Illumina® TruSeq® RNA Access Library Prep Kit Automated on the Biomek FX^P Dual-Hybrid Liquid Handler

David Horvath, M.S., Senior Applications Scientist, Beckman Coulter, Inc.; Tim Hill, Scientist, Illumina, Inc.

Introduction

The Biomek automated Illumina TruSeq® RNA Access Library Prep method is designed to address challenging workflows such as RNA-Seq and target capture when using degraded and FFPE derived RNA. It was developed specifically to optimize throughput and provide robust and reproducible results from the Illumina TruSeq® RNA Access Library Prep Kit Sets A and B (RS-301-2001 or RS-301-2002, respectively). The automated method supports processing up to 2 full RNA Access kits per run (96 pre-hybridization libraries and 24 4-plex hybridization capture pooled libraries), allowing for complete TruSeq® RNA Access library preparation in as little as 3 days, providing a significant increase in productivity as compared to manual library preparation. This technical note describes the automation of the Illumina TruSeq® RNA Access Library Prep Kit on the Beckman Coulter Biomek FX^P Dual Hybrid automated liquid handler (96-well Multichannel Pipettor and Span-8 Pipettor).

The Illumina TruSeq® RNA Access Library Prep kit overcomes the challenges of FFPE sample gene expression profiling studies by combining RNA Seq with exome enrichment. By focusing on the coding region of RNA, RNA Access requires less input (10 ng for fresh/frozen and 20 ng for FFPE samples) and fewer reads, thereby increasing the number of samples per run for more cost-effective transcriptome analysis.

The automated method contains 5 distinct modules designed around the Illumina recommended safe stopping points which provide users with substantial flexibility in planning their experiments. The desired module, as well as module specific options, is selected via a guided Software User Interface to assist with run configuration from 1 to 96 samples. Reagent Calculators help reduce setup errors by providing the user clear instructions for preparing master mixes and reagents. The method provides options to perform incubations and/or thermal cycling either off-deck using an external thermal cycler or on-deck with a Biometra TRobot thermal cycler integrated to the Biomek FX^P liquid handler deck, providing true walk-away capability. The method also employs 8 individual Span-8 probes which enable individual well pipetting and reduce the required dead volume when transferring precious enzymes or reagents by allowing pipetting from any labware format. A static Peltier unit ensures that enzyme master mixes are kept cool during the course of the run. Cleanup, wash, elution and sample transfers are efficiently performed utilizing the multi-channel 96 pipetting head.

Protocol

This automated method utilizes the Biomek FX^P automated liquid handler in conjunction with the Illumina TruSeq® RNA Access Library Prep Kit protocol (15049525 Rev. A). Automation consumables, instrument configuration and other details can be found at the end of this document. The Illumina TruSeq® RNA Access Library Prep Biomek Method is divided into 5 Modules to allow for greater workflow flexibility. Each Module is designed to be run individually and may contain Sections and various Section Options selectable through a pop-up Software User Interface which allows for more specific run parameter configuration. All Illumina Safe Stopping Points are supported (Figure 1).

The automated Illumina TruSeq® RNA Access Library Prep method utilizes an HTML-driven Software User Interface (UI) that allows the user to customize their workflow by offering a number of different options (Figure 2). The user may select any number of samples between 1 and 96 for the cDNA Synthesis, Library Construction and Quant/Norm/Pooling Modules and between 1 and 48 samples for the Hybridization and Capture

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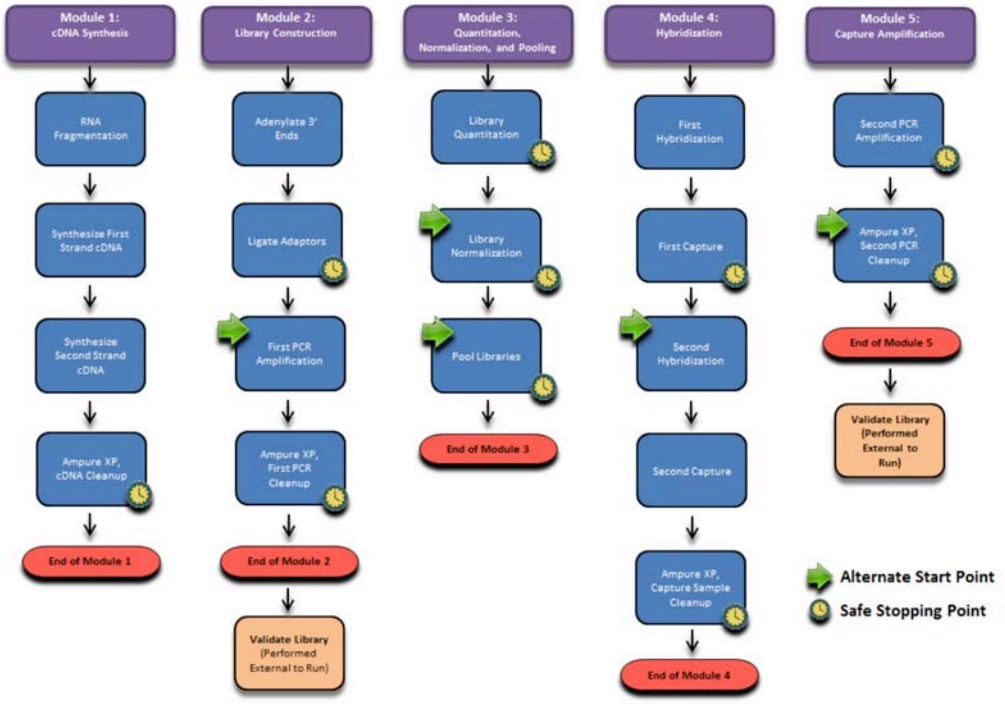

Figure 1: Overview of the automated Illumina TruSeq® RNA Access Library Prep workflow and corresponding Illumina protocol steps.

Amplification Modules. In addition, the user can choose to perform off-deck thermal cycling and incubations or perform on-deck thermal cycling and incubations using an integrated Biometra T-Robot thermal cycler. Other options include FFPE or non-FFPE tissue and the ability to utilize alternate start points within a module. Several options are provided around the addition of the TruSeq® adaptors to the deck, including an optional pause step to allow for addition of the adaptors immediately prior to use and the ability to perform custom TruSeq® adaptor transfers based on a user-supplied .csv formatted file. Additionally, the user can specify the type of labware containing the TruSeq® adaptors, including tubes or a variety of 96 well PCR plate types.

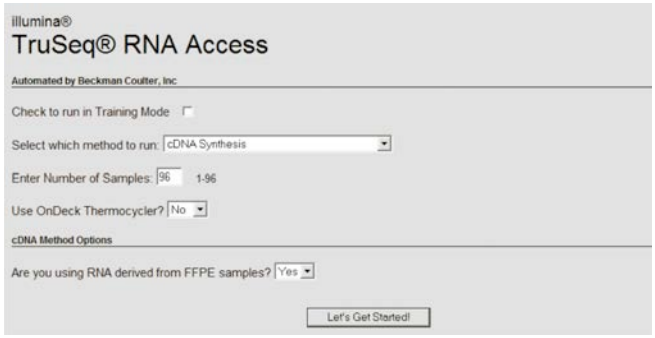

Figure 2: Example screenshot of the Automated Illumina TruSeq® RNA Access Library Prep User Interface.

In addition to the User Interface, the automated Illumina TruSeq® RNA Access Library Prep method contains an HTML-driven Reagent Calculator that provides the user with the recipes and total volumes of all required master mixes and reagents required for the run based upon the number of samples to be processed and the Module / Section that the user has selected to run via the UI (Figure 3).

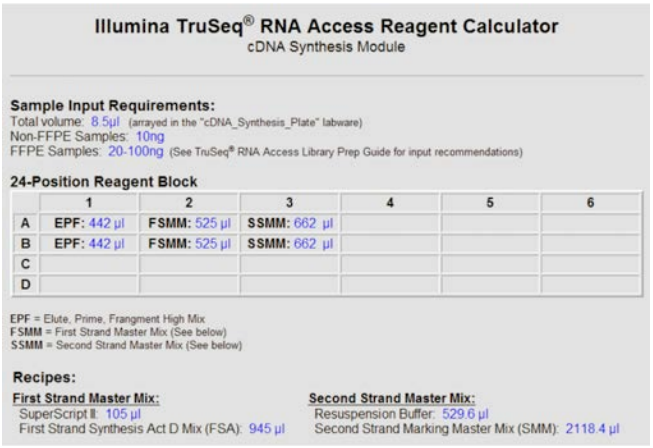

Figure 3. Example screenshot of the automated Illumina TruSeq® RNA Access Library Prep Reagent Calculator

Experimental Design and Results

Universal Human Reference (UHR) RNA (Agilent P/N 74000), FirstChoice® Human Brain Reference RNA (Life Technologies P/N AM6050) and FFPE Total RNA from Human Adult Normal Liver (Bio-Chain P/N R2234149) were selected as control samples for this experiment. Each RNA control sample was normalized to 50ng/ul and run on the 2100 Bioanalyzer (Agilent) using an RNA 6000 Nano (Agilent) chip (Figure 4). The recommended sample input amount for each control was determined based on DV_{200} values (Table 1) and 8 technical replicates were arrayed for each control into a 96-well BioRad Hard-Shell PCR plate and normalized to a total starting sample volume of 8.5µl.

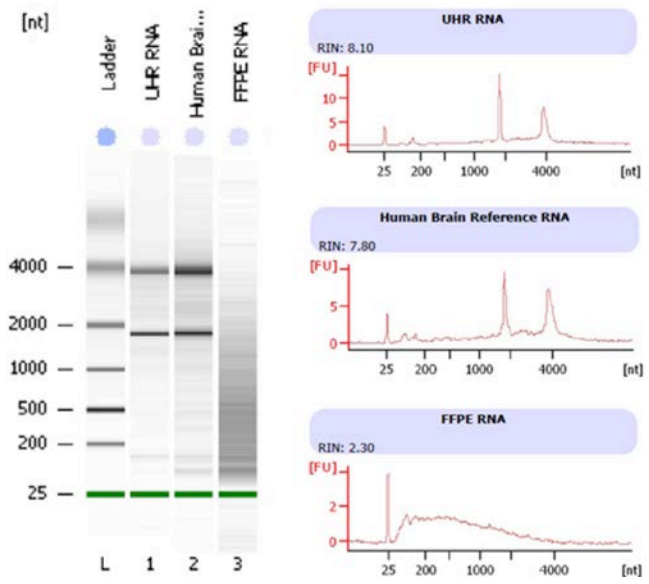

Figure 4. Gel image and electrophoretic data of RNA control samples run using an RNA 6000 Nano chip on a Bioanalyzer 2100 instrument and analyzed using the 2100 Expert software.

Control Sample	DV_{200} Value	Recommended Input
UHR RNA	96%	10 ng
Brain RNA	86%	10 ng
FFPE RNA	70%	20 ng

Table 1: DV_{200} values Recommended Input amounts of each RNA Control Sample.

The samples were processed using the automated Illumina TruSeq® RNA Access Library Preparation method on the Biomek FX^P automation platform through the cDNA Synthesis and Library Construction Modules. The samples were assayed following the first PCR enrichment on the Bioanalyzer 2100 using a DNA 12000 chip (Figure 5). Quantitation assays were prepared using the Quantitation module with Pico-Green assay (Life Technologies). Sample concentrations were then analyzed on a Paradigm (Beckman Coulter) fluorescent plate reader (Figure 6).

Results of the quantitation were used to normalize the samples to a concentration of 20 ng/µl followed by creation of (2) 4-plex pooled samples for each 8 sample control set using the Normalization and Pooling options of the Quant/Norm/Pool Module. Each pool contained 200 ng of library from each sample component in the pool for a total of 800 ng of prepared library in a final volume of 40 µl.

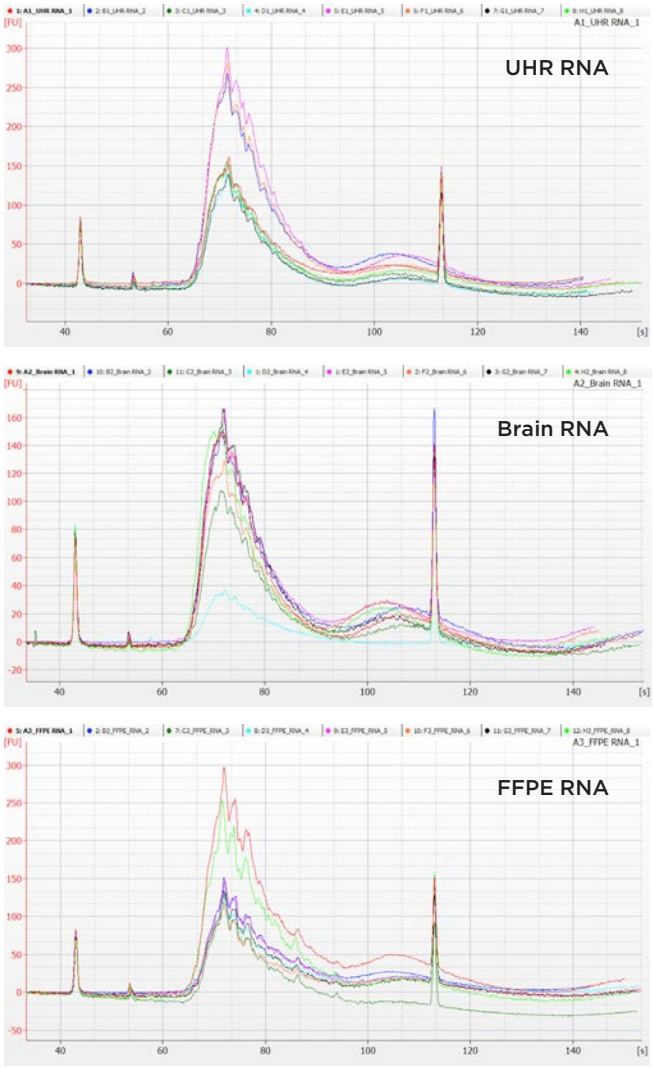

Figure 5: Electrophoretic data of cDNA libraries following first PCR enrichment. Samples were processed using a DNA 1000 chip on a Bioanalyzer 2100 instrument and analyzed using 2100 Expert software (Agilent).

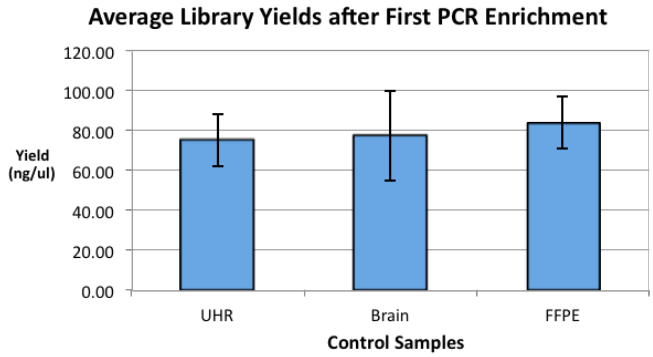

Figure 6: Pico-Green quantitation results showing average library yields for each control set following the first PCR enrichment.

Samples were then processed through the Hybridization and Capture Amplification modules. The samples were assayed following the second PCR enrichment on the Bioanalyzer 2100 using a High Sensitivity DNA Chip (Figure 7). Quantitation assays were prepared for the pooled libraries using the KAPA SYBR Fast Universal 2X qPCR Master Mix library quantification kits (PN# KK4824, KAPA Biosystems) and assayed on a 7900 HT (Applied Biosystems) real-time PCR Instrument (Figure 8).

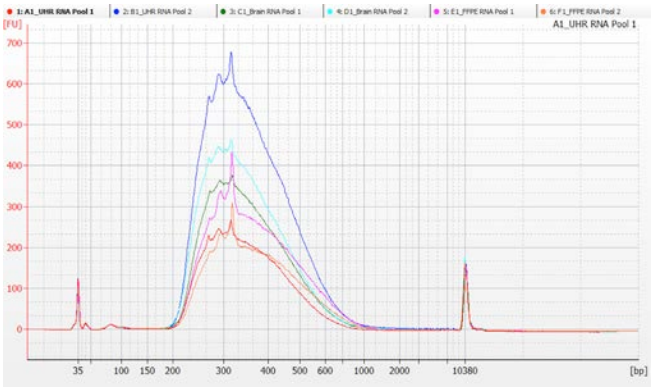

Figure 7: Electrophoretic data of cDNA library pools following second PCR enrichment. Samples were processed using a DNA High Sensitivity chip on a Bioanalyzer 2100 instrument and analyzed using 2100 Expert software (Agilent).

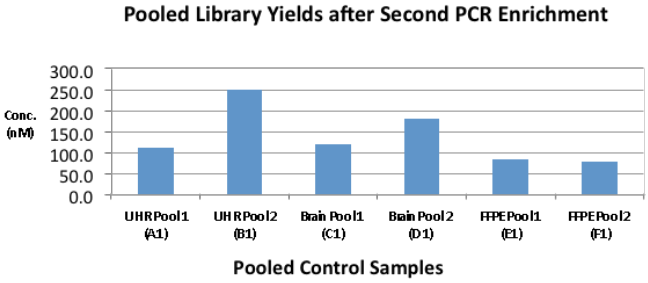

Figure 8: qPCR library pool yields for each control set following the second PCR enrichment.

Each pooled sample was prepared in quadruplicate for a run on a HiSeq 2500 Sequencer (Illumina) using v3 sequencing chemistry. Samples were sequenced in a 2 x 75 cycle, paired end run and mapped to the human reference genome (hg19) using TopHat alignment parameters implemented via BaseSpace (https://basespace.illumina.com). The TruSeq® RNA Access pooled libraries generated an average of > 12 million pass filter reads, average % Abundant of < 3% and average Ribosomal RNA reads of < 2.1% (Table 2).

Alignment distribution analysis demonstrated that the TruSeq® RNA Access libraries were highly enriched for coding transcripts with an average of > 75% alignment to coding regions for each of the pooled libraries (Figure 9). The automated protocol produced highly reproducible results for % duplicates and median insert size (Figure 10).

Sample	# of Reads	% Total Aligned	% Unaligned	% Abundant	% Ribosomal	% Median CV Coverage Uniformity	% Stranded
A1-1	11,236,271.00	98.2%	1.8%	1.9%	1.4%	96.5%	98.9%
A1-2	15,407,099.00	98.2%	1.8%	2.0%	1.5%	95.0%	99.1%
A1-3	9,806,134.00	98.1%	1.9%	1.7%	1.3%	95.5%	99.1%
A1-4	15,333,806.00	98.1%	1.9%	1.9%	1.4%	96.0%	99.0%
average	**12,945,827.50**	**98.1%**	**1.9%**	**1.9%**	**1.4%**	**95.8%**	**99.0%**
B1-1	28,650,424.00	98.2%	1.8%	2.3%	1.8%	94.5%	99.0%
B1-2	12,338,409.00	98.2%	1.8%	1.4%	1.0%	96.0%	98.8%
B1-3	15,203,520.00	98.2%	1.8%	1.7%	1.3%	95.5%	98.4%
B1-4	15,366,472.00	98.3%	1.7%	2.1%	1.6%	98.0%	98.8%
average	**17,889,706.25**	**98.2%**	**1.8%**	**1.9%**	**1.4%**	**96.0%**	**98.7%**
C1-1	20,534,042.00	98.0%	2.0%	2.0%	1.2%	108.5%	98.3%
C1-2	14,857,353.00	97.8%	2.2%	4.0%	3.1%	104.5%	98.5%
C1-3	15,760,871.00	97.8%	2.2%	3.6%	2.6%	104.0%	98.5%
C1-4	6,128,368.00	97.2%	2.8%	2.1%	1.3%	108.0%	98.5%
average	**14,320,158.50**	**97.7%**	**2.3%**	**2.9%**	**2.1%**	**106.3%**	**98.4%**
D1-1	16,044,626.00	97.9%	2.1%	2.1%	1.3%	105.0%	98.5%
D1-2	21,508,042.00	97.9%	2.1%	2.3%	1.5%	106.0%	98.4%
D1-3	18,873,105.00	97.9%	2.1%	1.6%	1.0%	106.0%	98.5%
D1-4	16,903,908.00	97.9%	2.1%	2.3%	1.5%	107.5%	98.4%
average	**18,332,420.25**	**97.9%**	**2.1%**	**2.1%**	**1.3%**	**106.1%**	**98.4%**
E1-1	23,311,844.00	97.9%	2.1%	1.7%	1.3%	99.5%	99.8%
E1-2	23,528,647.00	97.8%	2.2%	2.2%	1.7%	96.5%	99.8%
E1-3	16,540,492.00	97.8%	2.2%	2.5%	2.0%	97.5%	99.8%
E1-4	20,860,340.00	97.8%	2.2%	2.2%	1.7%	97.0%	99.8%
average	**21,060,330.75**	**97.8%**	**2.2%**	**2.2%**	**1.7%**	**97.6%**	**99.8%**
F1-1	13,306,255.00	97.7%	2.3%	1.9%	1.0%	97.5%	99.8%
F1-2	10,749,932.00	97.8%	2.2%	2.0%	1.5%	98.0%	99.8%
F1-3	14,746,032.00	97.9%	2.1%	1.4%	0.9%	97.0%	99.8%
F1-4	22,856,236.00	97.9%	2.1%	1.6%	1.1%	100.5%	99.8%
average	**15,414,613.75**	**97.8%**	**2.2%**	**1.7%**	**1.1%**	**98.3%**	**99.8%**

Table 2: High level summary of paired end 75 bp HiSeq 2500 sequence runs.

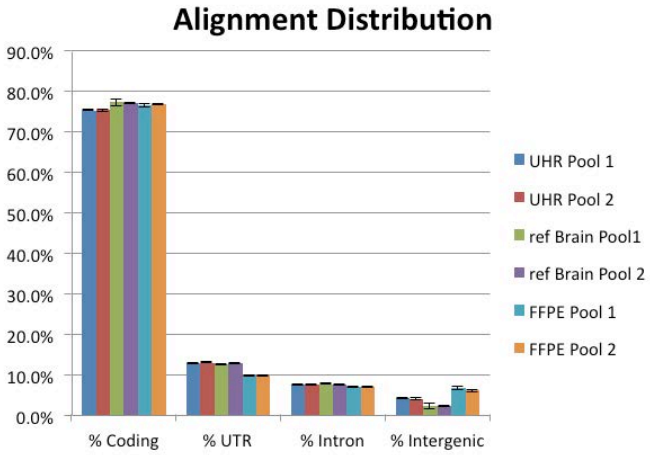

Figure 9: Percent of reads aligned to region. TruSeq® RNA Access enriches for reads aligning to coding region.

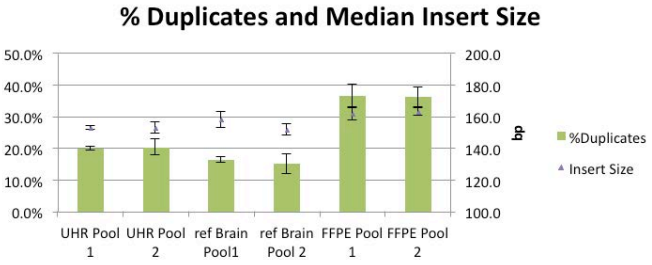

Figure 10: Automated protocol produces highly reproducible results for % duplicates and median insert size. Duplicates will vary depending on sample quality.

Conclusion

Coupling the Illumina TruSeq® RNA Access Library Prep Kit with the Beckman Coulter Biomek FX^P automated liquid handler increases the power of your research objectives by providing robust and reliable RNA Seq / Exome Enrichment results with walk-away capability.

The Biomek FX^P Automated TruSeq® RNA Access method has been demonstrated to generate highly enriched exome libraries. Sequencing analysis further demonstrated high quality, robust RNA-Seq data, yielding an average of >12 million pass filter reads for each 4-plex library pool with a minimal % Abundance and % Ribosomal component. The automated protocol produced >75% of reads aligned to coding regions with highly reproducible results for % duplicates and median insert size.

In summation, the TruSeq® RNA Access Library Prep Kit automated on the Beckman Coulter Biomek FX^P Dual Hybrid automated liquid handler (96-well Multichannel Pipettor and Span-8 Pipettor) delivers a demonstrated, robust, flexible and efficient walk-away RNA Seq / Exome Enrichment workflow that is capable of generating 96 RNA Seq libraries from low input samples which are suitable for sequencing on all Illumina platforms in as little as 3 days.

TruSeq RNA Access Library Prep Automated Method Standard Deck Layout

Required ALPs/ Devices

PART NUMBER	QTY	MANUFACTURER	DESCRIPTION
719948	1	Beckman Coulter	4x3 ALP kit
379448	1	Beckman Coulter	Orbital Shaker ALP, Single Position
719357	2	Beckman Coulter	Static 1x1 ALP Platform
719361	1	Beckman Coulter	Static Peltier ALP
A93942	1	Beckman Coulter	Shaking Peltier ALP
719590	1	Beckman Coulter	Span-8 Disposal ALP
719356	1	Beckman Coulter	Disposable Tip Loader ALP
719654	1	Beckman Coulter	Span-8 wash ALP
719363	1	Beckman Coulter	Wash Station including pump and tubes
719366	1	Beckman Coulter	Biomek FX Device Controller
846-050-991	1	Biometra	(Optional) TRobot 96 Thermocycler

Required Labware

PART NUMBER	QTY	MANUFACTURER	DESCRIPTION
B01124	6	Beckman Coulter	Biomek Span-8 P1000 Tips, Pre-sterile with Barrier
379503	6	Beckman Coulter	Biomek Span-8 P250 Tips, Pre-sterile with Barrier
717253	7	Beckman Coulter	Biomek AP96 P250 Tips, Pre-sterile with Barrier
A21586	20	Beckman Coulter	Biomek P50 Tips, Pre-sterile with Barrier
534681	3	Beckman Coulter	Reservoir, Half
372790	14	Beckman Coulter	Reservoir, Quarter
372792	2	Beckman Coulter	Reservoir, Quarter, Divided by Width
372795	1	Beckman Coulter	Reservoir, Frame
A32782	1	Beckman Coulter	Agencourt® SPRIPlate® 96R - Ring Super Magnet Plate
A83054	1	Beckman Coulter	BCI Tube Block (Aluminum Reagent Block)
373661	1	Beckman Coulter	Beckman Coulter BCI_TubeRack_2ml_Tubes
AB-1127	9	Fisher Scientific	Abgene 96-Well Storage Plate, Square Well, 1.2 mL
HSP-9641	18	Bio-Rad	Hard-Shell® Thin-Wall 96-Well Skirted PCR Plates
655076	2	VWR	Greiner Bio-One Black Pico Green Assay Plate
16466-042	56	VWR	2mL Screw Cap Microcentrifuge Tubes- Conical Bottom
ML-5010	2	Phenix Research	Phenix Research Black Universal Lid
MSL-2022	1	Bio-Rad	Arched Auto-Sealing Lids. Optional - for use with On-Deck T-Robot thermal cycling only

User-Supplied Consumables

PART NUMBER	MANUFACTURER	DESCRIPTION
AB00138-01000	American Bioanalytical	Ethanol
FC-121-3001 (LT-A) FC-121-3002 (LT-B) FC-121-3003 (96)	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)

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

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