A flexible automation solution for genotyping by sequencing in plant breeding to maximize sample throughput

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ABSTRACT

Marker assisted breeding using targeted genotyping by sequencing (GBS) is gaining traction as an effective tool for advanced breeding. We have developed and validated a 1,536-barcode set for multiplexed sequencing using AgriSeq™ targeted GBS technology. While 1,536 barcodes provide a tremendous potential sample throughput, the logistics of handling four 384-well plates of barcoded samples can be arduous and time consuming to perform manually.

The use of a traditional liquid handler can reduce hands-on-time, however, the number of tips required for processing large numbers of samples can be negatively impactful, both economically and environmentally. To mitigate this impact, we have incorporated a MANTIS® liquid handler from Formulatrix® into the AgriSeq workflow.

The Mantis is a positive air-displacement system that precludes the use of disposable tips and delivers the necessary volumes for repetitive dispersals of enzyme mixes, binding solutions, and washes; saving cases of pipet tips and reducing reagent 'dead' volume while easing operator fatigue and potential for technical errors. Capable of delivering a full 384-well plate's worth of reagents in 5-10 minutes, this non-contact dispenser results in significant time savings as well.

We have verified that performing the library preparation protocol with the MANTIS® provides results that are equivalent to or better than a purely manual workflow or one using a more traditional liquid handler while lessening our environmental impact through reduction of tips used. Sequencing 1,536 unique libraries on a single chip in less than 3 hours on the Ion GeneStudio™ S5 Prime system makes AgriSeq targeted GBS technology more efficient and affordable.

INTRODUCTION

Single nucleotide polymorphisms (SNPs) are genetic markers with a generally low mutation rate. As such, they have emerged as the most widely used genotyping markers in agricultural applications such as trait monitoring, marker-assisted breeding selection, marker-assisted backcrossing, trait introgression and strain purity [1]. With the implementation of targeted sequencing approaches in next generation sequencing technologies, genotyping-by-sequencing (GBS) provides mid-density (hundreds to a few thousand markers) SNP genotyping, providing an attractive alternative to traditionally more costly arrays for monitoring marker sets of these sizes.

The AgriSeq GBS workflow (**Figure 1**) is a high-throughput workflow, designed to amplify and sequence up to 5000 genetic markers in a single multiplexed reaction. It offers an automation friendly, low-cost, reproducible and robust solution to deliver up to 1536 samples per chip. As demand for higher throughput and faster turnaround time increases, more and more labs are turning to smaller automation systems that don't required skilled operators and costly maintenance packages, but that are cost-effective and easy to use.

Here we demonstrate the use of the Mantis liquid handler in the AgriSeq GBS workflow. It utilizes a positive-displacement mechanism with a pipette tip as a reagent reservoir to provide both faster dispensing and a huge reduction in expenses for plastic consumables.

Figure 1. Complete AgriSeq GBS Workflow

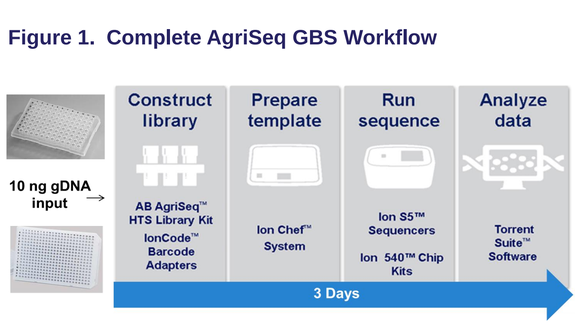

Figure 1. Requiring only 10 ng of genomic DNA, amplicon libraries can be constructed using the AgriSeq HTS Library Kit reagents in either 96-well or 384-well format for faster processing and compatibility with automated liquid handling platforms. Compatible with DNA barcoding, different barcoded adapters are used for each library, which allows them to be pooled for simultaneous sequencing of hundreds of samples on the Ion S5 sequencing platform. Once constructed, AgriSeq libraries are placed on the Ion Chef overnight, for template preparation and chip loading, followed by sequencing on the Ion S5 system the next day. The complete GBS workflow takes as little as three days from DNA to results.

MATERIALS AND METHODS

Incorporation of the Mantis liquid handler (**Figure 2**) into the AgriSeq GBS workflow was validated using a custom designed AgriSeq corn genotyping panel targeting 500 markers.

Figure 2. The Mantis liquid handler from Formulatrix.

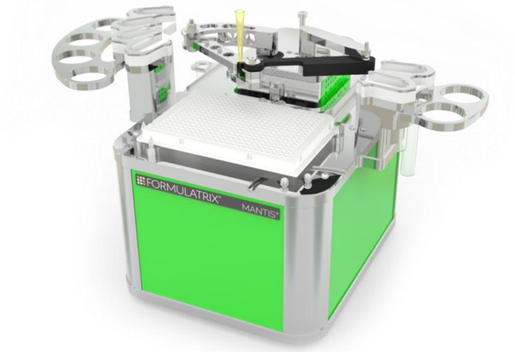

Figure 2. The Mantis liquid handler is a positive air displacement system that uses a microfluidic silicone chip that can fill and dispense reagents as fast as 20 times per second. This system is compatible with both 96 or 384-well plates. Using a single pipette tip as a reagent reservoir, the Mantis only requires 6 µL of dead volume making this an ideal consumable-free system for expensive reagents.

DNA isolated from 384 corn field samples was used to generate both manual and automated libraries using the Mantis liquid handler following the 384-well AgriSeq HTS library preparation protocol (**Figure 3**), which can be downloaded from the Thermo Fisher Scientific website.

Figure 3. AgriSeq Library Prep workflow

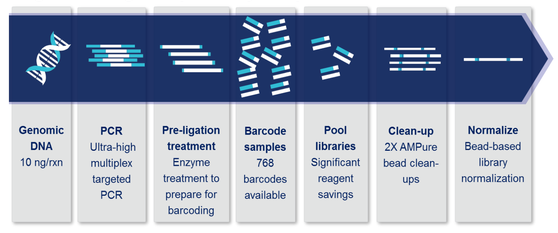

Figure 3. Using the AgriSeq HTS Library Kit, 10ng/rxn of corn DNA was amplified using a custom AgriSeq corn panel. Each sample was then treated with a Pre-ligation Enzyme to remove residual primer dimers allowing for more efficient sequencing. Samples were ligated with unique barcoded adapters allowing them to be pooled for subsequent clean-up and sequencing while retaining traceability to the original sample during analysis for significant cost savings. Libraries were cleaned-up by a two-round AMPure purification. A final bead-based normalization step helps ensure each library is at a consistent final concentration suitable for direct input into template prep on the Ion Chef™ instrument.

The Mantis liquid handler was used to replace three steps upfront in the AgriSeq library prep workflow, including the addition of the amplification master mix, pre-ligation enzyme mix and the barcoding mix. These steps all involve dispensing a single reagent to an entire 384-well plate.

In addition, 384 corn DNA samples were tested in quadruplicate using the Mantis workflow to evaluate sample concordance between different IonCode™ Barcode Adapter kits. A total of 1536 IonCode barcode adapters were used, of which 1152 barcodes are commercially available. A small benchtop PIPETMAX® 268 liquid handler from Gilson® that uses PIPETMAN® technology was used for the addition of barcode adapters to each amplicon library.

Each 384-well barcoded library plate was pooled separately. In addition, all four 384-well libraries were pooled together to generate a single library containing all 1536 barcoded samples. Manual and mantis workflow libraries were sequenced on separate chips. All libraries were sequenced on the Ion S5 XL sequencing system using Ion 540 Chips. Utilizing this system, up to 1536 samples can be barcoded and run on a single sequencing run allowing for up to 3072 samples to be tested per day (**Table 1**).

Table 1. Sample Throughput Capability

No. of Markers	No. of samples per chip	No. of samples per day
5000	140	192
3645	192	384
1822	384	768
911	768	1536
607	1152	2304
455	1536	3072

Table 1. Sample scalability depends on the density of the chip used and the number of markers in the panel tested. This table shows the maximum recommended number of samples that can be analyzed at different marker densities per Ion 540 chip or per day, assuming an average of 70 million reads/chip to achieve 100X average base coverage.

Data were analyzed using the Torrent Variant Caller plugin available as part of the Torrent Suite software package, to determine the genotype calls for each marker and sample tested. Sample call rate and genotype concordance was determined between workflow methods and between samples barcoded using barcodes from different IonCode Barcode Adapter Kits.

RESULTS

Table 2. The AgriSeq library steps replaced using the Mantis liquid handler

Library Step	Method	Time (m:s)	Volume (µl)	No. Tips Used
Amplification Mix	Manual	7:00	3226	384
	Mantis	4:21	2710	1
Pre-Ligation Enzyme	Manual	7:00	921	384
	Mantis	2:14	783	1
Barcoding Mix	Manual	12:00	1382	384
	Mantis	9:33	1174	1

Table 2. Two plates of 384 corn samples were used to generate amplicon libraries; one plate was prepared by hand and the other was prepared using the Mantis liquid handler. Delivery of three different reagents, including the addition of the amplification mix, pre-ligation enzyme mix and the barcoding mix were compared. The Mantis can deliver a full 384-well plate's worth of reagents faster than doing it by hand, while easing operator fatigue and potential for technical errors. It requires less reagent volume for repetitive dispersals of enzyme mixes, saving previous reagents. As a non-contact dispenser that does not require the use of disposable tips, the Mantis saves cases of pipet tips, reducing environmental waste.

Figure 4. Equivalent call rate between manual and Mantis workflows

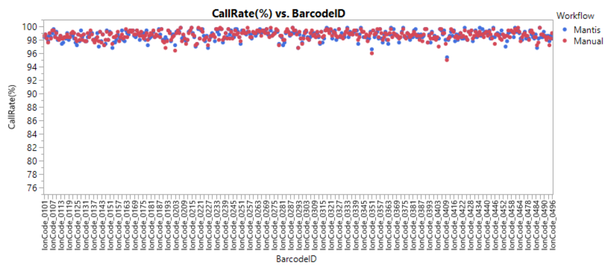

Figure 4. 384 amplicon libraries were generated with both the manual and Mantis workflows using DNA from corn field samples. The call rate, the number of markers generating a genotyping call for each sample, was calculated for each workflow. The mean call rate for both workflows was 98.6%. This demonstrates the Mantis workflow produces results equivalent to libraries prepared manually.

Figure 5. High genotype concordance between manual and Mantis workflows

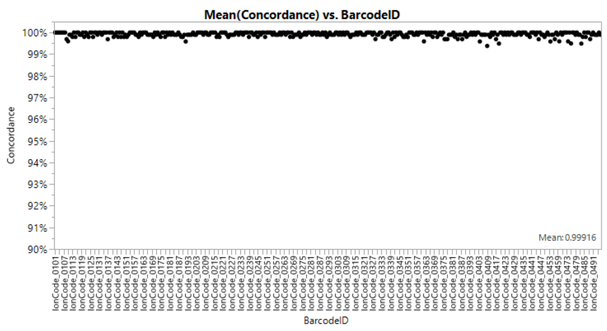

Figure 5. Genotype concordance is calculated as the percentage of markers that give identical genotypes for replicate samples. The graph above shows the concordance between the manual and Mantis workflows for 384 corn samples. Mean genotype concordance was 99.9% demonstrating high performance obtained using the Mantis liquid handler with the AgriSeq GBS workflow.

Figure 6. Equivalent call rate between barcode kits

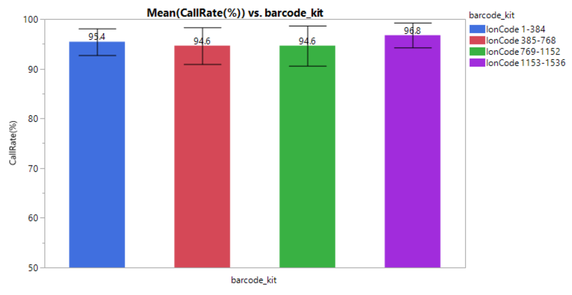

Figure 6. Barcode kit performance was assessed using 384 corn samples tested in replicates of n=4 with the AgriSeq 384-well workflow and a corn panel targeting 500 markers. Each sample was uniquely barcoded using IonCode barcode adapters from kits 1-384, 385-768, 768-1152 and 1153-1536. All 1536 barcoded libraries were pooled and sequenced on a single Ion 540 chip. The average mean read depth for all 1536 samples was 89.8%. The four different barcode adapter kits resulted in mean call rates of 95.4% ±2.7, 94.6% ±3.7, 94.6% ±4.0 and 96.8% ±2.5. A oneway analysis of call rate by barcode kit showed that barcode kit 1152-1536 was statistically higher than the other three kits, however the difference was not practically significant (2%). The mean call rate for all 1536 samples was 95.4%.

Figure 7. High sample genotype concordance between barcode kits

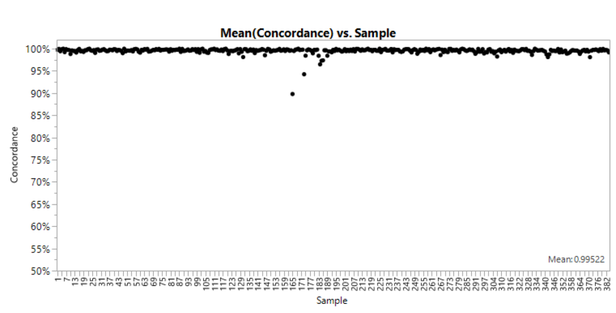

Figure 7. Barcode kit performance was further assessed by calculating genotype concordance between uniquely barcoded technical replicates of 384 corn samples processed through the AgriSeq workflow. Mean genotype concordance was 99.5%, demonstrating highly robust and repeatable results obtained through the AgriSeq workflow using the Mantis liquid handler and different IonCode Barcode Adapter kits.

CONCLUSIONS

High throughput library preparation can be a tedious process that requires skilled pipetting and tremendous focus to perform manually. The use of a traditional liquid handler can be a cost-effective means to increase throughput while increasing consistency and reducing technical errors [2]. The Mantis liquid handler from Formulatrix, with its small footprint and lower price point (compared to traditional liquid handlers) is ideal for use with the AgriSeq GBS workflow.

Using a single pipette tip as a reagent reservoir, this noncontact dispenser delivers repetitive dispersals of master mixes and other reagents of various viscosity, saving cases of pipet tips and reducing reagent 'dead' volume while easing operator fatigue and potential for technical errors. Capable of delivering a full 384-well plate's worth of reagents in less than 10 minutes, the Mantis results in significant time savings as throughput increases.

We have verified that performing the AgriSeq 384-well library protocol with the MANTIS provides results that are equivalent to a manual workflow while lessening our environmental impact through the reduction of consumables used. With four IonCode Barcode Adapter Kits available that offer equivalent performance, sequencing 1,536 unique libraries on a single chip makes AgriSeq targeted GBS technology more efficient and affordable.

REFERENCES

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TRADEMARKS/LICENSING

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