mL of fresh 1X PBS to re-fill the centrifuge tube and the 100,000 x g, 70 min ultracentrifugation step was repeated once more to remove co-precipitated free protein, such as BSA. This final exosome-containing pellet was re-suspended in ~100 µL of fresh 1 X PBS and a small aliquot (1µL) was diluted x5 and lysed (RIPA Lysis Buffer, Upstate® Cat.#20-188) for protein concentration measurement by BCA assay

AAG-839APP03.15-A

(Pierce® BCA Protein Assay Kit, Cat.#23225). A second aliquot was diluted 1500x in freshly filtered (via 0.1 µm Nylon syringe filter) PBS and exosome size distribution and number concentration measured by Nanoparticle Tracking Analysis (NTA, NanoSight LM10) at room temperature. 10µL of the frozen purified exosome (protein concentration 1.5mg/mL) was digested with 63 µL of lysis buffer and 3µL of proteinase K for 30 min at room temperature and RNA was extracted using an RNAdvance Cell v2 kit (Beckman Coulter, A47942). Two samples were used for miRNA extraction using the RNAdvance Cell v2 miRNA supplemental protocol without DNase treatment (Beckman Coulter, AAG-850SP03.15-A). Samples were eluted in 25µL of nuclease free water in the final elution step. 1µL of RNA was analyzed using an Agilent RNA 6000 Pico chip and a small RNA chip (Agilent Technologies, 5067-1513 and 5067-1548) on the 2100 Bioanalyzer (Agilent Technologies). miRNA (let7c, miR16, miR21, miR155 and RNU44) gene expression was determined using a Taqman microRNA assay (Life Technologies, assay ID000379, 000391, 000397, 002623 and 001094 and ID001006). 1µL of eluted RNA was used for the reverse transcription reaction using the TaqMan micro RNA Reverse Transcription Kit (Life Technologies, 4366596) and 1.33µL of cDNA was used per 20 µL PCR reaction in triplicate using Taqman Universal Master Mix II (Life Technologies, 4440038). For messenger RNA gene expression, 1µL of eluted RNA was used for cDNA synthesis using a random primer (Life Technologies, 4368814), and 1.33 µL of the cDNA was used for a 20µL PCR reaction using prime time qPCR assays (Integrated DNA Technologies). The primer probe assay ID's used for the ACTB, B2M, GAPDH and HPRT1 were Hs.PT.39a.22214847, Hs.PT.39a.22214845, Hs.PT.39a.22214836 and Hs.PT.39a.22214821 respectively.

Results and Discussion

Size distributions of exosomes isolated from IMR-90 and Jurkat cells.

Nanoparticle Tracking Analysis (NTA) was used to confirm the size range of the isolated exosomes. Ultracentrifuge pelleted exosomes were diluted serially in increments of 10 (in 1X PBS, freshly sterile filtered through 0.01um nylon syringe filters several times) to reach an optimal particle concentration as measured by NTA in the range of 10^6 to 10^8 particles/mL. The size distribution of exosomes from IMR90 and Jurkat are presented in Figure 1, and cover the range of 30-100 nm as expected for exosomes.

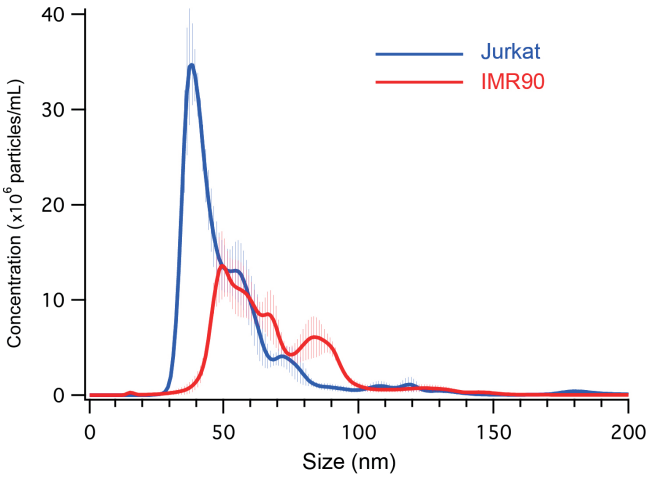


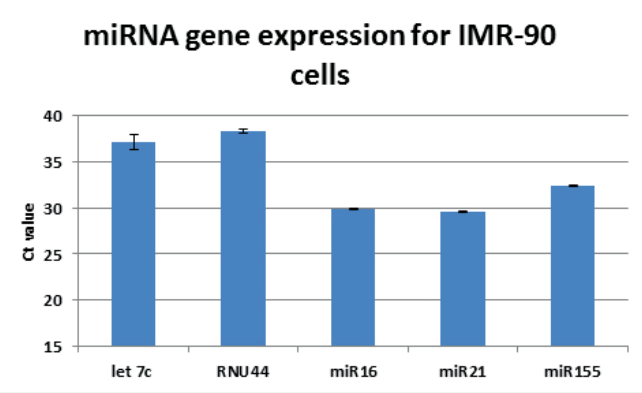
Figure 1: Size distribution of exosomes using Nanoparticle Tracking Analysis.

MiRNA gene expression data demonstrates that miRNA was successfully extracted from exosome samples.

1µL of eluted nucleic acid was used for let 7c, RNU44, miR16, miR21 and miR155 gene expression. The average cycle threshold (Ct) was calculated from triplicate samples. The average Ct values for let 7c, RNU44, miR16,miR21 and miR155 target gene expression in IMR-90 cells were 37.13+/-0.784, 38.32+/-0.21, 29.85+/-0.08, 29.60+/-0.05 and 32.39+/-0.06 respectively (Figure 2 Left). The average Ct values for let 7c, RNU44, miR16,miR21 and miR155 target gene expression in Jurkat cells were 36.97+/-0.63, 37.02+/-0.07, 27.52+/- 0.07, 33.37+/-0.38 and 35.29+/-0.37 respectively (Figure 2 Right). This result indicates that miRNA was successfully extracted from exosomes harvested from cell culture medium utilizing the RNAdvance Cell v2 kit. The results showed that miR16 was the most highly expressed genes and let 7c and RNU44 were lowest expressed genes in these exosomes.

RNA gene expression data demonstrates that messenger RNA was successfully extracted from exosome samples.

1µL of eluted nucleic acid was used for ACTB1, B2M, GAPDH and HPRT gene expression to determine messenger RNA was successfully



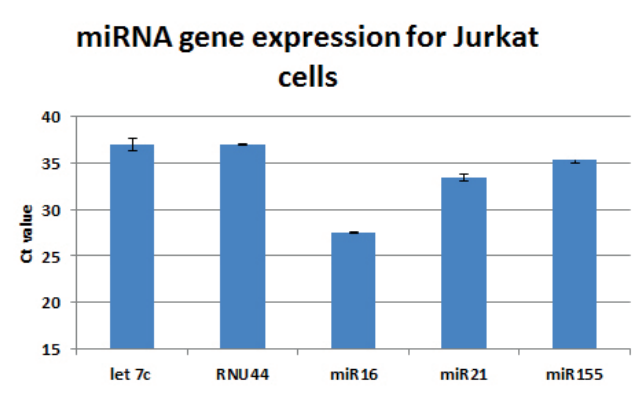
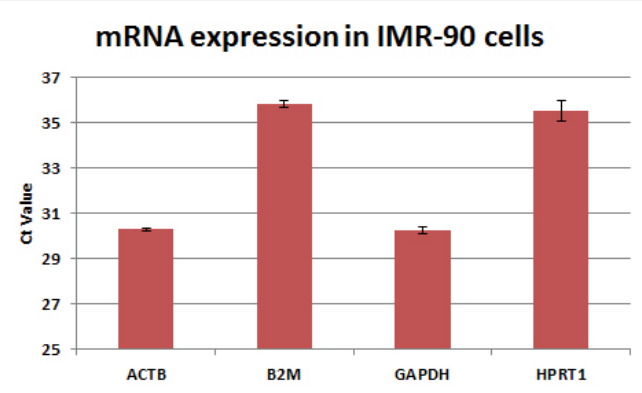


Figure 2: miRNA gene expression from cultured medium of IMR-90 cells (Left) and Jurkat cells (Right).



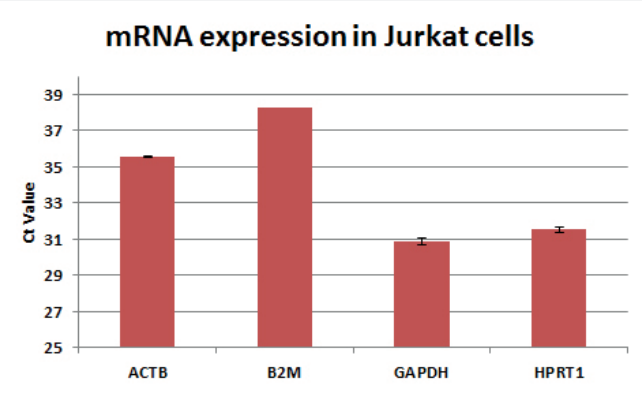


Figure 3: mRNA gene expression from cultured medium of IMR-90 cells (Left) and Jurkat cells (Right).

extracted from exosomes. The average cycle threshold (Ct) was calculated from triplicate samples. The average Ct values for ACTB, B2M, GAPDH and HPRT1 gene expression in IMR-90 cells were 30.27±0.075, 35.79+/-0.157, 30.25+/-0.161 and 35.50+/-0.442 respectively (Figure 3, Left). The average Ct values for ACTB1, B2M, GAPDH and HPRT1 gene expression in Jurkat cells were 35.53±0.016, 38.30, 30.86+/-0.187 and 31.51+/-0.179 respectively (Figure 3, Right). This result indicates that messenger RNA was successfully extracted from exosomes harvested from a cultured medium of cell cultures utilizing the RNAdvance Cell v2 kit. Among these genes, B2M has the lowest amount detected and GAPDH showed the highest expression in both cell lines.

Conclusions

The data from this study shows that the Beckman's RNAdvance Cell v2 Kit can be used for miRNA and RNA extraction from exosomes The magnetic bead based extraction protocol provides scalable throughput and it is automation-friendly.

Acknowledgement

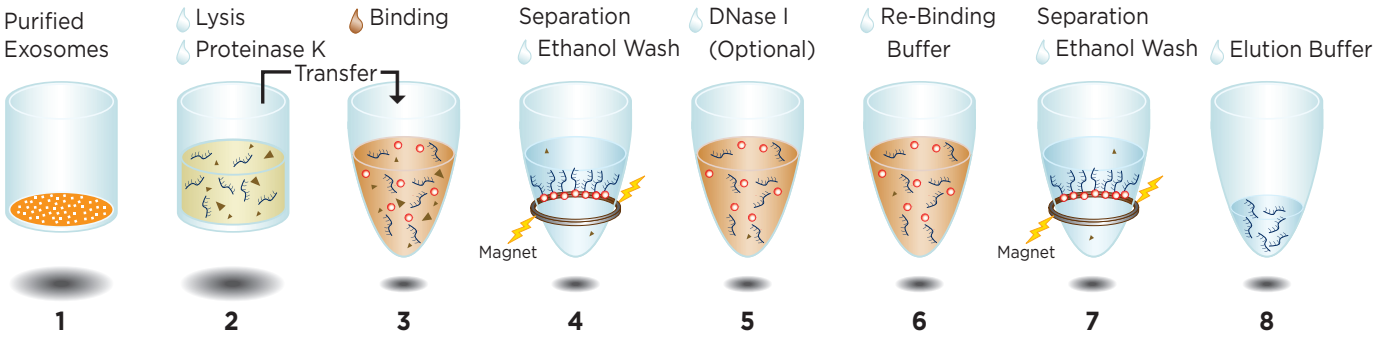
Exosomes were provided by UC Davis. Randy P. Carney would like to acknowledge financial support from the T32 HL07013 training grant.

BECKMAN COULTER
Life Sciences

AAG-839APP03.15-A

Agencourt® RNAdvance® CELL v2 Kit Supplemental Protocol for miRNA and total RNA Isolation from Exosomes

Process Overview



Introduction

The Agencourt RNAdvance Cell v2 purification kit utilizes Beckman Coulter's patented Agencourt SPRI® paramagnetic bead-based technology to isolate total RNA from cultured eukaryotic cell lines. The protocol is modified to isolate micro RNA (miRNA) and total RNA from 10-50 µL of exosomes isolated from cultured medium or body fluids in 96 well or 1.7 mL tube formats.

Notice for miRNA and total RNA Isolation

- If it is planned to use the RNAdvance Cell v2 kit for miRNA plus total RNA isolation, **100% Isopropanol must not be added directly to the Wash Buffer bottle**. See Reagent Preparation, step 4 for wash buffer preparation. A 1:2 ratio of Wash buffer: 100% Isopropanol is used for miRNA plus total RNA isolation.
- Binding buffer conditions: 80µL of Bind buffer + 250µL of Isopropanol. Use 330µL in the Binding step.
- Use 85% ethanol for all ethanol washing steps.

Materials Supplied by the User

Consumables and Hardware:

- Agencourt SPRIPlate 96R – Ring Super Magnet Plate (Beckman Coulter Life Sciences, A32782) or Agencourt SPRIStand – Magentic 6-tube Stand (for 1.7 mL tubes) (Beckman Coulter Life Sciences, A29182)
- ABgene 1.2mL 96-Well Storage Plate, Square Well, U-Bottomed (ABGene #1127) for plate format
- 1.7 mL microcentrifuge tubes (Fisher Scientific, NC9448938) for tube format

Reagents:

- 100% Isopropanol; American Bioanalytical AB07015 or equivalent
- 85% ethanol; freshly prepared/diluted from 100% ethanol (American Bioanalytical, AB00138 or equivalent)
- DNase I (RNase-free); Ambion AM2222 or AM2224
- Reagent grade water, nuclease-free; Ambion AM9932

BECKMAN COULTER
Life Sciences

General Remarks on Handling RNA

RNases are ubiquitous and general precautions should be followed in order to avoid their introduction to the sample during the Agencourt RNAdvance Blood procedure. The most common sources of RNase contamination are hands, dust particles, and contaminated laboratory instruments, solutions and glassware. When working with RNA, the following procedures should be followed to limit RNase contamination:

- Always work with gloved hands and change gloves frequently.
- Use RNase free, filtered pipette tips for pipetting whenever possible.
- Use dedicated RNase free equipment, e.g. pipettes, pipette tips, gels boxes, etc.
- Avoid using reagents, consumables and equipment that are in common use for other general lab processes.
- When available, work in a separate room, fume hood or lab space.
- Use plastic, disposable consumables that are certified RNase free.
- Purchase reagents, such as commonly used buffers and water, that are certified RNase free.
- Prepare small individual aliquots of such buffers to avoid repeated transfer out of stock buffers. This lowers the risk of contaminating the stock solution.
- Wipe down work surfaces with commercial RNase inhibiting surfactant solutions or 85% ethanol before starting work.

Reagent Preparation

1. For each new kit, assemble Proteinase K once. Mark each tube or bottle with the date of assembly. Mix components by inverting the tube/bottle several times. To avoid foaming, do not vortex. The solution will appear cloudy immediately after mixing – let the solution sit for 5 minutes to clear prior to using. **Store the Proteinase K solution at -20° C when not in use.**

	Proteinase K Solution (50 mg/mL) Volume of PK Buffer to add to lyophilized Proteinase K
96 Prep kit #001354 / A47942	400 µL
960 Prep kit #001355 / A47943	4 mL
Storage Condition	-20˚C

2. Wash Buffer Preparation:

For miRNA and total RNA isolation: Add 100% Isopropanol to the Wash Buffer in a proportion of 1:2 (Wash Buffer: isopropanol). To make 10 mL of wash buffer solution, add 6.67 mL of 100% Isopropanol with 3.33 mL of Wash Buffer in a 15 mL conical tube and vortex thoroughly for 10 seconds.

For total RNA isolation only: Add 100% Isopropanol to the Wash Buffer in a proportion of 1.5: 1 (Wash Buffer: isopropanol). To make 10 mL of wash buffer solution, add 4 mL of 100% Isopropanol with 6 mL of Wash Buffer in a 15 mL conical tube and vortex thoroughly for 10 s.

3. Prepare fresh 85% ethanol with nuclease free water. (Note: 85% ethanol is hygroscopic. Fresh 85% ethanol should be prepared for optimal results).

4. Prepare Bind Buffer Solution – Prepare this solution fresh and discard any unused solution.

330 µL of Bind/Isopropanol solution are required per sample. Vortex the tube containing the Bind Buffer magnetic beads for at least 30 seconds to fully resuspend the beads. Combine 80µL Bind Buffer with 250 µL 100% isopropanol and mix thoroughly.

5. Prepare DNase Solution – Prepare this solution fresh-discard any unused solution.

25 µL of 1X DNase solution are required per sample. Combine 20 µL nuclease-free water with 2.5 µL 10X DNase buffer, and 2.5 µL of DNase I and mix thoroughly by pipetting or inverting the tube.

6. Prepare Lysis/PK Solution – USE WITHIN 30 MINUTES.

63 µL of Lysis/PK of solution are required per sample. Combine 3 µL PK (50 mg/mL) with 60 µL of Lysis Buffer, for a total of 63 µL Lysis/PK Solution. Mix gently to avoid creating bubbles.

Procedure

1. Add 63 µL of Lysis/PK Solution (see Reagent Preparation, step 6) **to each sample either in a tube or 96-well plate.** Gently pipette tip mix 10 times at the bottom of the well to resuspend the exosomes suspension.

2. Incubate the samples for 30 minutes at room temperature to complete the lysis and digestion.

3. Add 330 µL of Bind Buffer Solution to each sample and pipette tip mix 10 times or until homogeneous.

4. Incubate the samples for 5 minutes at room temperature to bind nucleic acids.

5. Place the sample plate on an Agencourt SPRIPlate 96R – Ring Super Magnet Plate or Magnetic Stand for 5 minutes or wait for the solution to turn completely clear.

Carefully aspirate and discard the supernatant while the plate is situated on the magnet.

When aspirating, place the pipette at the center of the well to avoid disturbing the magnetic beads.

6. Take the plate off the magnet. Add 300 µL of Wash Buffer. (See Reagent Preparation, step 4.) **Pipette tip mix 10 times, or until the magnetic particles are fully resuspended.**

It is normal for a few bead clumps to remain after resuspension.

7. Place the plate back on the magnet for 5 minutes, or wait for the solution to turn completely clear. Fully remove and discard the supernatant while the plate is situated on the magnet.

When aspirating, place the pipette at the center of the well to avoid disturbing the magnetic beads.

8. Take the plate off the magnet. Add 300 µL of 85% ethanol. Gently pipette tip mix 5 times, or until beads are fully resuspended.

9. Place the plate back on the magnet for 5 minutes, or wait for the solution to turn completely clear. Thoroughly remove and discard as much of the ethanol wash as possible.

Excess ethanol can reduce the activity of DNase during the next steps.

OPTIONAL DNAse treatment: Skip steps 11-15 if DNase treatment is not required.

11. Take the plate off the magnet. Add 25 µL of DNase Solution (see Reagent Preparation, step 5) **and pipette tip mix 10 times, or until the beads are fully resuspended.**

The addition of aqueous DNase releases DNA and RNA from the beads. DNA will be digested and the RNA will need to be re-bound to the beads later in the protocol.

12. Incubate the sample plate at room temperature for 15 minutes to complete the DNase digestion.

13. DO NOT REMOVE THE DNase SOLUTION. Add 165 µL of Wash Buffer to each sample and pipette tip mix 10 times, or until homogeneous.

During this step, Wash Buffer re-binds RNA to the beads. Additionally, the Wash Buffer helps to dissolve and rinse away proteins and other contaminants.

14. Incubate the plate at room temperature for 5 minutes to bind.

15. Place the plate on the magnet for 5 minutes or wait for the solution to turn completely clear. Remove and discard the supernatant.

16. Take the plate off the magnet. Wash the beads by adding 300 µL of 85% ethanol.

Pipette tip mix 5 times, or until beads are fully resuspended.

Ethanol washes remove salt, Wash Buffer and any residual contaminants.

17. Place the sample plate on the magnet for 5 minutes or wait for the solution to turn completely clear. Remove ethanol and discard.

18. Repeat steps 16-17 one more time for a total of 2 ethanol washes.

19. Remove as much of the final ethanol wash as possible. Allow the beads to dry for 3-5 minutes at room temperature while the sample plate is on the magnet.

Any droplets or puddles of liquid should be gone before continuing to the next step.

20. Take the plate off the magnet. Elute the RNA by adding 25-40 µL of nuclease free water. Pipette tip mix 10 times and incubate at room temperature for 5 minutes to complete elution.

21. Place the plate back on the magnet for 2 minutes, wait for the solution to turn completely clear. Transfer the clear RNA solution to a new plate or new tubes for storage (-20° C).

If beads are aspirated during the transfer, dispense the eluant back into the well and let the beads settle longer on the magnet to better compact the bead ring.

For Beckman Coulter's worldwide office locations and phone numbers, please visit www.beckmancoulter.com/contact

AAG-850SP03.15-A

Automation of Micro RNA and Total RNA Purification from Tissues Using the Agencourt RNAdvance Tissue Kits and Biomek Span-8 Liquid Handler

Summary

Micro RNAs are small, naturally occurring non-coding ribonucleic acids with sizes between 18 and 40 nucleotides (nts) that have been demonstrated to play a significant role in the regulation of gene expression. As a result, interest in smaller RNA species such as miRNA has increased. This application note describes the purification of miRNA and total RNA from fresh-frozen tissue samples using the Beckman Coulter SPRI (Solid Phase Reverse Immobilization) magnetic bead-based chemistry and the Biomek automated extraction method. The RNAdvance miRNA Tissue 96 demonstrated method enables automated purification of total RNA, including miRNA and other small RNAs, from 1 to 96 samples on a Biomek Span-8 Workstation. Total RNA and miRNA can be purified from very small amounts of animal tissue as starting materials. The Biomek automated SPRI method is an easy, high-yielding and robust nucleic acid purification process that does not require centrifugation and vacuum filtration steps. Purified nucleic acids are easily eluted from the magnetic beads under aqueous conditions, which provide maximum flexibility for downstream applications. The data shows that the samples extracted using the Biomek gave comparable RNA yield, miRNA and messenger RNA gene expression as compared to samples extracted manually.

Materials and Methods

Rat liver tissue was homogenized using a Precellys 24 (Bertin) homogenizer. Eighty to 100 mg of tissue was homogenized in a CK28-7 mL tube (Bertin Technologies, KT03961-1-302.7) containing 1 mL of RNAdvance Tissue lysis buffer (Beckman Coulter Life Sciences, A32646) and anti-foaming Dx Reagent (Qiagen, 19088). Tissue was homogenized at 6,500 RPM for 20 seconds at the first cycle and 6,000 RPM for 20 seconds at the second cycle. Lysate was adjusted to 10 mg per 400 µL lysis buffer and then digested with proteinase K in a 96-Well Riplate®-2.2 mL (Ritter Medical, 43001-0020) at 37°C for 25 minutes. RNA was extracted according to the instructions for the RNAdvance Tissue miRNA protocol (AAG-230A07.14-A) using the Agencourt RNAdvance Tissue miRNA 96 (A35555 with miRNA) demonstrated method (Beckman Coulter Life Sciences). Purified RNA was eluted with 40 µL of nuclease-free water in a Hard-Shell Thin-Wall 96-Well Skirted PCR Plate (BioRad, HSP-9611). Eluted RNA concentration and purity were measured with a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific). The RNA purity was determined by the OD260/OD280 and OD260/OD230 ratios. One µL of the diluted RNA sample was analyzed on an Agilent RNA 6000 Pico chip (Agilent Technologies, 5067-1513) using the 2100 Bioanalyzer (Agilent Technologies) to determine RNA integrity. Let-7c miRNA expression was determined by a TaqMan® micro RNA assay (Life Technologies 4427975, assay ID000379). B2M cDNA was synthesized using a reverse primer (CCT GGG CTT TCA TCC TAA CA). PCR products were amplified using a primer probe mix cocktail (Forward primer TGA TCT TTC TGG TGC TTG TCT C, Reverse primer TAG CAG TTG AGG AAG TTG GG, probe CGG TGG ATG GCG AGA GTA CAC TTG). 50 ng of total RNA was used for the reverse transcription reaction using the TaqMan® micro RNA Reverse Transcription kit for let-7c and B2M

BECKMAN COULTER
Life Sciences

gene expression (Life Technologies, 4366596) and 1.33 µL of cDNA was used per PCR reaction in triplicate using TaqMan® Universal Master Mix II (Life Technologies, 4440038). The detail of the RT and PCR setup was described in IB-17265A[1]. For consumables and tools used for a Biomek NX^P Span-8 automated workstation, see Table 1.

Table 1. Biomek NX^P Configuration (Tools and Consumables).

Type	Quantity	Description	Beckman Coulter Part No.
Devices	1	Orbital Shaker	379448
	1	Span-8 Passive Wash	719654
ALPS	1	Biomek NX^P Span-8 4x3 ALP Kit	989839
Magnet Plate	1	Agencourt SPRIPlate 96R—Ring Super Magnet Plate	A32782
Reservoirs	1	Reservoir Frame	372795
	1	Half Reservoir	534681
	2	Full Reservoir	372784
	2	Quarter Reservoir	372790
Consumables	8	Biomek AP96 P1000 Tip Boxes	B01123
	1	96-Well Riplate—2.2 mL	See Materials and Methods
	1	Hard-Shell Thin-Wall 96-Well Skirted PCR Plate	See Materials and Methods

Results and Discussion

Summary of RNA Yields and Purity from 96 Samples Using the Biomek RNAdvance miRNA Tissue 96 Extraction Method

An average of 10 mg of liver tissue from 96 replicates was used to evaluate RNA yield and purity using the automated RNAdvance Tissue extraction method for miRNA (Beckman Coulter Life Sciences). The RNA was eluted in 40 µL of nuclease-free water. The average concentration from 96 samples was at 1,066 ng/µL with CV % of 8.6. The calculated average yield for 10 mg of tissue was at 42.6 µg (Table 2). The OD260/OD280 ratio for 96 samples ranged from 2.04 to 2.15 with an average ratio of 2.1. The OD260/OD230 ratio for 96 samples ranged from 1.80 to 2.2 with an average ratio of 2.1 (Table 2). Figure 2 shows the examples of RNA profiling and gel view.

Table 2. Average Yield and Purity From a Total of 96 Replicate Liver Samples.

Average Conc. per 10 mg (ng/µL) ±CV%	Average Yield per 10 mg (µg)	Average OD260/OD280 Ratio	Average OD260/OD230 Ratio
1,066 ±8.6%	42.6	2.1	2.1

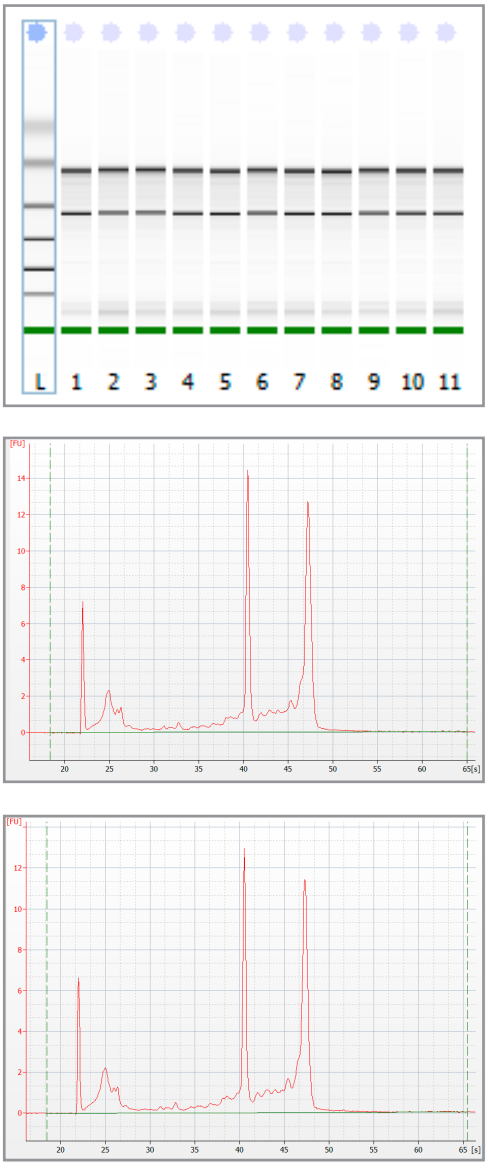


Fig. 1. Example of total RNA profiling on gel view and electropherograms. 1:1,000 dilution of RNA samples was analyzed on an RNA Pico Chip.

Biomek Automated Extraction Gave Comparable RNA Yields as Compared to Manual Extraction

To compare the RNA yield between manual extraction and Biomek automated extraction, the average RNA yield and purity was calculated from 3 batches of liver lysate prepared using the Biomek Workstation from 3 different runs. Figure 2 shows that automated extraction gave comparable RNA yields as compared to manually extracted samples.

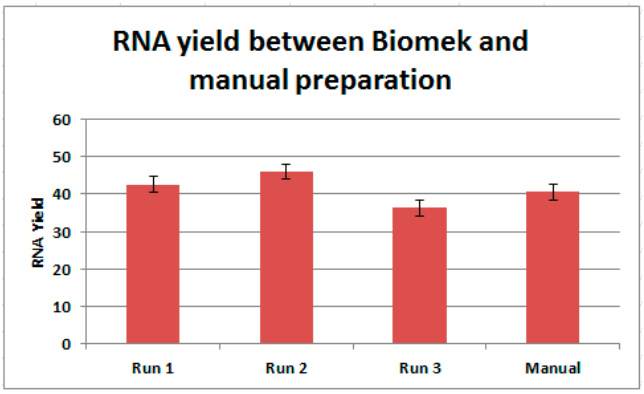


Fig. 2. Average RNA yields for Biomek automation and manual extraction methods. Y axis represents RNA yield (µg). X axis represents 3 different Biomek-extracted samples and manually extracted samples.

Biomek Automated Extraction Gave Comparable miRNA and Total RNA Recovered Efficiency as Compared to Manual Extraction

50 ng of total RNA was used to determine let-7c miRNA and B2M gene expression from either manual or Biomek automation purified RNA samples. The average cycle threshold (Ct) was calculated for each method. The average Ct value for let-7c gene expression from the Biomek-extracted samples was 23.85 ±0.05 and the manually extracted samples showed a Ct value of 24.17 ±0.024. The average Ct value for B2M gene expression from Biomek-extracted samples was 23.62 ±0.014 and the manually extracted samples showed a Ct value of 23.50 ±0.005. The result indicates that both extraction methods gave comparable miRNA and messenger RNA extraction efficiency (Figure 3). The minus RT and controls with no template showed no amplification, indicating that the amplification resulted from miRNA and mRNA alone (data not shown).

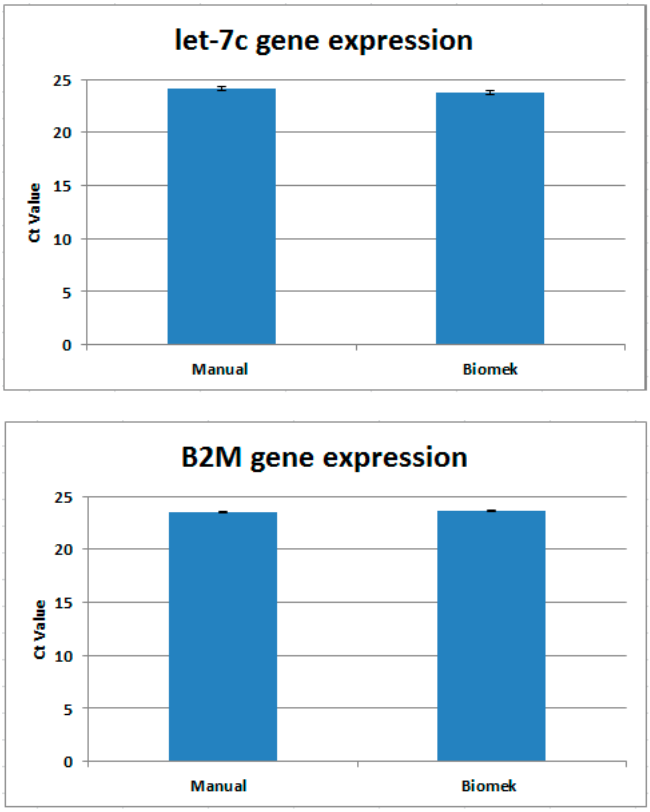


Fig. 3. Average Ct value for the let-7c miRNA and B2M gene expression in a 50 ng reaction. Results of TaqMan® gene expression assay for B2M (bottom) and microRNA assay for let-7c (top), comparing the eluates generated from the manual and automation.

Conclusions

The data from this study shows that the RNAdvance Tissue Kit provides high-purity RNA. The automated extraction and manual extraction protocols show no difference in RNA yields or miRNA and messenger RNA gene expression profiling. The Biomek RNAdvance miRNA Tissue 96 demonstrated method is an easy, robust automated nucleic acid protocol that can process from 1 to 96 samples in a 96-well plate format. It provides a streamlined workflow for downstream assays such as qPCR, microarray and NGS-RNA sequencing applications.

Authors

Bee Na Lee, PhD

John R. Palys

Christian Croisetiere

Isabel Gautreau

Beckman Coulter Life Sciences, Indianapolis, IN, USA

Reference

1. Highly-efficient miRNA Isolation using the Agencourt FormaPure and RNAdvance Cell v2 Kits and Biomek Automated Extraction Methods.

Life Sciences

For Beckman Coulter's worldwide office locations and phone numbers, please visit "Contact Us" at www.beckmancoulter.com

AAG-453APP08.14-A

Automation of Micro RNA and Total RNA Purification from Formalin-fixed paraffin-embedded tissues (FFPE) using the Agencourt FormaPure Kit and Biomek NX^{P} Span 8 Laboratory Automation Workstation

Bee Na Lee, Ph.D., Staff Application Scientist and John R. Palys, Systems Engineer II
Beckman Coulter Life Sciences

Summary

Micro RNAs are small, naturally-occurring non-coding ribonucleic acids of approximately 22 nucleotides in length that function in gene silencing and post-transcriptional regulation of gene expression. A single microRNA (miRNA) can target and regulate several (maybe even hundreds) of transcripts, and can involve multiple biological networks or pathways. As a result, interest in miRNA biomarker research has increased. This application note describes the purification of miRNA and total RNA from FFPE tissue samples using the Agencourt Formapure paramagnetic bead based chemistry automated on the Biomek NX^{P} Laboratory Automation Workstation. The method enables automated purification of total RNA, including miRNA and other small RNAs, from 8–96 samples on a Biomek Span 8 workstation. Total RNA and miRNA can be purified from 10 micron FFPE slices as starting materials.

Automating SPRI (Solid Phase Reversible Immobilization) chemistry provides an easy, high yielding and robust nucleic acid purification process that does not require centrifugation and vacuum filtration steps. Purified nucleic acids are easily eluted from the magnetic beads under aqueous conditions, which provide maximum flexibility for downstream applications. The data shows that the samples extracted using the Biomek gave comparable RNA yield, miRNA and messenger RNA gene expression compared to samples extracted manually.

Materials and Methods

FFPE samples (10 micron thick slices) were de-paraffinized, lysed with proteinase K, and then extracted using the Beckman Coulter Agencourt FormaPure Kit (part number A33342). RNA was extracted according to the instructions for the FormaPure miRNA protocol AAG-666SP11.14-A (Reference 1-2) using the Agencourt FormaPure 96 Biomek NX^{P} Span8 method (A35556, Reference 3) with the modified buffer volume in binding and washing steps to recover miRNA. Purified RNA was eluted with 40 µL of nuclease-free water in a hard-shell thin-wall 96-well skirted PCR Plate (BioRad, HSP-9611). Eluted RNA concentration and purity were measured with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). The RNA purity was determined by the OD260/OD280 and OD260/OD230 ratios. 1 µL of the diluted RNA sample was analyzed on an Agilent RNA 6000 Pico chip (Agilent Technologies, 5067-1513) using the 2100 Bioanalyzer (Agilent Technologies) to determine RNA quality. Let-7c miRNA expression was determined by a Taqman microRNA assay (Life Technologies 4427975, assay ID000379). For messenger RNA gene expression, cDNA was synthesized using a B2M or ACTB gene specific reverse primer (TCTGCTCCCCACCTCTAAGT and CACCTTCACCGTTCCAGTTT respectively). PCR products were amplified using a primer probe mix cocktail. B2M (forward primer, GGACTGGTCTTTCTATCTCTTGTAC; reverse primer, ACCTCCATGATGCTGCTT AC; probe CTGCC TGTGAACCATGTGACTTTG). ACTB (forward primer ACAGAGCCTCGCCTTTG, reverse primer CCTTGCACATGCCGGAG, probe TCATCCATGGTGAGCTGGCGG). 50 ng of total RNA was used for the reverse transcription reaction using the TaqMan micro RNA Reverse Transcription kit for let-7c and 250ng for B2M and ACTB gene expression (Life Technologies,

BECKMAN COULTER
Life Sciences

TYPE	QUANTITY	DESCRIPTION	PART NO.*
Devices	1 1 1	Orbital Shaker Span 8 Passive Wash Static Peltier	379448 719654 A93938
ALPS	1	Biomek NX Span-8 4x3 ALP Kit	989839
Magnet Plate	1	Agencourt SPRIPlate 96R-Ring Super Magnet Plate	A32782
Reservoirs	1 3 1 1 1	Reservoir Frame Half Reservoir Full Reservoir Quarter Reservoir Quarter Reservoir, divided by length	372795 534681 372784 372790 372788
Consumables	8 1 1	Biomek AP96 P1000 Tipboxes 96-Well Riplate-2.2 mL Hard-Shell Thin-Wall 96-Well Skirted PCR Plate	B01123 See Materials and Methods See Materials and Methods

Table 1: Biomek NX^{P} configuration (Tools and consumables). *Beckman Coulter Life Sciences.

4366596) and 1.33 µL of cDNA was used per PCR reaction in triplicate using Taqman Universal Master Mix II (Life Technologies, 4440038). The detail of the RT and PCR set up is described in AAG-700APP11.14-B (Reference 2). For consumables and tools used for the Biomek NX^{P} Span 8 automated workstation, see table 1.

Results and Discussion

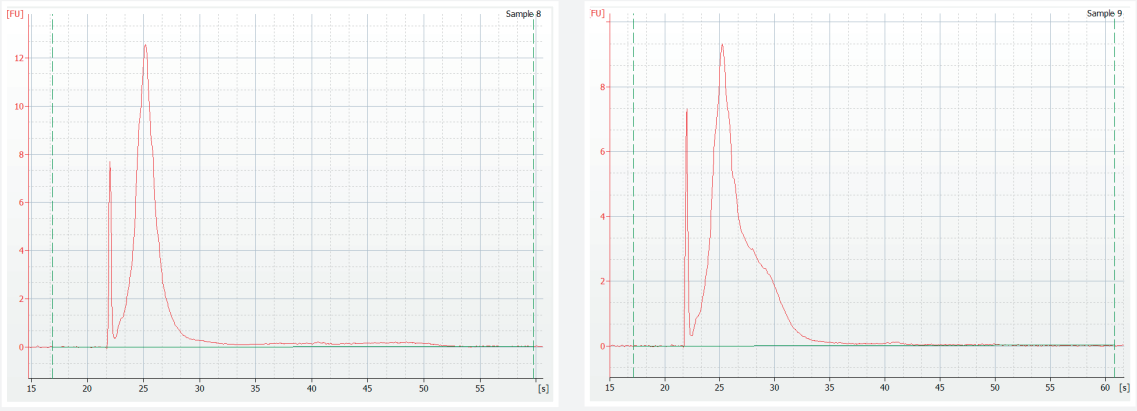
Summary of RNA yields and purity from 48 samples using the FormaPure 96 Biomek NX Span8 NX^{P} method.

48 samples (10 micron per slice) were used to evaluate RNA yield and purity. The RNA was eluted in 40 µL of nuclease-free water. The average concentration from 48 samples was 186ng/µL (Table 2). The calculated average yield for 10 micron of FFPE was 7.46 µg (Table 2). The OD260/OD280 ratio for 48 samples ranged from 1.97-2.02 with an average ratio of 2.0. The OD260/OD230 ratio for 48 samples ranged from 1.61-2.0 with an average ratio of 1.86 (Table 2). Figure 2 shows an example of the RNA profile.

Average conc. per 10 micron (ng/µL)+/-STD	Average yield (µg) per 10 micron	Average OD260/ OD280 ratio	Average OD260/ OD230 ratio
186.62+/-54	7.46	2.0	1.86

Table 2: The average yield and purity was calculated from a total of 48 different FFPE slices from the same FFPE block.

Figure 1: Example of total RNA Profiling on electropherograms. 1:30 dilution of RNA samples was analyzed on an RNA Pico Chip.



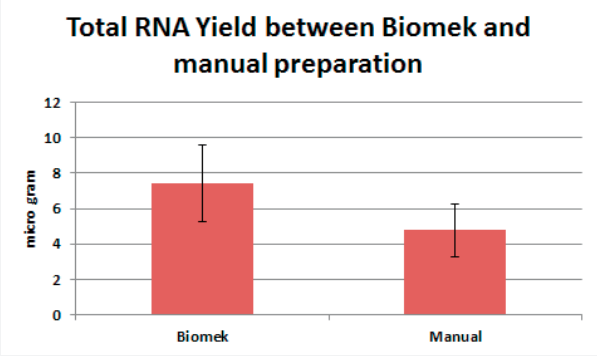


Figure 2: Average RNA yields for Biomek automated and manual extraction methods.

Biomek automated extraction gave slightly higher RNA yields compared to manual extraction.

To compare the RNA yields from manual extraction and Biomek automated extraction, the average RNA yield and purity was calculated from two batches of FFPE lysate prepared using the Biomek workstation and manual extraction. Figure 2 shows that the automated extraction gave an average of 7.4+/-2.16 µg of total RNA whereas manual extraction gave an average of 4.76+/-1.46 µg of total RNA per 10 micron individual FFPE sample. The data indicated slightly higher RNA yields for Biomek automated preparation compared to manual extraction. This is most likely due to less bead loss with extraction by the Biomek liquid handler compared to manually pipetting in multiple washing steps. Since each individual slice of FFPE samples might contain a different amount of tissue, we also observed large variation of RNA yields between samples, using either manual or Biomek extraction methods.

Gene expression data demonstrates comparable miRNA and total RNArecovery from Biomek and manual extraction methods.

50 ng of total RNA was used to determine let-7c miRNA gene expression from either manual or Biomek automation purified RNA samples. The average cycle threshold (Ct) was calculated for each method. The average Ct value for let-7c gene expression from the Biomek-extracted samples was 23.722+/- 0.011 and the manually-extracted samples showed a Ct value of 23.842+/-0.010. Similarly, 250 ng of total RNA was used to determine regular gene expression from either manual or Biomek automation purified RNA samples. The average Ct value for ACTB gene expression from Biomek-extracted samples was 26.06+/- 0.178 and the manually-extracted samples showed a Ct value of 26.14+/-0.019. The average Ct value for B2M gene expression from Biomek-extracted samples was 26.42+/- 0.117 and the manually-extracted samples showed a Ct value of 26.60+/-0.062. The result indicates that both extraction methods gave comparable miRNA and messenger RNA extraction efficiency (Figure 3). The minus RT and controls with no template showed no amplification, indicating that the amplification resulted from miRNA and mRNA alone (data not shown).

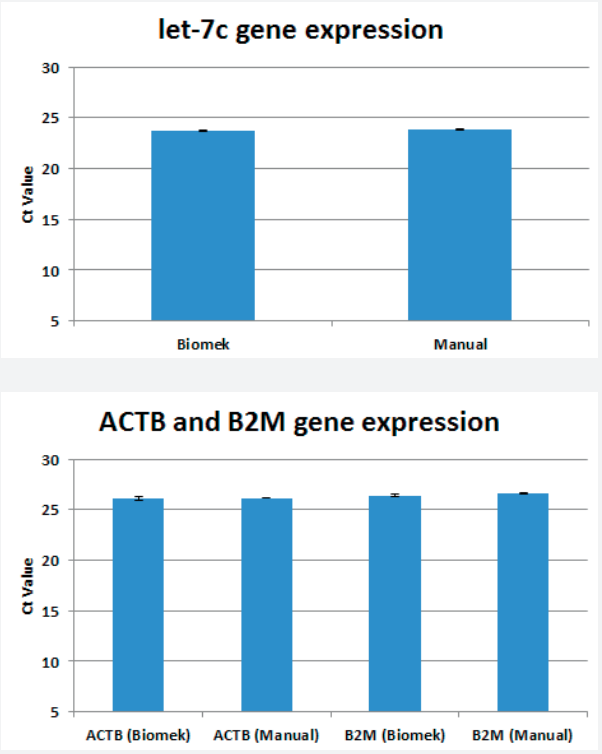


Figure 3: Average Ct value for the let-7c miRNA and regular gene expression. Results of theTaqman gene expression assay for microRNA assay for let 7c (top) and ACTB and B2M (bottom) comparing the eluates generated from the manual and automation extraction methods.

Conclusions

The data from this study shows that the FormaPure Kit provides high quality RNA. The automated extraction and manual extraction protocols show similar miRNA and messenger RNA gene expression profiling. The FormaPure 96 Biomek NX^{P} Span8 method is an easy, robust automated nucleic acid protocol that can process from 8 to 96 samples in a 96-well plate format. It provides a streamlined workflow for downstream assays such as qPCR, micro-array and NGS-RNA sequencing applications.

Reference

1. FormaPure® Kit Supplemental Protocol for microRNA and total RNA Isolation from Formalin-Fixed, Paraffin-Embedded Tissue (AAG-666SP11.14-A).
2. Highly efficient microRNA and total RNA purification from Formalin-fixed paraffin-embedded tissues (FFPE) using Agencourt FormaPure Kit (AAG-700APP11.14-B).
3. Automated TruSeq RNA sample preparation from FFPE tissue specimens utilizing the Biomek FX^{P} liquid handler. IB-17929A

BECKMAN COULTER

Life Sciences

AAG-747APP01.15-A

MicroRNA Extraction from Laser Capture Micro-Dissected (LCM) FFPE Tissue Using Beckman Coulter's Agencourt FormaPure Kits

Summary

Obtaining specific populations of cells from heterogeneous tissue samples is a major challenge in studying gene expression profiles of specific cell types. However, laser capture micro-dissection (LCM) enables the selection and capture of individual cells or groups of cells from discrete morphological structures by cutting away unwanted cells. This enriches histologically pure cell populations and therefore it can specifically isolate cancer cells from normal tissues. MicroRNA (miRNA), RNA, DNA or proteins can then be extracted from these micro-sections for gene expression analysis. LCM sampling can be applied to different samples, including formalin-fixed, paraffin-embedded (FFPE) tissues or fresh frozen tissues (FFT). This application note describes the purification of miRNA and total RNA from LCM FFPE samples using the Beckman Coulter Agencourt FormaPure kit. The method uses Beckman Coulter's SPRI (Solid Phase Reverse Immobilization) magnetic bead-based chemistry, which provides an easy, rapid, high yielding, robust and automation-friendly nucleic acid purification procedure, that does not require vortexing, centrifugation or filtration steps. The data shows that miRNA was successfully extracted from LCM FFPE samples.

Materials and Methods

Tumor and normal cells from a prostate cancer FFPE tissue block were selected and micro-dissected using a digital pathology-guided approach to identify the regions of interest (ROIs). Three representatives matching LCM scanned sections were selected for study using the ArcturusXT™ LCM Instrument (Life Technologies) and Arcturus® Paradise® FFPE Tissue LCM Staining Kit (Life Technologies, KIT0312-J). The micro-dissected sections were then digested with lysis buffer and proteinase K overnight using a FormaPure Kit (Beckman Coulter, A33342). Six samples (triplicates of tumor and benign samples) were used for miRNA extraction following the FormaPure Kit miRNA supplemental protocol (Beckman Coulter, AAG-666SP11.14-A). Samples were eluted in 20 µL of nuclease-free water in the final elution step. The concentration of the RNA was measured by a Quant-iT™ RiboGreen® RNA assay kit (Life Technologies, R11490). One µL of RNA was analyzed by an Agilent RNA 6000 Pico chip and a small RNA chip (Agilent Technologies, 5067-1513 and 5067-1548) using the 2100 Bioanalyzer (Agilent Technologies). miRNA (miR146a and RNU48) gene expression was determined by a TaqMan® microRNA assay (Life Technologies, assay ID001097 and ID001006). 10 ng of RNA was used for the reverse transcription reaction using the TaqMan® microRNA Reverse Transcription Kit (Life Technologies, 4366596), and 1.33 µL of cDNA was used per PCR reaction in triplicate using TaqMan® Universal Master Mix II (Life Technologies, 4440038).

Results and Discussion

RNA Yields From LCM FFPE Samples Using FormaPure Kits

Three pairs of tumor/benign LCM samples prepared from the FFPE block were used to extract total RNA and miRNA. The RNA was eluted in 20 µL of nuclease-free water. The RNA concentrations were between 2 ng/µL to 3.6 ng/µL (data not shown). The

BECKMAN COULTER
Life Sciences

RNA yields were between 41 ng to 72 ng (Figure 1). All samples show similar fragmented RNA quality from FFPE samples (Figure 2).

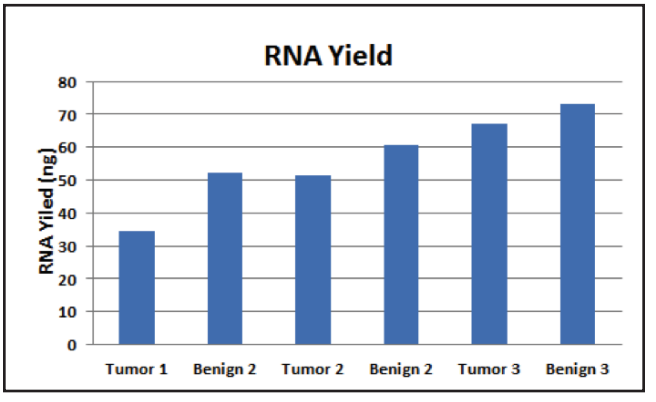


Fig. 1. Summary of RNA yield from 3 pairs of tumor/benign samples.

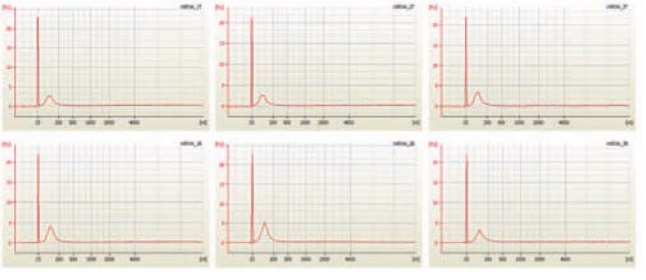


Fig. 2. Typical fragmented RNA from micro-dissected FFPE samples.

MicroRNA Gene Expression Indicating that miRNA was Successfully Extracted from LCM Samples

10 ng of total RNA was used for miR146a and RNU48 gene expression to determine miRNA gene expression. The average cycle threshold (Ct) was calculated from triplicates of each tumor/benign pair. The average Ct value for miR146a target gene expression in benign cells was 32.22±0.11 and 31.67±0.22 in tumor cells. The average Ct value for RNU48 miRNA reference gene expression was 24.17±0.16 in benign cells and 24.14±0.013 in tumor cells (Figure 3). This result indicates that downstream RT-PCR of target and reference miRNAs is feasible with robust Ct values derived from RNA, extracted utilizing the FormaPure method from tumor and benign cells micro-dissected from FFPE tissue blocks. Differential expression of the target miR146a, and the reference RNU48, was observed across the samples.

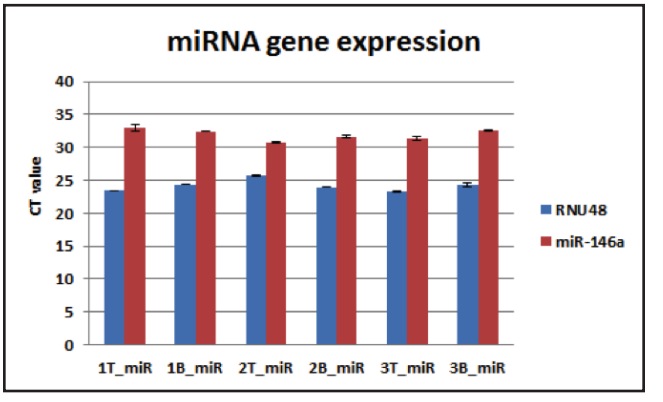


Fig. 3. miR146a and RNU48 miRNA gene expression.

Conclusions

The data from this study shows that the Beckman Coulter FormaPure Kit can be used for low-input sample extraction such as LCM from FFPE tissue blocks. The magnetic bead-based extraction protocol provides automation-friendly, scalable throughput and does not require vortexing, centrifugation, or filtration steps.

Acknowledgement

The data used in this application note was provided by a customer.

Author

Bee Na Lee, PhD, *Staff Application Scientist*
Beckman Coulter Life Sciences

Highly Efficient MicroRNA and Total RNA Purification from Formalin-Fixed Paraffin-Embedded Tissues (FFPE)

USING AGENCOURT® FORMAPURE® KIT

Bee Na Lee, Ph.D. Staff Application Scientist, Beckman Coulter Life Sciences

Introduction

Extracting miRNA and total RNA from highly degraded archival formalin-fixed, paraffin-embedded tissue (FFPE) can be challenging due to combinations of fixation conditions, age, temperature, and storage. In the past few years, interest in the identification, detection and use of small RNA molecules from FFPE samples has rapidly expanded. The small RNA molecules refer to small interfering RNAs (siRNA), non-coding small RNA and regulatory microRNAs (miRNAs). These small RNA molecules, which range between 15-40 nucleotides in length, are not efficiently extracted by traditional precipitation or solid phase methodologies. This technical note describes how the Agencourt SPRI (Solid Phase Reverse Immobilization) magnetic bead based method is superior to the column purification method for purifying miRNA from formalin-fixed paraffin-embedded tissues (FFPE). The SPRI method is an easy, rapid, high yielding, robust and automation-friendly nucleic acid purification procedure that does not require centrifugation or filtration steps. This method uses carboxyl-coated magnetic particles that reversibly bind nucleic acids in the presence of binding buffers and crowding reagents. Typically, there are three basic steps in the extraction/purification procedure. In the first step, nucleic acids are immobilized onto the SPRI beads, leaving contaminants in solution. In the second step, a magnetic field is used to attract the micro-particles with bound nucleic acids out of the solution. Contaminants are then aspirated and the micro-particles with bound nucleic acids are thoroughly washed with molecular biology-grade ethanol. In the third step, purified nucleic acids are easily eluted from the micro-particles under low salt aqueous conditions, which provide maximum flexibility for downstream applications. The results show that the SPRI extraction method provides 5 times higher miRNA yield than the column purification method.

Materials and Methods

FFPE samples (four 10 micron thick slices) were deparaffinized, lysed with proteinase K digestion, and then extracted using either the Agencourt FormaPure Kit (Beckman Coulter, A33342) or miRNeasy FFPE Kit (Qiagen, 217504). The lysate was prepared and the purification steps were performed manually according to the vendor instructions for the miRNeasy kit and the FormaPure miRNA protocol described in the application note (www.beckman.com, AAG-666SP11.14-A) using the tube format (Beckman Coulter Life Sciences, A29182). In order to have a fair comparison of the RNA yields between these two methods, all sixteen samples were eluted in 40 µL of nuclease free water in the final elution step. The concentration and purity of the RNA was measured by using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). Purity was determined by the ratio of the OD260/OD280 and OD260/OD230. 1 µL of total RNA was analyzed by using the Agilent RNA 6000 Pico chip (Agilent Technologies, 5067-1513) and the 2100 Bioanalyzer (Agilent Technologies). microRNA gene expression was determined by Taqman microRNA assay (Life Technologies 4427975, assay ID000379). 50 ng and 100 ng of total RNA was used for the reverse transcription reaction using the TaqMan micro RNA Reverse Transcription kit (Life Technologies, 4366596) and 1.33 µL of cDNA was used per PCR reaction in triplicate using Taqman Universal Master Mix II (Life Technologies, 4440038).

BECKMAN COULTER
Life Sciences

Results and Discussion

Comparison the RNA yield: FormaPure purification kit gave a higher RNA yield than column purification kits.

To compare the miRNA and RNA yield between the FormaPure and Qiagen miRNeasy purification methods for the FFPE samples, eight of the FFPE samples were processed using the FormaPure kit and an additional eight were processed using the Qiagen miRNeasy FFPE kit. The result from Table 1 shows that the SPRI bead purification method produced average yields 5 times higher than the column purification method. The average yield prepared from the FormaPure kit was 10 µg and the average yield from the miRNeasy FFPE kit was 2 µg. The average yield per 10 micron of FFPE sample for the FormaPure kit was 2.5 µg whereas miRNeasy FFPE kit produced only 0.5 µg per 10 micron of FFPE sample. The average RNA concentration for the FormaPure kit was 257 ng/µL and the average concentration of RNA for the miRNeasy FFPE kit was at 48 ng/µL (Table 1).

Table 1: Summary of the average RNA yield:

Method	Average Conc. (ng/µL)	Average Yield (µg)	Elution Volume (µL)
FormaPure	257.0	10.0	40
miRNeasy	48.0	1.92	40

A total of 8 liver samples were evaluated per method.

Comparison of RNA purity: Similar RNA purity and quality were produced by both FormaPure and the column purification kits.

Figure 1 shows the typical fragmented FFPE RNA profile extracted using both methods. For the FormaPure extracted samples, the total RNA was diluted to 1:50 and miRNeasy samples were diluted 1:10 in order to get equivalent signals. All samples have similar typical FFPE RNA profiling with the low RIN between 2.3-2.4. The RNA purity was evaluated from a total of 24 samples (12 samples per method), showing that the RNA purity was consistent between these two methods. The OD260/OD280 ratio for both methods was between 1.8-2.0, with the average ratio above 1.8. The OD260/OD230 ratio for both methods was between 1.0-2.10 with the average ratio above 1.6 (Tables 2-1 and 2-2).

Table 2-1: Summary of RNA purity:

Method	Average OD260/OD280	Range OD260/OD280
FormaPure	1.89	1.83-2.04
miRNeasy	1.87	1.76-1.96

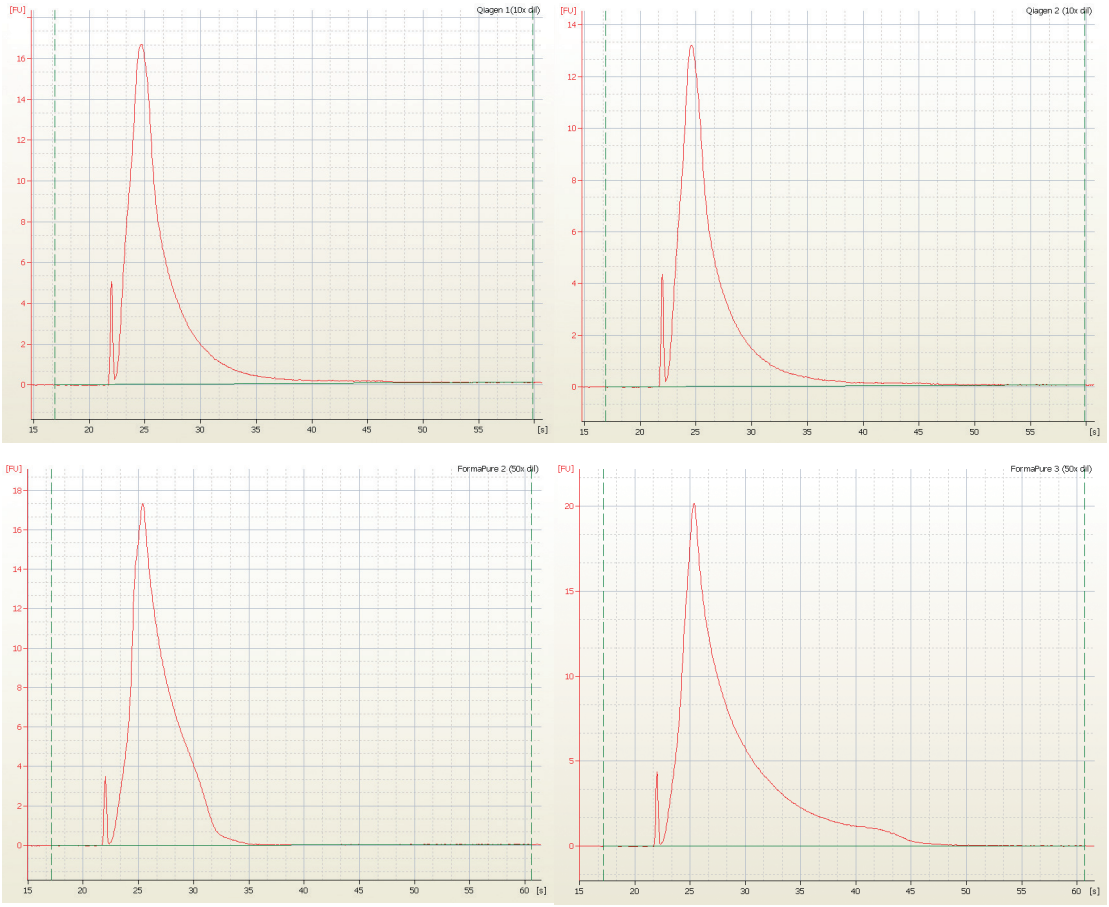
A total of 12 samples were evaluated per method. Sample type includes liver, colon and breast.

Table 2-2: Summary of RNA purity:

Method	Average OD260/OD230	Range OD260/OD230
FormaPure	1.65	1.02-2.10
miRNeasy	1.72	1.04-2.03

A total of 12 samples were evaluated per method. Sample type includes liver, colon and breast.

Figure 1. Typical fragmented FFPE RNA profiling:



RNA Pico Chip data: 1:10 dilution of the miRNeasy RNA samples (upper panel) and 1: 50 dilution of the FormaPure RNA samples (lower panel).

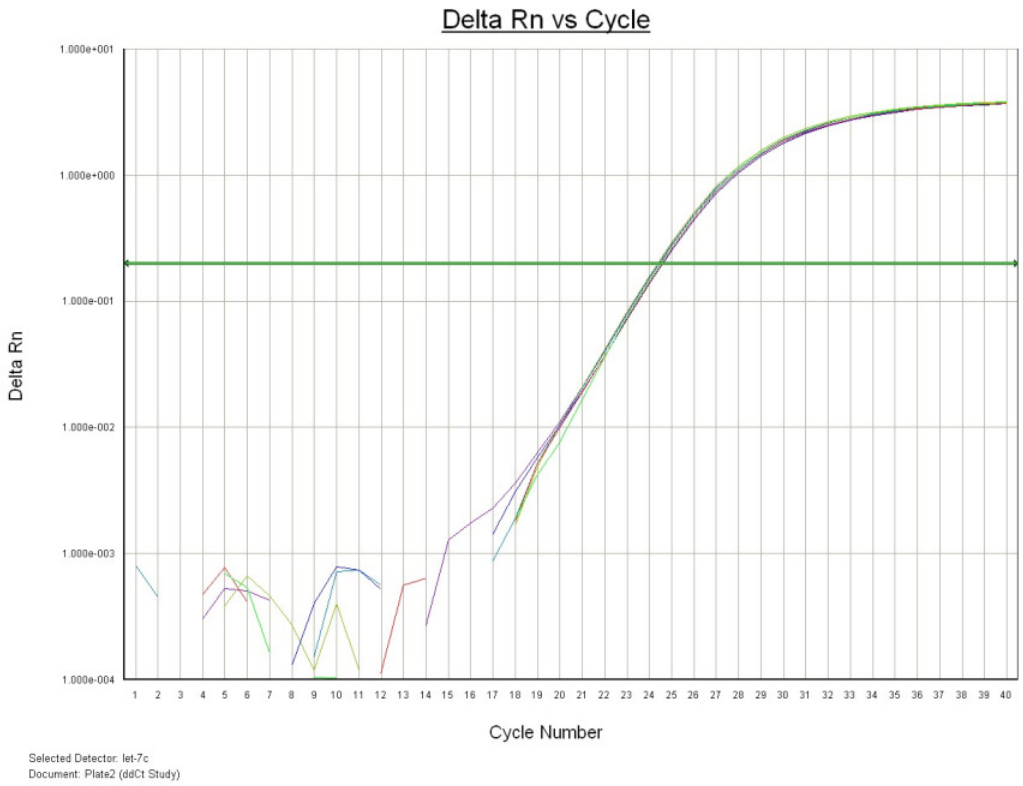
Comparison of miRNA yield: FormaPure purification showed higher miRNA extraction efficiency.

To determine the miRNA extraction efficiency, two different concentrations of total RNA from four liver samples extracted from both FormaPure and miRNeasy kits were used for let-7c gene expression. The results show the average threshold cycle value (Ct) for all samples was 23.5 Ct for 100 ng per reaction and 24.5 Ct for 50ng per reaction (Table 3 and Figure 2). The minus RT and controls with no template showed negative amplification, indicating that the amplification resulted from miRNA alone. This data confirmed that 5 times more miRNA was extracted using the FormaPure kit compared to the miRNeasy, because about 1/5 as much RNA was needed for cDNA synthesis in the reverse transcription reactions.

Table 3: Average Ct value for the let-7c gene expression

Kit Used	Amount of RNA Used	Average Ct value+/- Std Dev
FormaPure	100 ng	23.81+/-0.004
FormaPure	50 ng	24.57+/-0.029
miRNeasy	100 ng	23.45+/-0.035
miRNeasy	50 ng	24.62+/-0.035

Figure 2. Overlay of let-7c gene expression from liver FFPE samples extracted from FormaPure and miRNeasy kits.



The Taqman qPCR amplication plots showed that 1/5 as much RNA was needed from the miRNeasy extracted samples in order to achieve the same cycle threshold as those extracted using the Formapure kit..

Conclusions

The data from this study showed that miRNA and total RNA extracted from both the FormaPure kit and the miRNeasy kit had comparable purity and quality. However, the FormaPure kit gave RNA yields 5 times higher than the miRNeasy kit. Therefore, the FormaPure kit provides a more robust miRNA and total RNA extraction method for critical FFPE samples.

Acknowledgement

The author is grateful to the Danvers application team for providing the FFPE sliced samples.

PCR Reaction Setup Application

Technical Information Bulletin

Automation of PCR Reaction Setup Application Using the Biomek 4000 Laboratory Automation Workstation

Ruth Zhang, Ph.D., Amy Yoder, and William Godfrey, Ph.D., Beckman Coulter Life Sciences, Indianapolis, IN

Abstract

Polymerase chain reaction (PCR) assays continue to play an important role in research laboratories. The PCR Reaction Setup is offered on the Biomek 4000 Laboratory Automation Workstation, the newest addition to Beckman Coulter's research automation line. This application note guides users through the process of setting up a PCR reaction for 1 to 192 samples in 96-well plates with any combination of Master Mix, Primers, and Samples (Figure 1). It is the ultimate automation solution for complicated PCR reaction setup processes.

Instrument Setup Process and Key Features

1. Supports 1 to 192 high throughput configurations with up to two master mixes, two primers and two sample source labware including master mix made from single components (Figure 1).
2. Allows sample tracking by creating a sample ID file at setup (Figure 2).

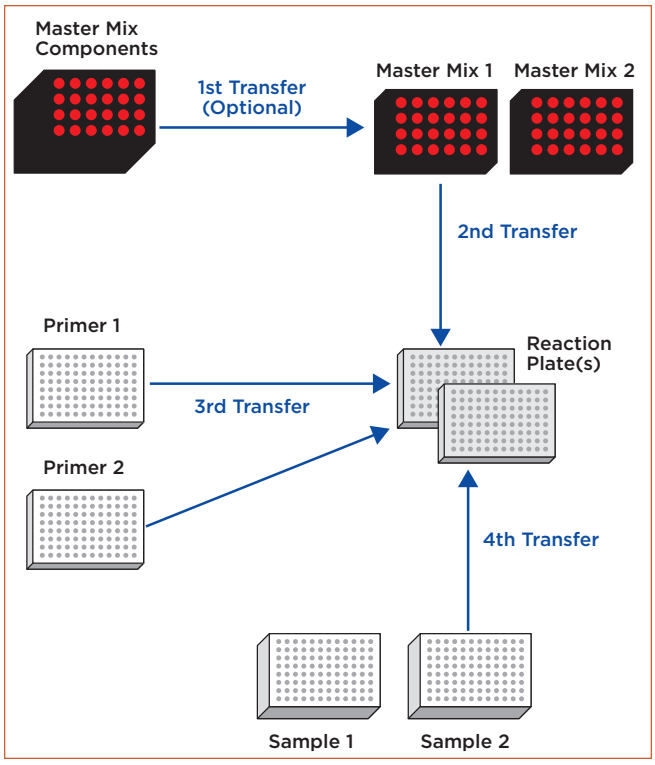


Figure 1. PCR Reaction Setup Process

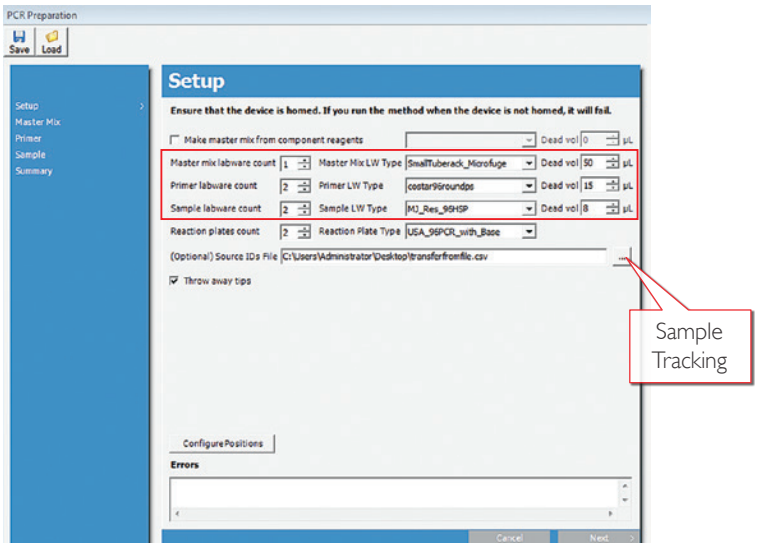


Figure 2. Up to two 96-well PCR reaction plates can be created in one reaction setup process.

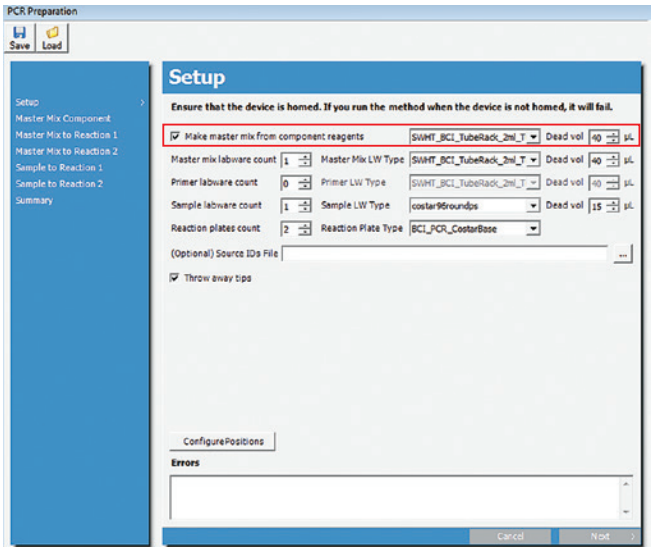
Discovery *in motion.*

BECKMAN COULTER Life Sciences

3. Supports both prepared PCR master mix and master mix made from individual PCR components (Figure 3).

4. Keeps track of tip usage and alerts the user if more tip boxes are needed (Figure 4).

3a



3b

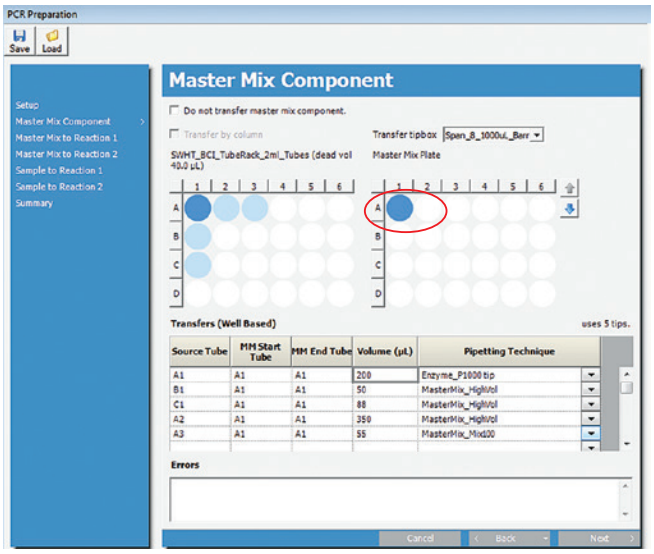


Figure 3. Master Mix can be made from individual PCR components, i.e., AmpliTaq DNA polymerase, dNTP, MgCl2, Triton X-100, Waters and Primers. All PCR components can be transferred from tubes of source labware to the tube of destination labware.

5. Allows saving and importing of setup conditions to repeat prior setup processes (Figure 5).

6. Provides standard pipetting templates and techniques which can also be customized by users.

7. Provides sample tracking report upon run completion.

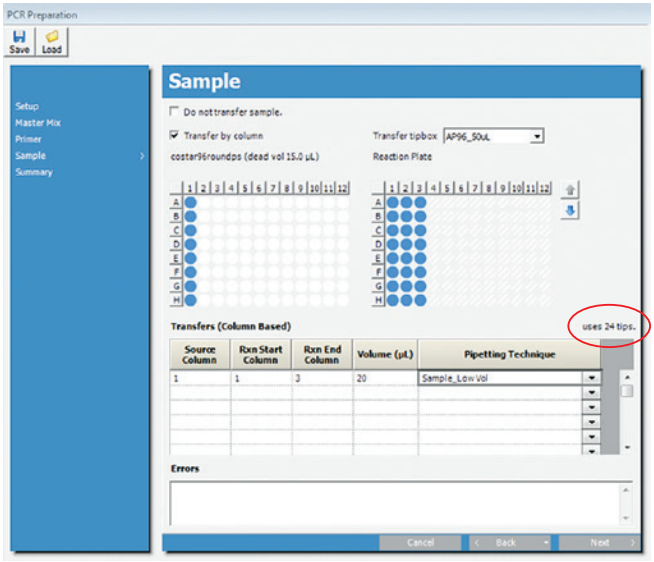


Figure 4. Tip usage is automatically tracked and calculated per number of samples processed.

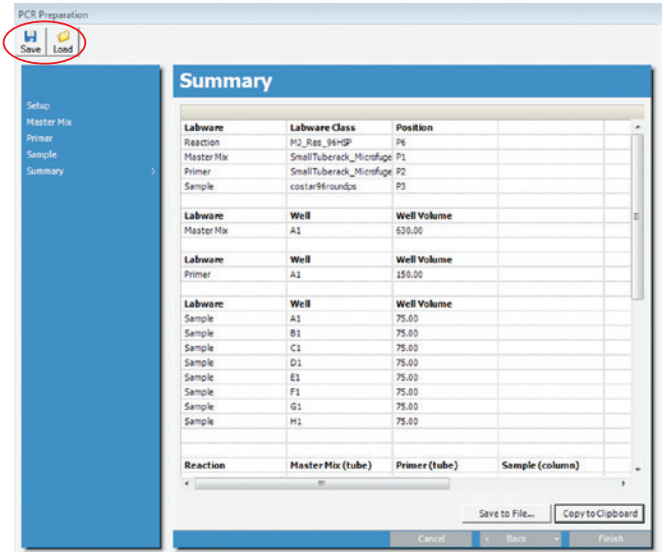


Figure 5. Click the "Save" button to save the setup condition. Click the "Load" button to import a saved file for repeat PCR reaction setup conditions.

B2012-13913-DG-1K

Results

The Biomek 4000 Laboratory Automation Workstation automated PCR Reaction Setup application offers flexible sample and reagent test combinations, limited only by the number of available tips and the amount of reagents on deck. This application can be used to prepare PCR and real-time PCR (qPCR). The system was used to prepare 32 replicate qPCR reaction plates from a single sample. This automated qPCR reaction setup data shows consistent amplification, with CVs of 1.74% for the 32 reactions (Figure 6). The PCR Reaction Setup Application was also utilized to prepare several PCR reactions on the Biomek 4000 Laboratory Automation Workstation which demonstrated no well-to-well cross-contamination from this automated PCR reaction setup process (Figure 7).

Automated Real-Time PCR Reaction Setup results in low CVs from 32 sample reactions

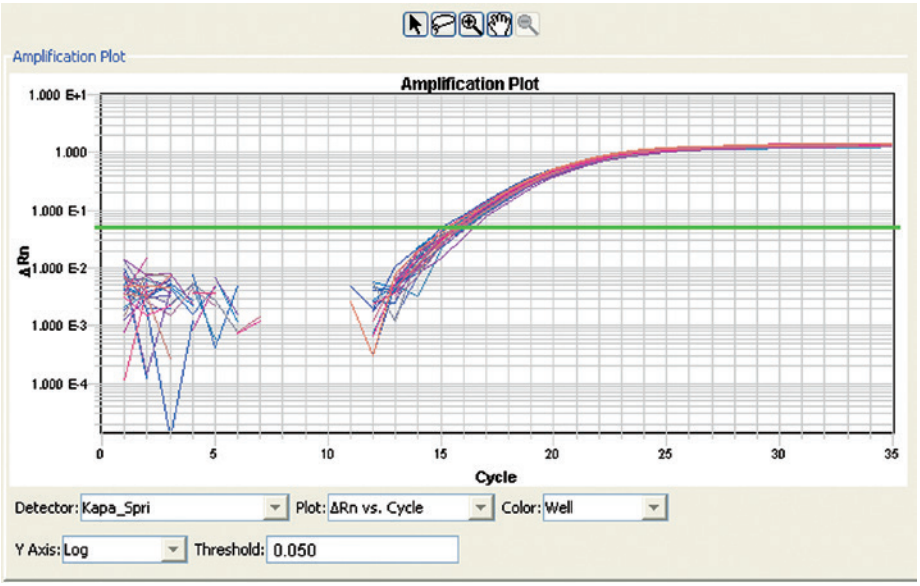


Figure 6. Real-Time PCR amplification (20 µL/reaction): using ABI 7900HT Fast Real-Time PCR System. The above data contains the β-actin real-time PCR reaction results from 32 samples (average Ct = 15.72 with 1.74% CV). Each β-actin qPCR reaction amplified 8 ng human genomic DNA (Promega), β-actin primer pairs (Promega), and KAPA SYBR FAST qPCR Master Mix (KAPABIOSYSTEMS).

Automated PCR Reaction Setup results no well-to-well cross-contamination

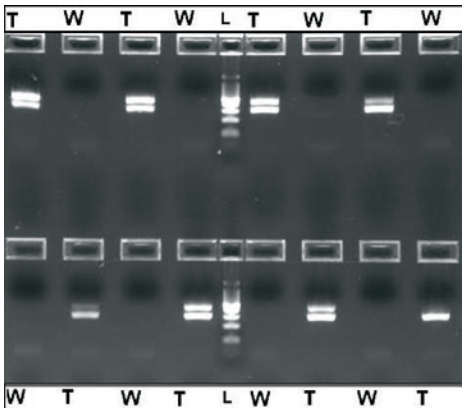


Figure 7. Cross-Contamination Results (25 µL/reaction): Human genomic DNA (24 ng/reaction), β-actin primer pairs and ready-to-use PCR master mix (Promega) were used for automated PCR cross-contamination tests. The test was conducted by amplifying gDNA template (T) and Water (W) in alternating wells. The absence of β-actin amplicons (285 bp) in negative wells indicates an absence of cross contamination. The middle lane is 100 bp DNA Ladder (L) (Promega).

Summary

With the ready-to-use PCR Reaction Setup Application for the Biomek 4000 Laboratory Automation Workstation, the user can set up a variety of PCR reactions, i.e., PCR and qPCR, using either default labware and techniques or user-modified labware and techniques by simply following the self-guided PCR User Interface. The application provides consistent results without the fear of cross contamination. The new P1000SL tool allows the creation of large volume master mix from individual PCR components located on the Biomek 4000 Laboratory Automation Workstation deck using the standard Beckman Coulter P1000 tips, that further facilitate the ease of use and flexibility in preparing PCR reactions.

B2012-13913-DG-1K

Space for
FSC Logo

Printed with soy ink

For Beckman Coulter's worldwide office locations and phone numbers, please visit www.beckmancoulter.com/contact

B2012-13913-DG-1K

PRINTED IN U.S.A.

PCR Reaction Setup and AMPure XP Application

Technical Information Bulletin

Reproducible and Efficient Automation of PCR Reaction Setup and Purification Using the Biomek 4000 Workstation and AMPure XP

Ruth Zhang, Li Liu, Amy Yoder, John Palys, Bryan Daniels, Isabel Gautreau and William Godfrey
Beckman Coulter Life Sciences, Indianapolis, IN

Abstract

The Biomek 4000 Laboratory Automation Workstation has been used to demonstrate highly reproducible real-time amplification (CV<2%) after automated reaction setup. In addition, the Agencourt AMPure XP reagent system has been automated on the Biomek 4000 Workstation to provide rapid amplicon purification (two 96-well plates in less than 90 minutes) with yield and purity equivalent to manual purification. Both procedures were demonstrated to be free of cross-contamination.

Introduction

Polymerase chain reaction (PCR) is a fundamental and essential technique for medical and biological research, as well as forensic identification. Its exceptional ability to accurately amplify and quantify extremely low levels of nucleic acids has driven the emergence of numerous applications, including genetic testing, tissue typing, infectious disease identification, genetic fingerprinting, DNA sequencing and cloning, and gene expression. It is now used in a wide range of formats, including RT-PCR, qPCR, Multiplex PCR, Inverse PCR, digital PCR, and Miniprimer PCR.

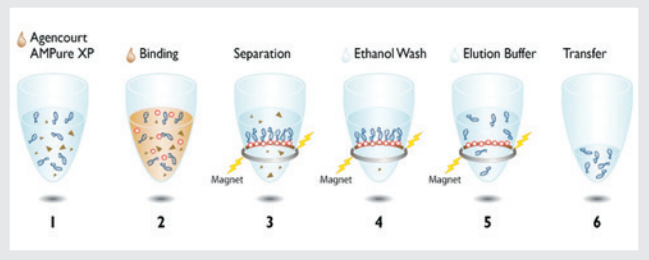


Figure 1. Schematic representation of the workflow for the six-step automated amplicon purification procedure.

Given the number of samples analyzed by PCR, the multitude of amplification formats used, and the need to reduce assay costs, a simple, easy-to-use, and high-throughput automation solution for PCR reaction setup is absolutely necessary. Automation of reaction setup can also eliminate both human error and contamination associated with manual processing, while assuring high quality results.

Efficient recovery of pure PCR amplicons is essential for downstream applications such as genotyping, cloning and primer walking. While many purification systems are commercially available, the Agencourt AMPure XP reagent system is a highly efficient, easily automated PCR purification system that delivers superior quality DNA with no salt or dNTP carryover. The AMPure XP reagent system uses an optimized buffer to selectively and reversibly bind PCR amplicons that are 100bp and larger to solid-phase paramagnetic beads. The excess primers, nucleotides, salts and enzymes from the reaction are removed using a simple Bind-Wash-Elute procedure (Figure 1). Requiring no centrifugation or vacuum filtration, the AMPure XP reagent system is highly amenable to automation.

This application note describes the use of the Biomek 4000 Laboratory Automation Workstation to automate PCR reaction setup, as well as amplicon purification using the AMPure XP system. The pipetting tools, updated software and Windows 7 compatibility improve the ease and speed of reaction setup. One to 192 PCR sample reactions can be setup in two 96-well PCR plates using up to two plates of master mixes, two primer sources and two sample sources, including master mix made from individual components. Amplification of a test template was highly efficient and reproducible, with yields equivalent to manual amplification. Efficient and reproducible automated AMPure XP purification can be completed in less than 90 minutes for 192 PCR samples (10 - 70 µL). Both procedures were shown to be free of cross-contamination between wells.

Discovery *in motion.*

BECKMAN COULTER Life Sciences

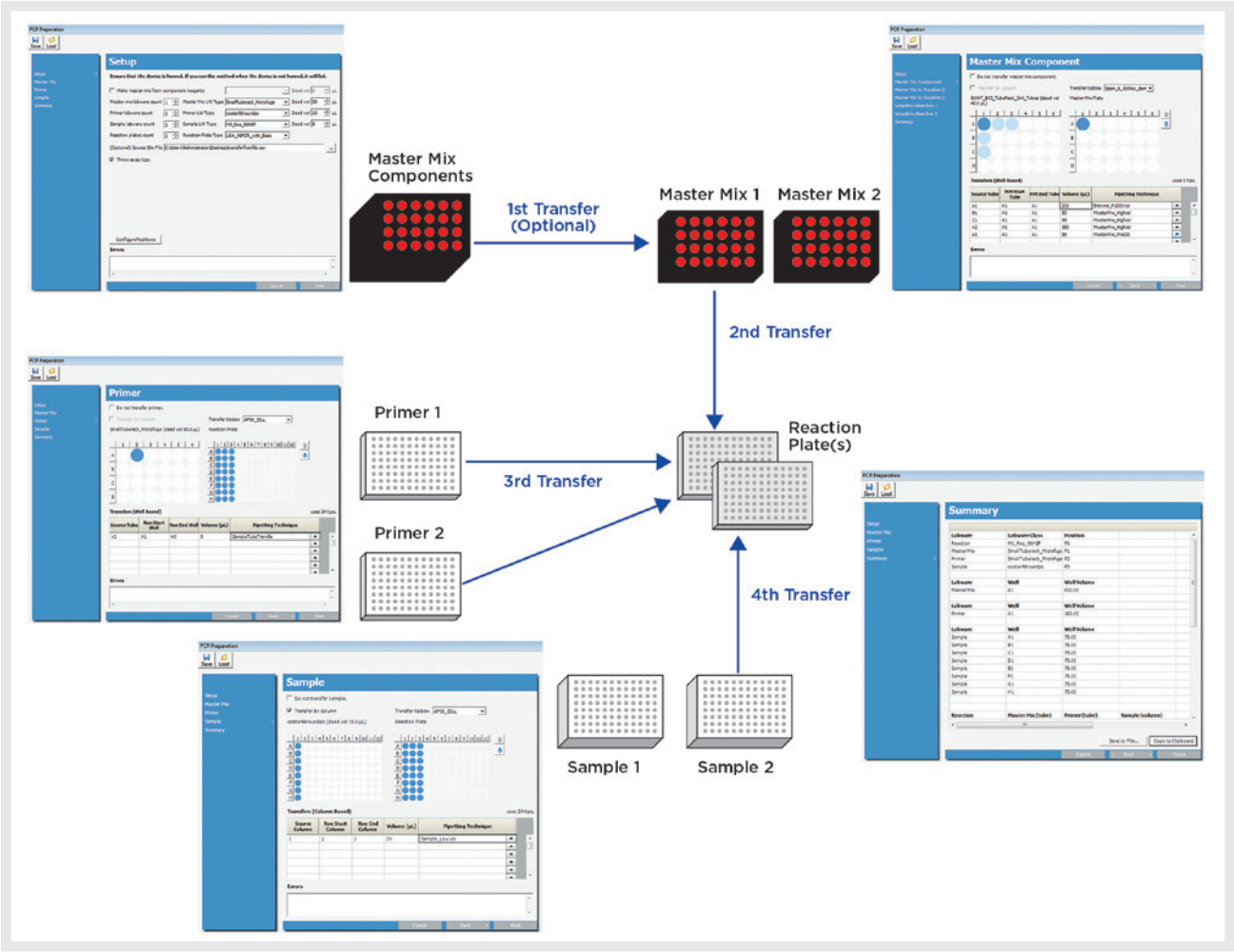


Figure 2. Overview of the automated PCR reaction setup process on the Biomek 4000 Workstation, illustrating the pop-up interface and two 96-well PCR reaction plates that can be set up from different reagent sources.

Materials

Reagent Source

- **Agencourt AMPure XP kit**, Beckman Coulter, Brea, CA (P/N A63882)
- **KAPA SYBR Fast Universal qPCR Kit**, KAPA Biosystems, Woburn, MA
- **AccuPrime Supermix I**, Life Technologies, Carlsbad, CA
- **pGEM DNA vector**, Promega, Madison, WI
- **M13 Forward Primer: 5'-TAA TAC GAC TCA CTA TAG GG-3'**, IDT, Coralville, IA
- **1000bp_pGEM Reverse Primer: 5'-TCT AGT GTA GCC GTA GTT AGG-3'**, IDT, Coralville, IA
- **Human Genomic DNA**, Promega, Madison, WI
- **β-actin Primer Pairs**, Promega, Madison, WI
- **PCR Master Mix**, Promega, Madison, WI
- **2% agarose gel**, Life Technologies, Carlsbad, CA
- **Nuclease-free Water**, Life Technologies, Carlsbad, CA
- **Denatured 70% Ethanol**, American Bioanalytical, Natick, MA

Instruments

- **Biomek 4000 Laboratory Automation Workstation** Beckman Coulter, Brea, CA (P/N B22638)
- **7900 HT Fast Real-Time PCR System**, Life Technologies, Carlsbad, CA

B2013-14053 IB-17985A

Results and Discussion

Reproducible and Reliable PCR Reaction Setup

The key feature for this application on the Biomek 4000 Workstation is flexibility. With the visual-well-selection user interface, a user can easily set up two 96-well PCR plates for various combinations of master mixes, primers, and samples, as well as create master mixes from single components (Figure 2). This flexibility enables automation of a variety of PCR formats and reaction setup conditions.

Human genomic DNA (24 ng/reaction), β-actin primer pairs and ready-to-use PCR master mix (Promega) were used for automated PCR setup employing default automation transfer techniques. The PCR results showed the absence of the β-actin amplicons (285 bp) in any negative control wells in which human genomic DNA template was replaced by water (Figure 3).

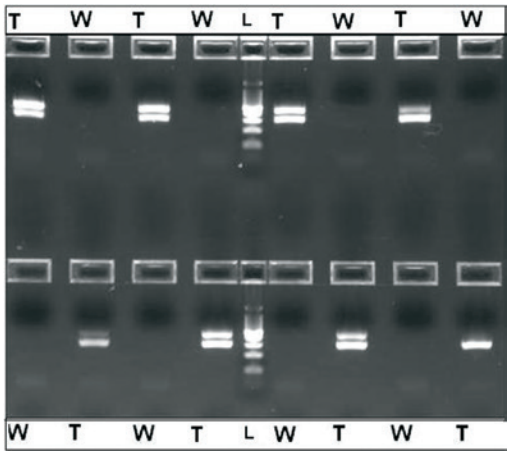


Figure 3. Electrophoretic separation on 2% agarose of amplified human genomic DNA (24 ng per 2 5µL reaction) using β-actin primer pairs and ready-to-use PCR master mix. 20 µL of the reaction volume was loaded onto the gel. The results show no presence of the β-actin amplicons (285bp) in any negative control containing no gDNA template (W), versus robust amplification in adjoining plate wells that contained the human gDNA template (T). The middle lane (L) is a 100bp DNA Ladder.

The Biomek 4000 Workstation was also used to automate setup of real-time amplification of the 285bp Human β-actin gene utilizing a ready-to-use KAPA SYBR qPCR master mix and default automation transfer techniques. The data showed highly consistent amplification with a coefficient of variation (CV) of 1.74% across 32 amplified samples (Figure 4).

Efficient and Reliable Automated AMPure XP Purification

The biggest advantage of automating PCR purification on the Biomek 4000 is speed, as two 96-well PCR plates of samples can be purified in less than 90 minutes for volumes between 10 to 70 µL. The Biomek 4000 AMPure XP method allows users to select the number of samples to be purified, as well as user ID, sample, and reagent lot number tracking via LIMS data collection. Plasmid DNA fragments

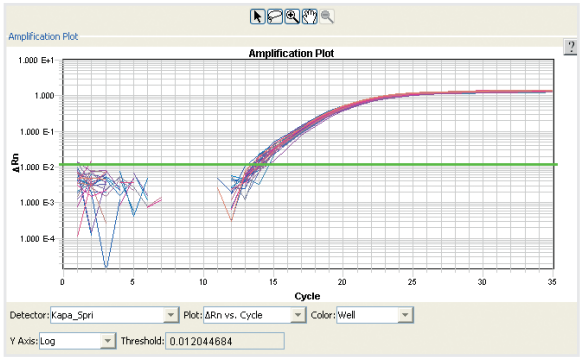


Figure 4. Real-time β-actin PCR reaction results from 32 samples. Each β-actin qPCR reaction amplified 8 ng of human genomic DNA using β-actin primer pairs and KAPA SYBR FAST qPCR Master Mix. The samples gave an average Ct value of 15.72 with 1.74% CV, illustrating highly consistent liquid transfers.

(1000 bp pGEM) produced by PCR were purified on the Biomek 4000 using this method, producing equivalent yield and purity (260nm/230nm ratio) to amplicons purified using the manual process (Table 1).

In order to measure cross-contamination during automated purification, the 452bp standard from the KAPA SYBR Fast Universal qPCR Kit was used as template to generate a large volume of PCR fragments. These were then purified using the Biomek 4000 AMPure XP method. The purification was performed on a 96-well plate containing the amplified 452bp fragments in one well and water in the adjacent well (Figure 5A). These purified samples were then transferred to a 384-well real-time PCR plate. Each purified sample (PCR fragment or water) was placed in the upper left corner of a four-well quadrant on the 384-well plate, with nothing added to two of the other wells, and a PCR calibration standard added to the fourth well (Figure 5B).

Real-time amplification was then performed using the ABI7900HT Fast Real-Time PCR system and the KAPA SYBR FAST master mix. True amplification was observed only in the wells containing the 452bp PCR fragments that had been purified using the automated AMPure XP system, and not in the control wells which contained water before purification (Figure 5C). The accuracy of quantitation using the automated dispensing of calibration standards was excellent, resulting in an R2 value of 0.9849. All of the re-purified fragments showed nearly identical amplification curves and Ct values, demonstrating the excellent reproducibility of the automated purification system.

	Automation (Column #1-3)	Manual (Column #4)	Automation/ Manual (%)
Yield (µg/mL)	5.5823	5.8491	95.4
Purity (260 nm/230 nm Ratio)	2.02	1.97	102.5

Table 1: Purification efficiency data using automated AMPure XP. 1000 bp pGEM plasmid DNA PCR fragments were purified using the Biomek 4000 AMPure XP method and a manual process. Yield (95.4%) and purity (102.5%) obtained in triplicate by automation using three columns were compared to those obtained from manual purification.

B2013-14053 IB-17985A

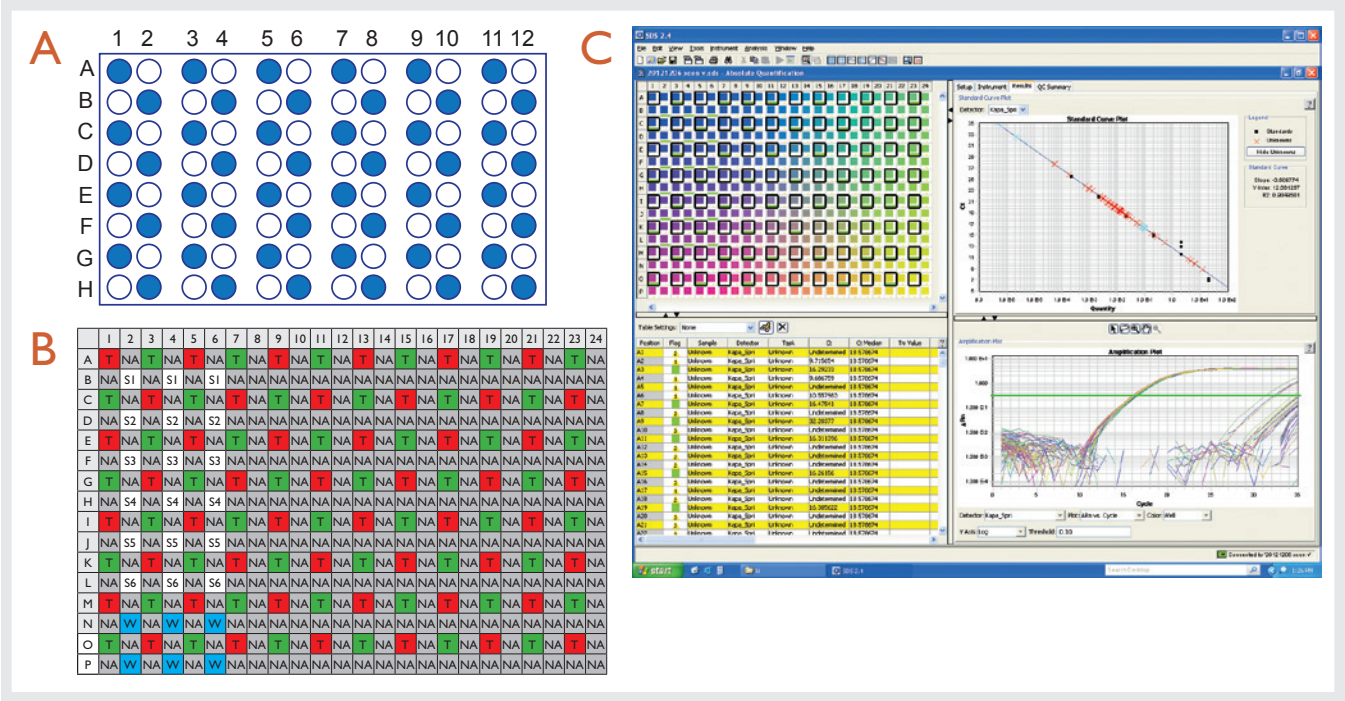


Figure 5. (A) A 96-well plate containing water (blue wells) and 70 µL of 452bp PCR fragments produced by manual amplification of the standard from the KAPA SYBR Fast Universal qPCR Kit (white wells), which was used to demonstrate the automated AMPure XP purification system.

(B) After purification, the samples were transferred to a 384-well real-time PCR plate in such a manner that each sample from A was represented by four wells on the real-time plate: one for the sample, one for a real-time PCR calibration standard, and two that are empty. T= AMPure reagent eluate samples; S = Calibration standards 1-6; W = Water blank controls for the real-time PCR reaction; NA = empty wells. Red wells are negative samples derived from the blue wells containing water in A, and green wells are positive samples derived by purification from the white wells containing 452bp fragments in A.

(C) The results of real-time amplification of the samples in B, illustrating true amplification of only the purified 452bp fragments, and no cross-contamination in samples derived from purification of the wells containing water in A. All of the re-purified fragments showed nearly identical amplification curves and Ct values, demonstrating the excellent reproducibility of the automated purification system. The noise under the amplification curve is derived from primer-dimer amplification.

Conclusion

Automation solutions for both PCR reaction setup and purification can help users from small, medium and high throughput laboratories improve efficiency and results. The Biomek 4000 Workstation and AMPure XP purification system improve reproducibility from day-to-day and across laboratories, reduce the opportunity for human error, and cut labor and reagent costs. The automation templates are simple, fast, and easy to use. The resulting amplification is highly reproducible, and the purification matches the yield and purity of manual procedures, while also delivering highly reproducible results. In addition, both processes are completed without cross-contamination, assuring reliable results.

BECKMAN COULTER
Life Sciences

Fast, Reliable Automated PCR Reaction Setup

Using the Biomek 4000 Laboratory Automation Workstation

A PCR Reaction Setup application is offered on the Biomek 4000 Laboratory Automation Workstation, the newest addition to Beckman Coulter's line of automated liquid handlers. This application guides the user through the process of setting up a PCR reaction for 1-192 samples in 96-well plates with any combination of Master Mix, Primers, and Samples. It is the ultimate automation solution for complicated PCR reaction setup problems.

Supports 1-192 high throughput configurations with up to two master mix, two primers, and two sample source labware (Figure 2).

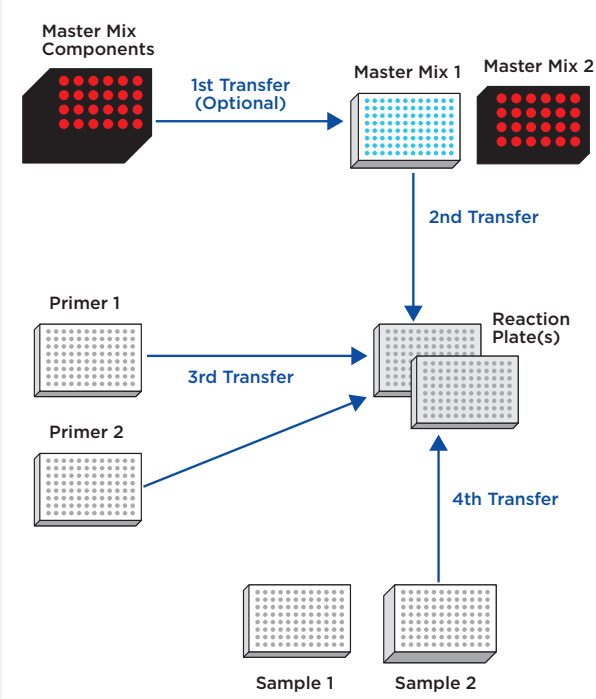


Figure 1. PCR Reaction Plate Setup Process.

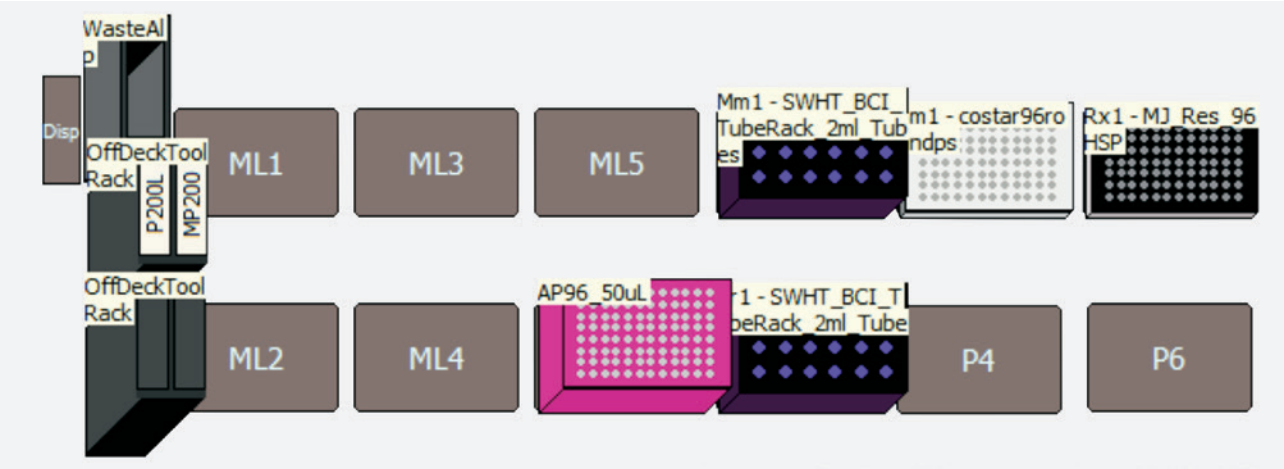
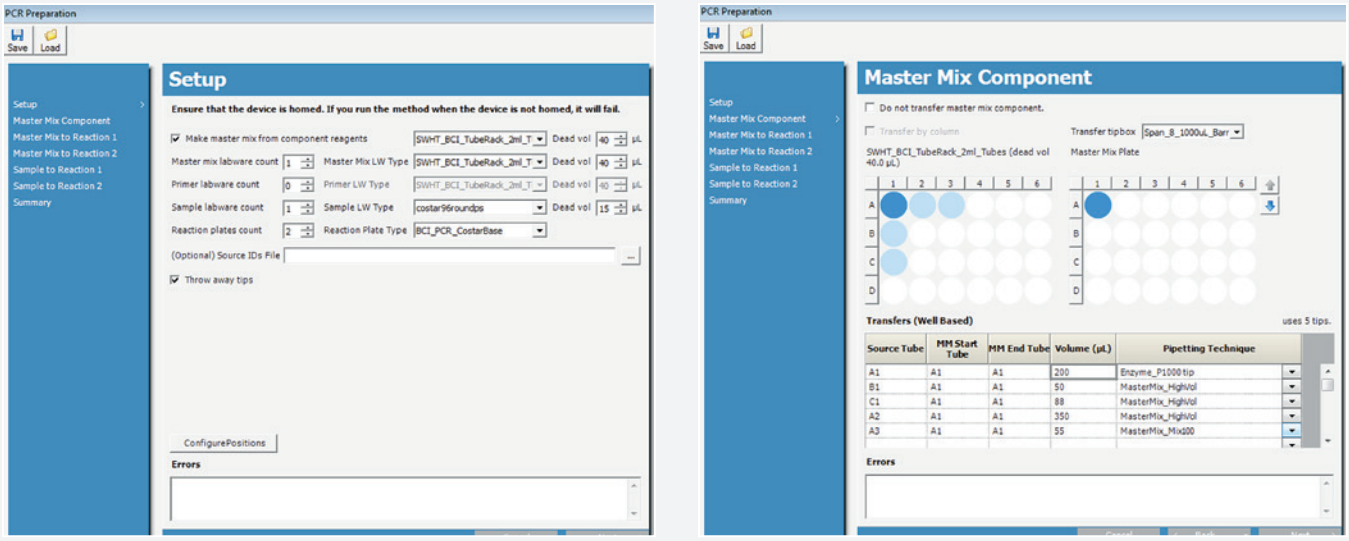


Figure 2. Up to two 96-well PCR reaction plates can be created in one reaction setup process.

Discovery *in motion.*

BECKMAN COULTER
Life Sciences

Supports both ready PCR master mix and master mix made from individual PCR components (Figure 3).



(3a) (3b)

Figure 3. Master Mix was made from individual PCR components, i.e., AmpliTaq DNA polymerase, dNTP, MgCl2, Triton X-100, water, and primers. All PCR components can be transferred from tubes from source labware (3a) to A1 tube of destination labware (3b).

RESULTS

The PCR reaction setup process has flexible sample and reagent test combinations, only limited by the number of available tips and reagent volumes. The result is you get excellent PCR products.

Using automation helps eliminate cross contamination in PCR reactions (Figure 5).

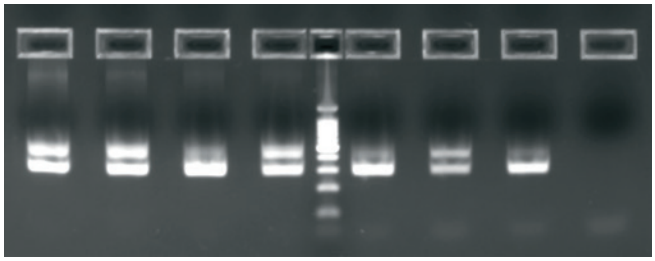


Figure 4. β-actin PCR products (285 bp) from human genomic DNA dilution series (Lane #1-7: 480 ng/µL to 7.5 ng/µL; Lane #8: water as negative control, and Middle Lane: 100 bp ladder).

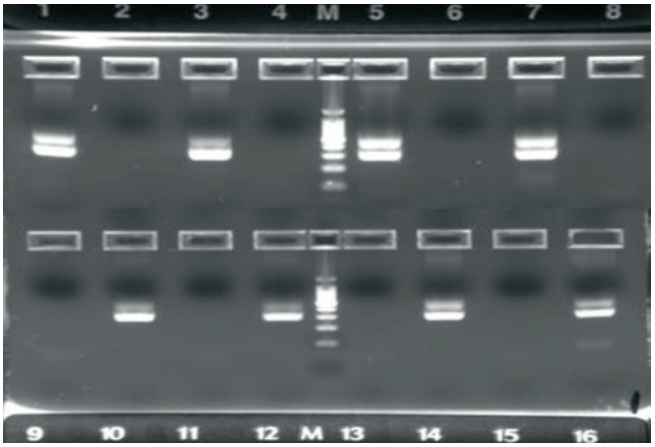


Figure 5. Cross-contamination test had shown no presence of the β-actin PCR products in the adjacent negative wells where human gDNA template was replaced with water.

BECKMAN COULTER

Life Sciences

For Beckman Coulter's worldwide office locations and phone numbers, please visit www.beckmancoulter.com/contact

B2012-13603-DG-1K

PRINTED IN U.S.A.