Fully Automated NEBNext Direct Genotyping Solution using Beckman Coulter Life Sciences Biomek i7 Workstation

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Abstract

The advancement of next generation sequencing technologies has enabled rapid genotyping of organisms for various applications such as plant and animal breeding. The increase of sequencing throughput in combination with the decrease of sequencing cost has allowed genotyping of large numbers of samples. The NEBNext Direct Genotyping Solution delivers cost-effective, high-throughput, NGS based targeted genotyping for plant and animal applications. It allows efficient genotyping of thousands of markers with high target coverage and uniformity. Automation has become crucial for the preparation of NGS libraries in high-throughput applications. Here, we automate the NEBNext Direct Genotyping Solution using the Beckman Coulter Biomek i7 Hybrid workstation. The automated method was carried out using 50ng of Tomato DNA per well in a 96-well-plate format, following the NEBNext Direct Genotyping Solution to capture 2286 SolCAP markers. The pooled libraries were then sequenced using an Illumina MiSeq. Expanded post-capture pool index strategy enabled processing of up to 9216 samples to obtain sufficient sequencing output. We observed equal distribution of post-filtered reads between 171.494 and 288.264 with an average of 226.551 post-filtered reads per sample.
The high quality of sequencing data was evident by >93.8% on-target coverage, a mean target coverage between 50x and 86x, and >97.7% uniformity across the samples. The automated workflow prepares sequencing ready libraries in 8 hrs, providing completely walk-away library preparation solution.

Introduction

Utilization of next-generation sequencing for genotyping organisms offers advantages in data cost and sample throughput for a wide range of applications including agricultural breeding for improved crop production, ornamental breeding, animal breeding and human sample identification. The NEBNext Direct Genotyping Solution was developed specifically to address sample preparation needs for targeted genotyping applications by offering pre-capture pooling of up to 96 samples, resulting in advantages in both throughput and cost.

Here, we describe an automated protocol for the NEBNext Direct Genotyping Solution using the Biomek i7 Workstation as a “core system” that can be adapted to various sample numbers with processing of up to 9,216 samples at a time. As such, the workflow can be used for medium-high throughput automation but can also be scaled up to suit ultra-high throughput projects such as plant and animal breeding programs.

(a) NEBNext Direct Genotyping Solution

The NEBNext Direct Genotyping Solution requires 25 – 250 ng of purified genomic DNA as input material dependent on the ploidy level, genome size, and the number of markers under investigation.

In the case described in this application note, 50 ng of tomato DNA is enzymatically fragmented and 5' tagged with an Illumina-compatible P5 adaptor. This adaptor incorporates an inline sample index to tag each sample prior to pooling and an inline Unique Molecular Identifier (UMI) to mark each unique DNA fragment within the samples. Subsequently, 96 samples are pooled prior to hybridization-based enrichment using biotinylated baits and streptavidin bead capture. Pools can be between 4 and 96 samples. Each pool is processed through ligation of a 3' adaptor, enzymatic removal of off-target sequence and PCR.

(b) NEBNext Direct Genotyping Solution - Automated workflow

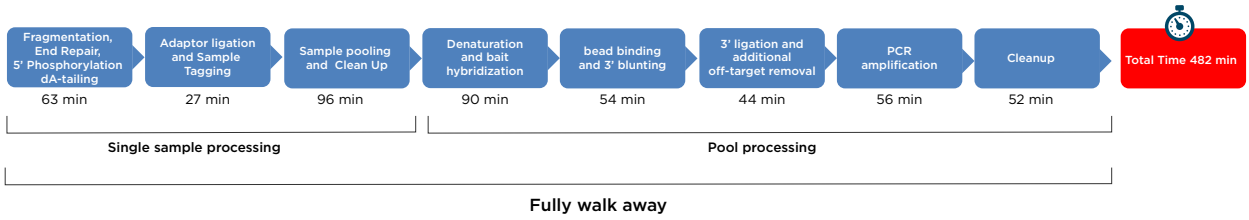

Figure 1. The complete workflow of the NEBNext Direct Genotyping Solution. Indicated below each process is the duration of the individual steps for the processing of 96 samples using 96 pre-capture sample indexes and 1 post-capture pool index.

The key benefits of the NEBNext Direct Genotyping Solution and its automation on the Biomek i7 workstation are:

- Automated 'walk-away' workflow produces sequencing-ready libraries in about 8 hours
- Dual-barcode system enables pooling of 9,216 samples into a single sequencing run
- Simultaneous identification of 100 – 5000 markers and contextual sequence information
- Customized and fully optimized bait design to maximize sequencing efficiency
- High specificity and coverage uniformity

(c) Sequencing strategy

The final NEBNext Direct Genotyping Solution libraries are designed to be sequenced on Illumina sequencing platforms with the following parameters (Figure 2):

- 20 cycles of Read 1 to sequence the 12 base UMI and the 8-base sample indices
- 8 cycles of Index 1 to sequence the pool index
- 75 cycles of Read 2 to sequence the target region. Read 2 can be elongated to generate additional data of flanking regions.

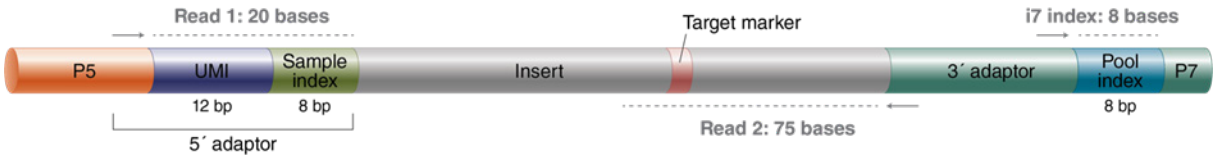

Figure 2. The sequencing read structure used for the NEBNext Direct Genotyping Solution.

(d) Biomek i7 Hybrid (Multichannel 96, Span-8) Workstation

The following system features of the Beckman Coulter Biomek i7 Hybrid workstation deliver reliability and efficiency to increase user confidence and walk-away time compared to manual operation.

- 300 µL or 1200 µL Multichannel head with 1-300 µL and 1-1200 µL pipetting capability
- Interchangeable head for 384 well tips
- Span-8 pod with fixed and disposable tips
- Enhanced Selective Tip pipetting to transfer custom array of samples
- Independent 360˚ rotating gripper with offset fingers
- High deck capacity with 45 positions
- Orbital Shakers, peltiers (heating and cooling), span-8 and 96 channel Tip washing for controlling sample processing
- Spacious open platform design to integrate on-deck and off-deck elements (e.g. thermo cyclers)
- Workflow optimization by in process scheduling for dual pipetting heads and resources
- Secure storage of final samples at 10°C
- Beckman Coulter Data Acquisition and Reporting Tool to collect experiment data in LIMS-friendly manner
- The Biomek Method Launcher (BML) providing a user-friendly interface to load the Biomek deck and run the methods

(e) The fully automated NEBNext Direct Genotyping Solution method

The NEBNext Direct Genotyping Solution method provides users with a single-day and fully automated walk-away workflow that can be applied to a minimum of 100 markers and a maximum of 5000 markers simultaneously. In combination with 96 pre-capture UMI-containing sample indexes and 8 post-capture pool indexes, this enables the user to prepare libraries of 768 samples in about 8 hours that can be pooled to facilitate sequencing of more than 3.8 million genotypes in a single Illumina sequencing run. The data presented in this technical note include the library construction using the Beckman Coulter Biomek i7 Hybrid automated liquid handler with 50 ng tomato DNA input per well in a 96-well-plate format using 96 pre-capture sample indexes and 1 post-capture pool index.

The Method Option Selector of the automated NEBNext Direct Genotyping Solution method enables the user to customize various parameters in each run providing a maximum in flexibility and adaptability. The automated method has a modular design that facilitates the user to select the desired library construction procedures for both pre- and post-capture. As a result, the user can choose to process different numbers of samples where up to 96 samples can be pooled into a single capture reaction and subsequently processed with the post-pooling library construction steps of the method (Figure 3).

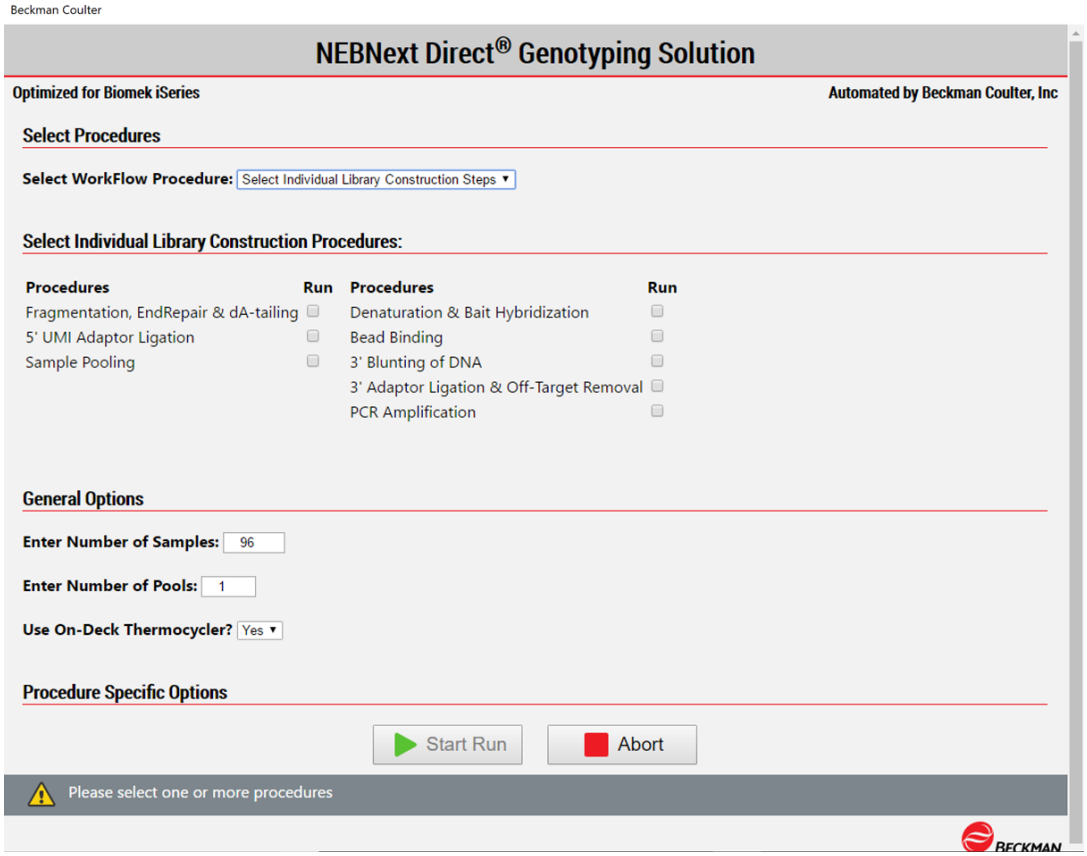

Figure 3. Biomek Method Options Selector: The user interface of the NEBNext Direct Genotyping Solution method provides various options to customize each run

The detailed Guided Labware Setup of the NEBNext Direct Genotyping Solution method guides the user through the whole deck setup procedure. Graphic and text interfaces are generated based on the selected run parameters and, based on the number of samples and pools, the required volumes for each reagent are calculated (Figure 4). To offer greater flexibility and appropriate setup of master mixes before starting the run, the user can also choose to print the detailed step-by-step instructions for the preparation of required reagents at the workbench.

Detailed instructions on the placement of the required labware and reagents on the deck of the Biomek i7 workstation, including an overview of the final deck layout (Figure 4), complete the Guided Labware Setup.

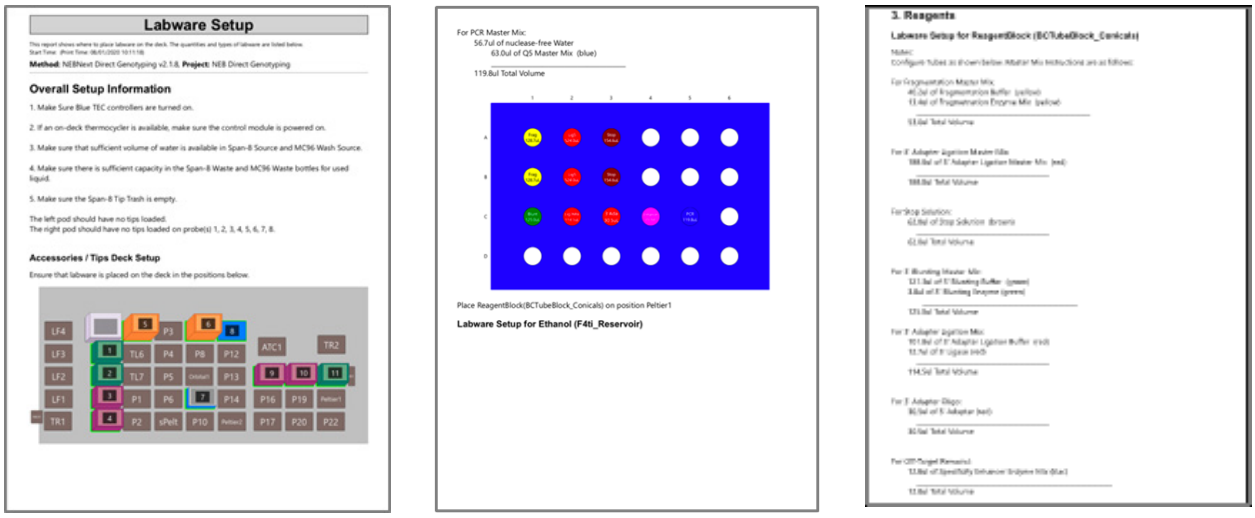

Figure 4. Example of labware setup for the preparation of 96 samples in 1 capture pool with the NEBNext Direct Genotyping Solution method. Detailed step by step instructions indicate the reagent volumes and guide the user through the whole deck setup procedure.

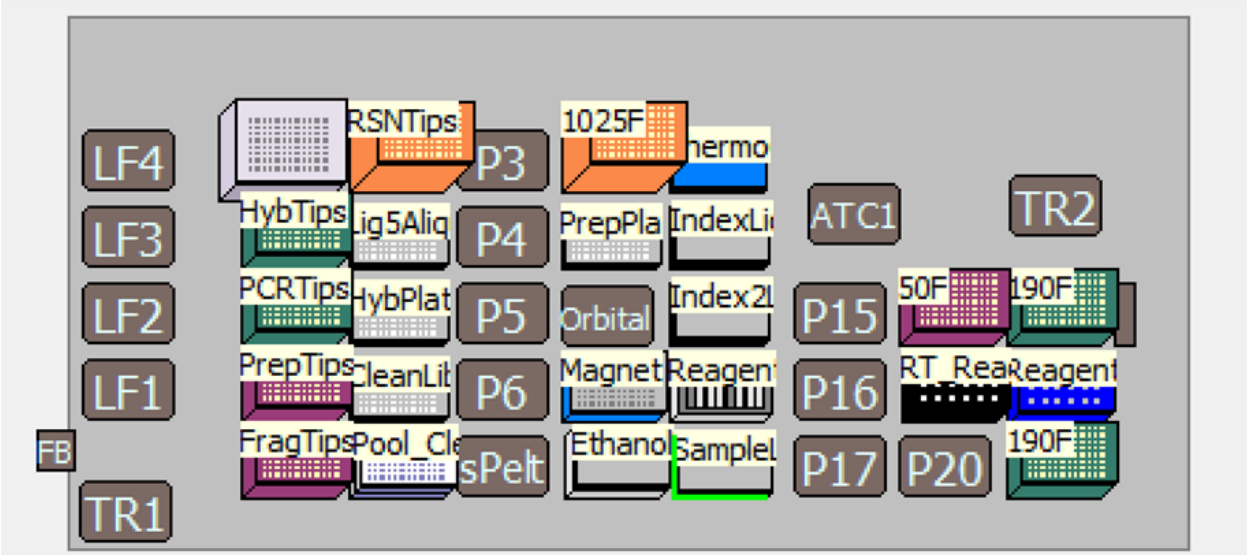

Figure 5. The final deck layout generated by the Guided Labware Setup for the preparation of 96 samples in 1 post-capture pool with the NEBNext Direct Genotyping Solution method

The NEBNext Direct Genotyping Solution method has been optimized to reduce plastic consumption to a minimum. The processing of 96 samples in a singe pool requires only 477 tips, 2 midi plates, 5 PCR plates and two plastic lids, making this not only a cost-effective, but also an environmentally friendly method (Figure 6).

	Plastic	96 samples in one pool	384 samples in four pools
Pre-Pool Consumables	Tip boxes p1000	120 tips	144 tips
	Tip boxes p200	8 tips	32 tips
	Tip boxes p50	304 tips	1,176 tips
	Midi plate AB_1127	1	1
	PCR plates	2	6
	plastic lid	2	5
Post-Pool Consumables	Tip boxes p1000	21 tips	29 tips
	Tip boxes p200	19 tips	72 tips
	Tip boxes p50	5 tips	20 tips
	Midi plate AB_1127	1	1
	PCR plates	3	3
	plastic lid	0	0

Reservoirs for both process steps: SPRI beads, wash buffers, TE, H_2O, EtOH

Figure 6. Plasticware and consumables required to prepare 96 samples in 1 post-capture pool and 384 samples in 4 post-capture pools, respectively.

Methods

The fully automated method was carried out using the NEBNext Direct Genotyping Solution workflow (#E9500, #E9530) with 50 ng Tomato DNA input per well in a 96-well-plate format using 96 pre-capture sample indexes and 1 post-capture pool index. The input DNA was fragmented for 15 minutes, the samples were indexed with a full 96 Indexed 5' adaptor plate and pooled into a single pool. Subsequently, hybridization was performed using Baits derived against a set of 2,286 SolCAP markers from tomato (#E9530B-L1AFA) and the pool was indexed with a pool index and amplified with 17 PCR cycles. A double bead cleanup was performed after PCR amplification and library yields and quality were assessed with an Agilent Bioanalyzer High Sensitivity DNA chip (Figure 7). The concentration of the library was 50 ng/µL (total yield 1.5 µg) and the electropherogram showed an average fragment size of 292 bp peaking at 216 bp.

A 9 nM final library pool was loaded onto an Illumina MiSeq Instrument according the manufacturer's instructions. The consumables and instruments used are listed in the tables 1-3.

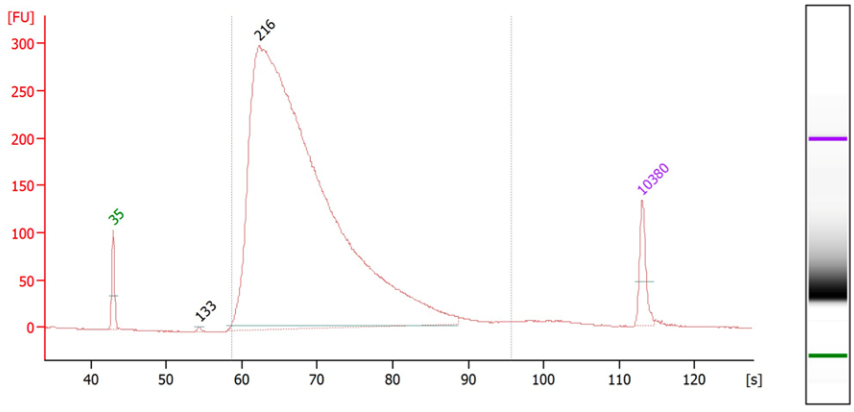

Figure 7. Electropherogram of the final library assessed with an Agilent Bioanalyzer High Sensitivity DNA chip.

Results

The NEBNext Direct Genotyping Solution combines a high degree multiplexing with capture-based enrichment to allow the processing of up to 9216 samples (96 sample indexes x 96 pool indexes) followed by sequencing on an Illumina platform that offers a sufficient output. As a consequence, an equal distribution of reads across all samples in the sequencing data is crucial. Key parameters extracted from the sequencing run with 96 samples in a single pool on an Illumina MiSeq Instrument highlight the equal distribution of post-filtered reads between 171.494 and 288.264 with an average of 226.551 post-filtered reads per sample (Figure 8). An on-target coverage of >93.8%, a mean target coverage between 50x and 86x and uniformity of >97.7% across the samples confirm high data quality for the 96 processed samples (Figures 9-11).

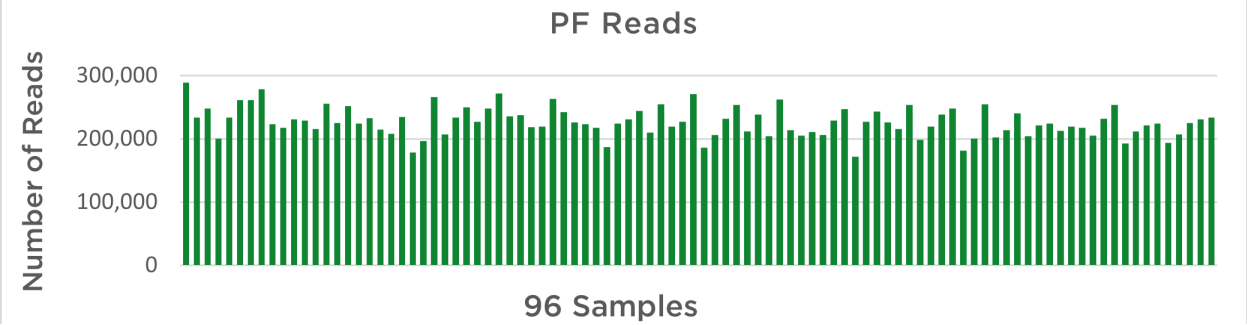

Figure 8. Number of reads passing Illumina's filter for each sample as measured by the Picard Alignment Summary Metrics tool. Sequencing on an Illumina MiSeq yielded post-filtered reads between 171.494 and 288.264 with an average of 226.551 post-filtered reads per sample.

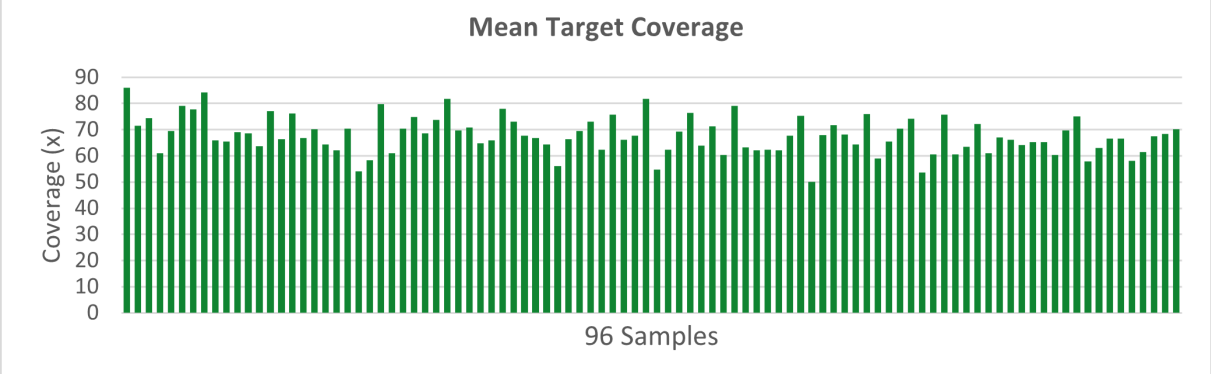

Figure 9. Mean coverage of each target calculated with the Picard tool CollectHsMetrics. Analysis of the sequencing data showed a mean target coverage between 50x and 86x.

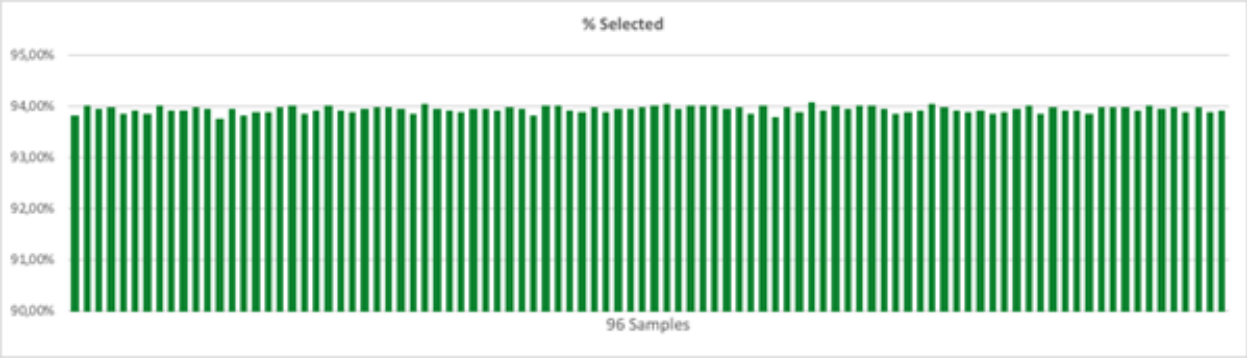

Figure 10. The percentage of bases located on or near a target calculated with the Picard tool CollectHsMetrics. Analysis of the sequencing data show percent selected is>93.8 across all samples..

Figure 11. Sequencing uniformity defined as the percentage of targets with coverage greater than 20% of the mean target coverage. Analysis of the sequencing data highlights the uniformity of >97.7% across the samples.

Summary

The implementation of the New England Biolabs NEBNext Direct Genotyping Solution on the Beckman Coulter Biomek i7 hybrid workstation resulted in a very effective method for generation of ready to-sequence libraries. The system matches a high number of primary input samples and provides a fully automated workflow. Each sample can be scanned with up to 5000 markers and 96 samples are condensed in one single pool very early in the process to minimize costs in chemistry and plastic consumables per sample. The current configuration with 96 well plates allows up to 384 samples per run.

High target coverage, >90% of on-target reads and >95% uniformity makes GS on the i7 a powerful tool for various applications, including agricultural breeding, ornamental breeding, animal breeding and human sample tracking.

This core system demonstrates the performance of the NEBNext Direct Genotyping Solution chemistry in combination with Biomek i7 hardware. The next steps will include developments to allow the native processing of 384 well plates and incorporation of additional i7 hardware components to extend the time of unattended operation and increase throughput. Additional advances towards miniaturization of reaction volumes could further decrease operation costs.

References

1. https://www.neb.com/protocols/2019/07/30/protocol-for-use-with-nebnext-direct-genotyping-solution-neb-e9500-e9530

2. Deschamps, S., Llaca, V., May, G. D. (2012) Genotyping-by-Sequencing in Plants, 1, 460-483; doi:10.3390/biology1030460

Materials

Equipment	Manufacturer
Bioanalyzer	Agilent

Table 1. Instruments used

Reagents	Manufacturer	Part Number
NEBNext Direct Genotyping Solution workflow	NEB	#E9500, #E9530
High Sensitivity D5000 Reagents	Agilent	5067-5593

Table 2. Reagents used

Consumables	Number	Manufacturer	Part Number
High Sensitivity D5000 ScreenTape	1	Agilent	5067-5592
1025µL PIPETTE TIPS, Sterile Filtered	2	Beckman Coulter	B85955
190 µL PIPETTE TIPS, Sterile Filtered	4	Beckman Coulter	B85911
50 µL PIPETTE TIPS, Sterile Filtered	3	Beckman Coulter	B85888
Frame for Reservoir	1	Beckman Coulter	372795
Quarter Reservoir, Sterile Polypropylene, 75ml	2	Beckman Coulter	372786
Quarter Reservoir, Sterile, Polypropylene, Divided by Length, 10ml/Section	2	Beckman Coulter	372788
Blue Heater/Chiller 24well block	1	Beckman Coulter	A83054
Alpaqua Magnum FLX Magnet Plate	1	Beckman Coulter	A000400
Tube Block, 24well	1	Beckman Coulter	373661
11mm Inserts	1	Beckman Coulter	373696
Hard-Shell Thin-Wall 96-Well Skirted PCR Plates, clear wells	7	Biorad	HSP-9641
ABgene Storage Plate, 96-well, 1.2 mL, square well, U-bottomed	2	ThermoFisher	AB1127
Universal Plastic Lid	4	4titude	4ti-0290
Reservoir Plate	1	4titude	4ti-0133
1.5ml ScrewCap Microcentrifuge Tubes, conical bottom	16	Thermo	21-403-196
Arched AutoSealing PCR Lid	1	BioRad	MSL-2022

Table 3. Consumables used per 96 sample run

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