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Life Sciences

High Throughput Preparation of High Quality Sequence-Ready Libraries from Cell-Free DNA (cfDNA) using Rubicon Genomics ThruPLEX® Plasma-seq Kit Automated on the Biomek FXP Laboratory Workstation

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Introduction

The ThruPLEX® Plasma-seq sample preparation kit takes advantage of ThruPLEX chemistry to generate high performance NGS libraries from cell-free DNA (cfDNA) isolated from plasma. Newly formulated repair and ligation reagents minimize PCR duplicates providing maximum library complexity and preserve the GC representation of the input DNA. Library construction is performed in a fast and simple, single-tube, 3-step process optimized for cfDNA inputs from < 1ng to 30ng. The streamlined workflow prevents sample loss and enhances positive sample identification. ThruPLEX Plasma-seq libraries can be used for research oncology applications in which circulating tumor DNA (ctDNA) is sequenced or in research involving the fetal fraction of maternal plasma. Libraries can be used for Copy Number Variant (CNV) analysis, whole genome sequencing applications or enriched using custom panels or leading target enrichment platforms.

The automated Biomek FXP method enhances the benefits of the Rubicon Genomics ThruPLEX Plasma-seq chemistry by providing high-throughput walk-away capability for all pipetting and liquid handling operations with optional on-deck integration of reaction incubations and thermal cycling. Temperature controlled reagent storage ensures consistently high quality libraries. Bead-based sample purification following PCR amplification is accomplished using trusted AMPure XP chemistry. The automated method is compatible with the 48S and 96D versions of the ThruPLEX Plasma-seq kits (Rubicon Genomics P/N, R400491 and R400492, respectively).

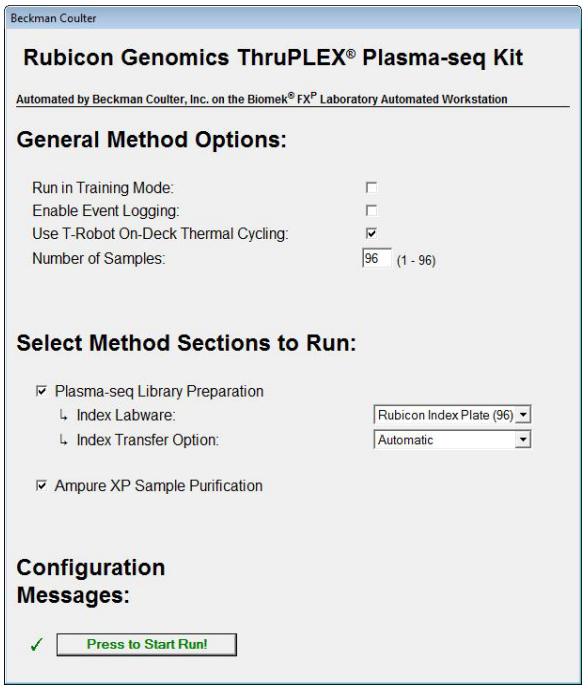

Figure 1. Example of user-defined method options selected via the Automated Rubicon ThruPLEX Plasma-seq method User Interface.

Automated Method Features and Key Benefits

The automated Rubicon ThruPLEX Plasma-seq Sample Preparation workflow features an intuitive User Interface (Figure 1) that allows for selection of customizable workflow options. The user may process any number of samples between 1 and 96. Users can execute the library preparation and sample purification processes separately or as a combined walk-away workflow allowing for maximum flexibility in planning their experiments. A full 96-sample library preparation run can be completed in < 3 hours including reagent and instrument setup (Figure 2). Various index labware options are available, including use of the ThruPLEX Plasma-seq index plates provided with the 48S and 96D kits. Index transfers can be accomplished either through pre-set well-mapping patterns or via a user-defined transfer file.Options selected via the User Interface dynamically update a Reagent Calculator which provides the user with instructions for preparation of reagent Master Mixes and reagent labware setup (Figure 3).

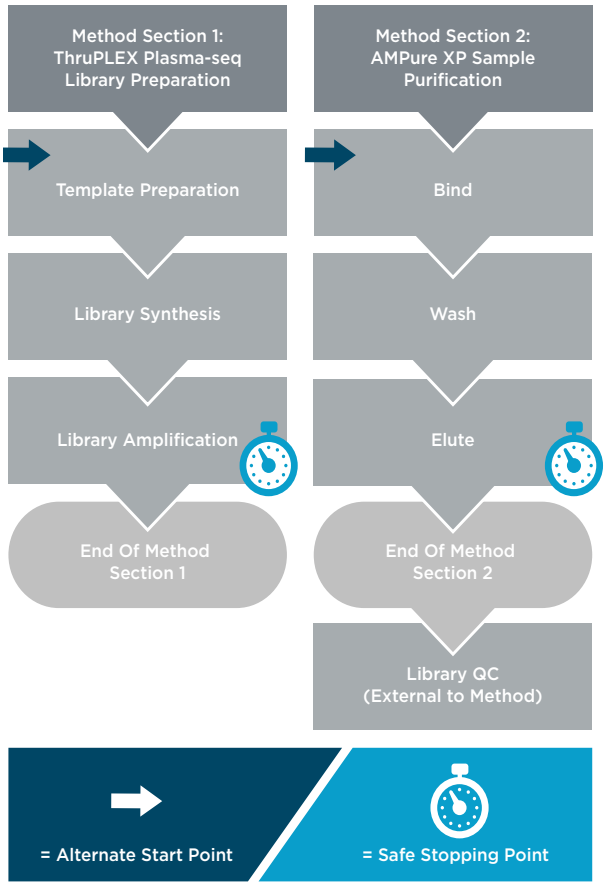

Figure 2A. Automated Rubicon ThruPLEX Plasma-seq workflow.

Method Section	Sample Number*/ Processing Time (h:m)			
ThruPLEX Plasma-seq Library Preparation	**12**	**24**	**48**	**96**
Method Setup	0:15	0:15	0:15	0:15
Method Run	2:24	2:30	2:34	2:42
Total	**2:39**	**2:45**	**2:49**	**2:57**
AMPure XP Sample Purification	**12**	**24**	**48**	**96**
Method Setup	0:15	0:15	0:15	0:15
Method Run	0:38	0:39	0:42	0:49
Total	**0:53**	**0:54**	**0:57**	**1:04**
Full Method (Library Prep + AMPure XP)	**12**	**24**	**48**	**96**
Method Setup	0:15	0:15	0:15	0:15
Method Run	3:02	3:09	3:16	3:31
Total	**3:17**	**3:24**	**3:31**	**3:46**

Figure 2B. Estimated processing times. The entire method may be performed with a single instrument setup or for added flexibility; each Method Section may be executed separately. *Any number of samples may be run between 1 and 96.

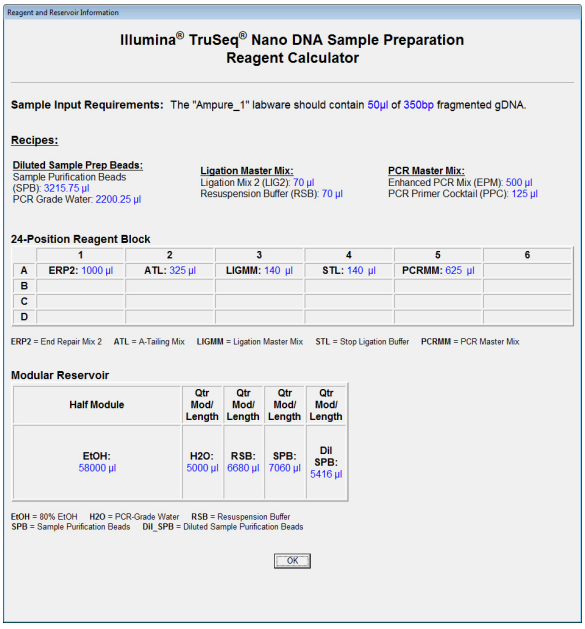

Figure 3. Example of Recipe and Reagent calculations provided by the Automated Rubicon ThruPLEX Plasma-seq Reagent Calculator.

The method employs 8 individual Span-8 probes as well as a 96-well multi-channel pipetting head to deliver the right balance of efficiency and reagent conservation for library preparation and sample cleanup steps. A Peltier unit ensures that enzyme master mixes are maintained at proper temperature during the course of the method. Optimized pipetting techniques deliver highly accurate and reproducible transfers of small reaction volumes ensuring robust reaction chemistry.

Experimental Design and Results

Low throughput and high throughput runs were performed with cfDNA of various inputs (0.1, 1, 5 and 10ng) along with replicates of non-template control (NTC) samples (PCR-grade H_2O). The samples were processed using Rubicon's ThruPLEX Plasma-seq protocol followed by AMPure XP sample purification automated on the Biomek FX^P Laboratory Automated Workstation. Library quality was assessed using an Agilent DNA High Sensitivity chip assayed on the Bioanalyzer 2100 instrument and analyzed with the Agilent 2100 Expert Software (Figure 4). Samples from the high-throughput run were quantified using the KAPA SYBR Fast Universal 2X qPCR Master Mix library quantification kit (PN# KK4824, KAPA Biosystems) and assayed on a CFX96 (Bio-Rad Laboratories) real-time PCR Instrument (Figure 4). Triplicate libraries from each input amount were sequenced on an Illumina NextSeq 500. NGS metrics of Median Insert Size, Estimated Library Size and % Duplication demonstrate similar metrics between manually prepared and Biomek prepared libraries. (Figure 5). Libraries also demonstrate low GC bias in GC% windows that constitute the majority of the human genome (Figure 6). In addition, extensive bioinformatics analysis demonstrates a highly robust process that is absent of any well-to-well cross-contamination (Figure 7).

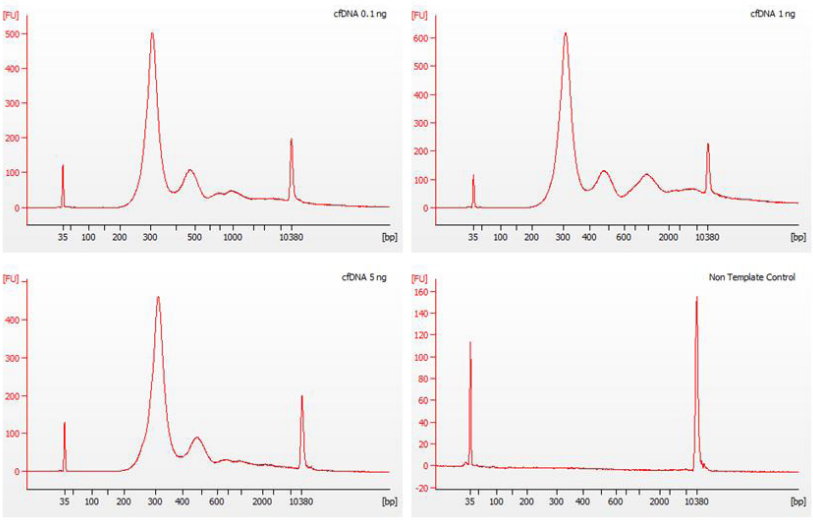

Figure 4A. Representative tracings of high quality cfDNA libraries generated from the automated Thru-PLEX Plasma-seq method showing Fluorescent Units (FU) over base pair fragment size (bp) assayed on an Agilent 2100 Bioanalyzer using a DNA High Sensitivity chip.

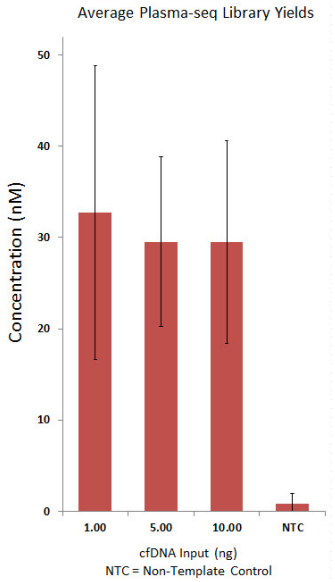

Figure 4B. Library yields from high-throughput run assayed with KAPA Biosystems Library Quantitation kit.

	Human cfDNA		Human cfDNA		Human cfDNA	
Input (ng)	0.1		1		10	
	Manual	**Biomek**	**Manual**	**Biomek**	**Manual**	**Biomek**
Median Insert Size	166	169	167	169	167	169
SD (n=3)	3.4	0.6	3.1	0.6	2.6	0.0
Estimated Library Size	5.1E+07	2.0E+08	1.7E+08	4.5E+08	2.5E+08	6.2E+08
% Duplication	4.5	6.6	2.8	2.3	1.9	2.4
SD (n=3)	0.9	1.4	0.4	0.2	0.2	0.1

Figure 5. Summary of critical NGS metrics to determine quality of cfDNA sequencing. Manually prepared and Biomek prepared libraries have similar metrics. Different samples and different degrees of multiplexing likely contribute to differences in Library size.

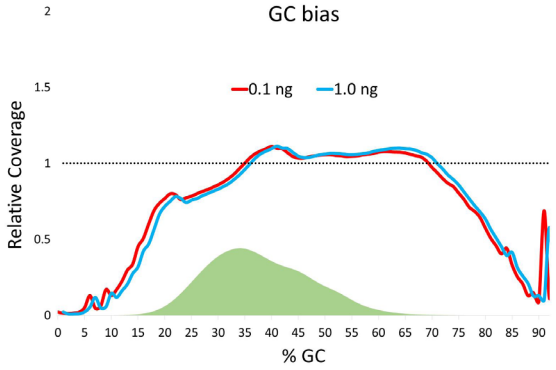

Figure 6. Biomek prepared libraries of 0.1 ng input (red line) and 1.0 ng input (blue line) demonstrate low GC bias in GC% windows that constitute the majority of the human genome (GC% relative distribution in entire human reference genome shown in green).

	Human cfDNA		Human cfDNA		Human cfDNA		*Arabidopsis* gDNA	
Input (ng)	0.1		1		10		10	
Ref. Genome	hg19	TAIR10	hg19	TAIR10	hg19	TAIR10	hg19	TAIR10
% Unmapped Reads	0.7	99.5	0.7	99.5	0.6	99.6	99.8	2.1
SD (n=3)	0.1	0.0	0.0	0.0	0.0	0.0	-	-

Figure 7A. NGS libraries generated from both human cfDNA and Arabidopsis gDNA were prepared on the same 96 well plate. Libraries exhibit a significant majority of unmapped reads when aligned to the inappropriate reference genome, indicating the absence of significant amounts of contaminating library from neighboring wells.

	Arabidopsis gDNA	Human cfDNA	NS	
Ref. Genome	TAIR10	hg19	TAIR10	hg19
Pass Filter Reads Aligned	2.40E+07	2.98E+07	502	239
SD	-	6.33E+06	5.4	3.2
	(n=1)	(n=9)	(n=78)	(n=78)

Figure 7B. Only select libraries prepared on the 96 well plate were loaded into the NextSeq for sequencing. However, all libraries present on the 96 well plate were included during demultiplexing, including those that were not loaded into the NextSeq (NS = not sequenced). NS libraries did not appear in a significant quantity when aligned to either reference genome, indicating the absence of indexes corresponding to libraries that were not loaded into the NextSeq.

Summary

Rubicon's ThruPLEX Plasma-seq Kit automated on the Biomek FX^P Laboratory Automated Workstation provides a feature-rich, robust, flexible and efficient walk-away library preparation workflow. The protocol has been demonstrated to prepare up to 96 high quality sequence-ready libraries per run from cfDNA inputs between 0.1ng and 10ng in < 3 hours which are suitable for sequencing on all Illumina platforms. Sequencing analysis demonstrates a highly accurate and reproducible automation protocol capable of generating high quality sequencing metrics which are similar to those of manually prepared libraries having low GC bias and absence of any well-to-well cross contamination.

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