Multiplex SNP Genotyping of Field Corn Crude Samples with Probe-Based Assays using the IntelliQube® from Douglas Scientific®

ABSTRACT

Single Nucleotide Polymorphism (SNP) genotyping must be accurate, reliable, cost-effective, and time efficient for agricultural marker-assisted selection and genomic selection programs to fully benefit from SNP analysis during the breeding process. To address these needs, Douglas Scientific has developed the IntelliQube, a fully integrated liquid handling and real-time quantitative PCR instrument, optimized for use with miniaturized reactions in 384- and 768-well Array Tape®. The physical characteristics of Array Tape paired with the IntelliQube provide the ability to dramatically decrease thermal cycling times. Additionally, the option to multiplex reactions using four different fluorescence channels allows for profound reagent and time savings. In this study, field corn seeds were genotyped for two high-quality, polymorphic SNPs using crude DNA extracts, Genotyping ToughMix® from Quanta BioSciences, and custom BHQplus® probe-based SNP genotyping assays.

INTRODUCTION

Plant and animal breeding programs rely heavily on marker-assisted selection (MAS) and genomic selection (GS) techniques. Single Nucleotide Polymorphisms, or SNPs, are genetic markers found in plant and animal genomes. They are stable from generation to generation and prevalent throughout the genome, making them ideal candidates for use in MAS and GS programs. The development of PCR-based SNP genotyping protocols has enabled researchers to analyze SNPs quickly and accurately. Additionally, improvements in laboratory automation have increased the capacity of MAS and GS laboratories, enabling high throughput sample screening, and thus a more rapid development of new agricultural products.

Field corn *(Zea mays)* is an economically important crop grown around the world. According to the USDA National Agricultural Statistics Service, 13.7 billion bushels of corn were produced in the United States alone in 2015, and 90 percent of the corn acres planted were biotech varieties (Honig). Corn is an important source of food globally and is used for many industrial applications. These uses, combined with the prevalence of biotech varieties, make corn one of the most widely tested crops in MAS and GS programs worldwide.

The IntelliQube from Douglas Scientific is a fully integrated laboratory instrument that combines liquid handling with real-time quantitative PCR (qPCR) analysis in miniaturized reaction volumes. The IntelliQube utilizes Array Tape in a unique and innovative 384- or 768- well format in place of standard 384-well microplates. Array Tape is a thin and flexible consumable that, in combination with miniaturized reaction volumes (1.6 µL), allows a dramatic decrease in thermal cycling times. The ability of the IntelliQube to also run multiplex reactions makes for profound reagent and time savings.

Described here is an experiment in which individual field corn seeds were genotyped using custom BHQplus SNP genotyping assays in miniaturized reactions under standard and fast thermal cycling conditions and in a multiplex format on the IntelliQube. The multiplex reactions consisted of two different SNP assays in a single well utilizing four fluorescence channels for the allele specific probes, and a fifth channel for a passive reference dye. Liquid handling, thermal cycling, and real time fluorescence detection were all performed inline on the IntelliQube. The two SNPs analyzed in this study were selected from a subset of 120 high-quality SNP markers identified through a

stringent statistical method described by Mammadov, et al., 2010. The SNPs were chosen for this study, not because of an association with specific traits, but because of their quality and polymorphic nature.

MATERIALS AND METHODS

Corn Samples: Post-harvest field corn seeds were donated by 11 different sources in central Minnesota, including local farmers and grain elevators. Samples were collected at random with a focus on geographic (not genetic) diversity. All farmers and elevators were asked not to provide any breeder, variety, or genetic information about their samples. Two individual seeds from each source were analyzed, totaling 22 samples.

DNA Extraction: A sodium hydroxide method was used to prepare crude corn DNA samples. Briefly, individual corn seeds were pulverized using a mini bead beater. A 0.25 M solution of sodium hydroxide was added to lyse the cells at 50 ˚C for 10 minutes. The samples were cooled to ambient temperature and neutralized with Tris-HCl buffer, pH 7.8. After centrifugation, the supernatant was collected and diluted 1:25 in water before use.

Assays and Reagents: Genomic information about the SNPs analyzed in this study can be found in Table 1. AccuStart™ Genotyping ToughMix (Quanta BioSciences) was used with an additional SNP enhancer provided by the manufacturer to genotype all samples with two SNP assays. An addition of 400 µM TAMRA™ reference dye was added to the 2X master mix for normalization. BHQplus probe-based SNP genotyping assays were designed using RealTimeDesign™ Software from LGC Biosearch Technologies™. Probe-based SNP genotyping assays are made up of two primers and two allele-specific probes with different fluorogenic dye labels, as described in Figure 1. The assay targeting SNP DZm2571611 utilized Quasar® 670 and CAL Fluor Red® 610 probe labels. The assay targeting SNP DZm2521843 used FAM and CAL Fluor Orange 560 dyes for the probes. The fluorescence probes and the associated detection channels on the IntelliQube are outlined in Table 2. The multiplex SNP assay consisted of the two individual SNP genotyping assays within one reaction well. BHQplus probes and primers were added at 2X concentration per assay to the 2X master mixes (400 nM and 1.8 µM, respectively) to achieve a final concentration in the PCR reaction of 200 nM of each probe and 900 nM primers, and 1X master mix.

SNP Name	Genbank Accession Number	Chromosome
DZm2571611	AC219032.4	4
DZm2521843	EU972344.1	10

Table 1: SNP information

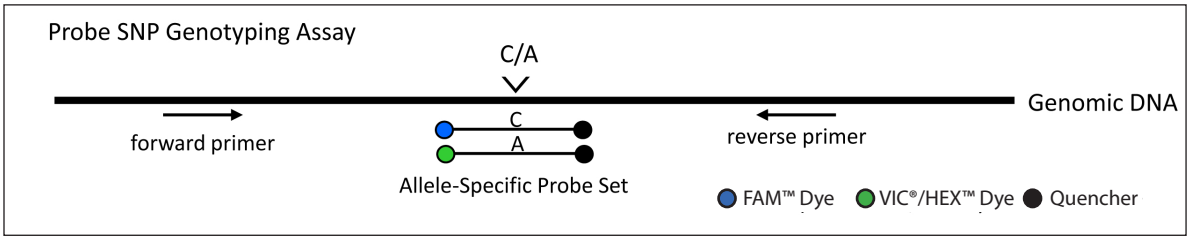


Figure 1: Assay design for probe SNP genotyping reactions. Probe-based SNP genotyping assays utilize forward and reverse primers to amplify a segment of genomic DNA surrounding a SNP. Two fluorogenic probes differentially bind to their allele-specific complement. Fluorescence signal is produced during each PCR cycle by separation of the dye and quencher, as probes are hydrolyzed through Taq exonuclease activity.

Fluorescent Probe	Channel	Excitation Filter (Center Wave Length/Band-nm)	Emission Filter (Center Wave Length/Band-nm)
FAM	FAM	480/20	510/20
CAL Fluor Orange 560	HEX	530/30	565/20
CAL Fluor Red 610	ROX	580/25	625/30
Quasar 670	Quasar 670	620/60	705/72

Table 2: Fluorescence probes and the associated detection channels on the IntelliQube.

Instrumentation: The IntelliQube exhibited in Figure 2 was used for reaction set-up, thermal cycling, and real-time fluorescence detection. DNA samples (800 nL) were dispensed into 384-well Array Tape with the Pipette Head from CyBi® product line. Master mix containing 2X BHQplus probe-based assays (800 nL) was dispensed with the non-contact dispense jet to create 1.6 µL total volume reactions. Thermal cycling conditions are highlighted in Table 3. Fluorescence values were obtained at the end of each cycle to generate real-time amplification curves. Real-time amplification curves and end-point cluster plots were generated by the IntelliScore® Software Suite.

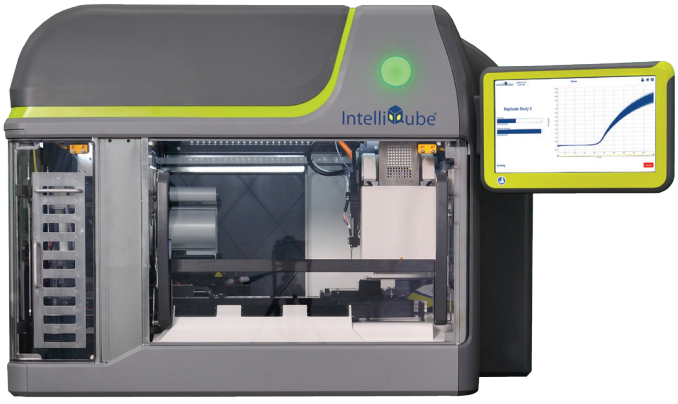


Figure 2: The IntelliQube is a fully integrated liquid handling and real-time quantitative PCR instrument optimized for use with miniaturized reactions in 384- or 768-well Array Tape.

A	Step	Time	Temp
	Enzyme Activation	2 min.	95 ˚C
40 Cycles	Denaturation	15 sec.	95 ˚C
	Annealing/Extension	60 sec.	60 ˚C

B	Step	Time	Temp
	Enzyme Activation	2 min.	95 ˚C
40 Cycles	Denaturation	1 sec.	95 ˚C
	Annealing/Extension	15 sec.	60 ˚C

Table 3: Thermal cycling conditions. Standard thermal cycling times (A) and fast thermal cycling times (B).

RESULTS

Field corn seeds were successfully genotyped for two high-quality, polymorphic SNPs using crude DNA extracts and custom BHQplus probe-based SNP genotyping assays. Concordance was observed between the results generated using the three conditions (standard thermal cycling, fast thermal cycling, and fast thermal cycling with multiplex SNP analysis). A comparison of the real-time curves and cluster plots generated by all three methods are shown in Figures 3, 4, and 5. The cluster plots generated with Array Tape contain all 22 seeds with four replicates of each seed, for a total of 192 data points—176 samples and 16 no template controls. A summary of the consensus calls are provided in Table 3, showing 100% concordance. Calculation estimates showed data points achievable in one eight-hour day were approximately 4,608 for standard run format, and 23,040 for Fast Multiplex format.

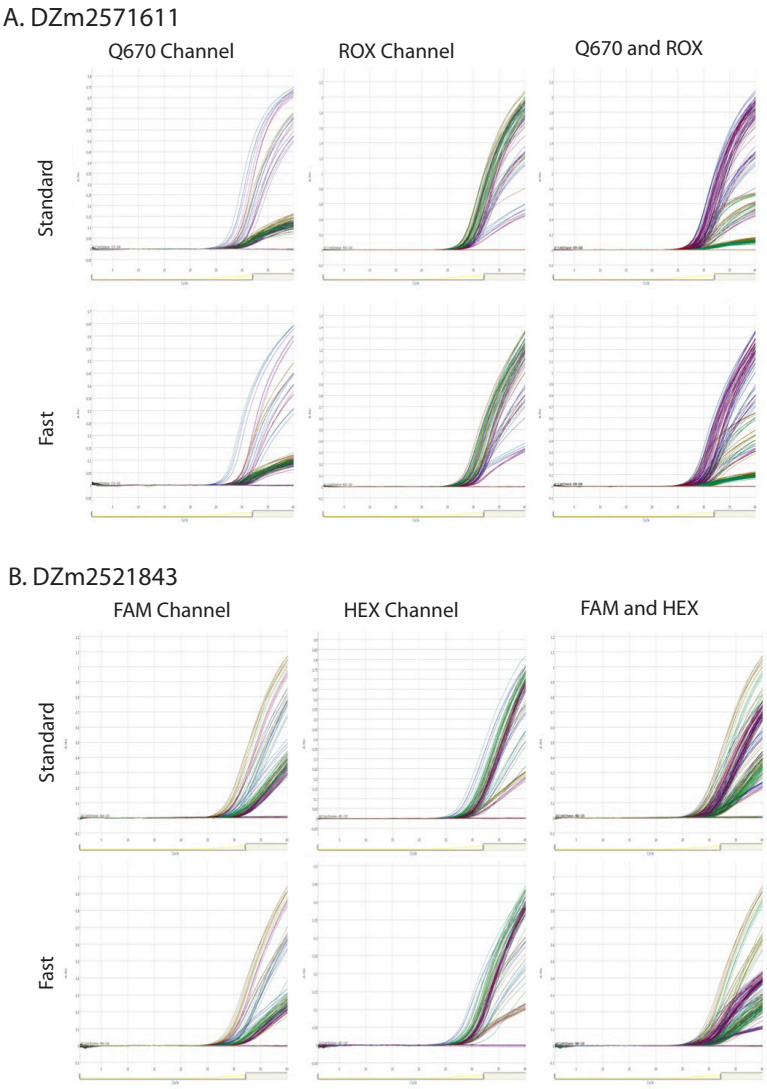
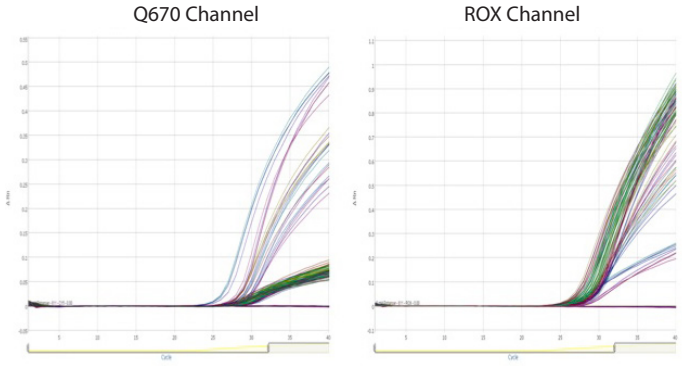
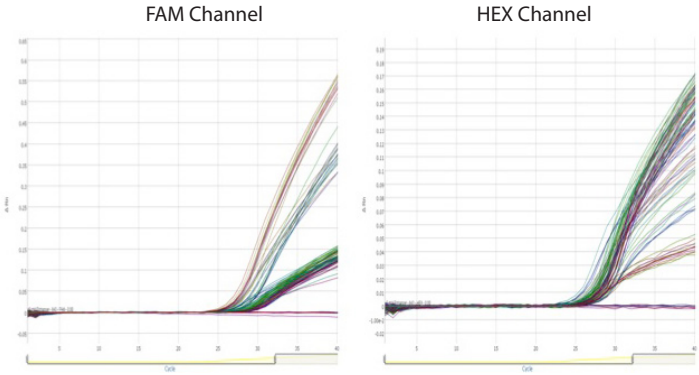


Figure 3: Real-time amplification curves. The real-time PCR curves for DZm2571611 (A) and DZm2521843 (B) are shown for both standard and fast protocols. The amplification curves are shown for the Quasar 670 and ROX channels for DZm2571611. The FAM and HEX channels are shown for DZm2521843. In both sets the curves are shown for each channel individually and combined.

A. DZm2571611



B. DZm2521843



C. DZm2571611 and DZm2521843

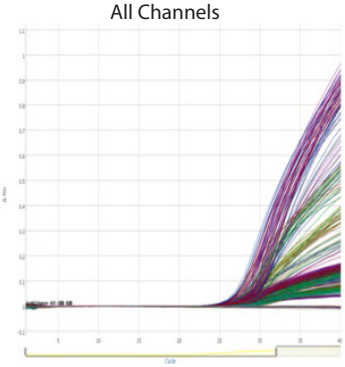


Figure 4: Real-time amplification curves. The real-time multiplex PCR amplification curves for DZm2571611 (A), DZm2521843 (B), and overlaid assays (C). The curves are displayed either as a single channel for their respective probe or as a combination of four channels required for multiplexing. The fast thermal cycling conditions were used for this run.

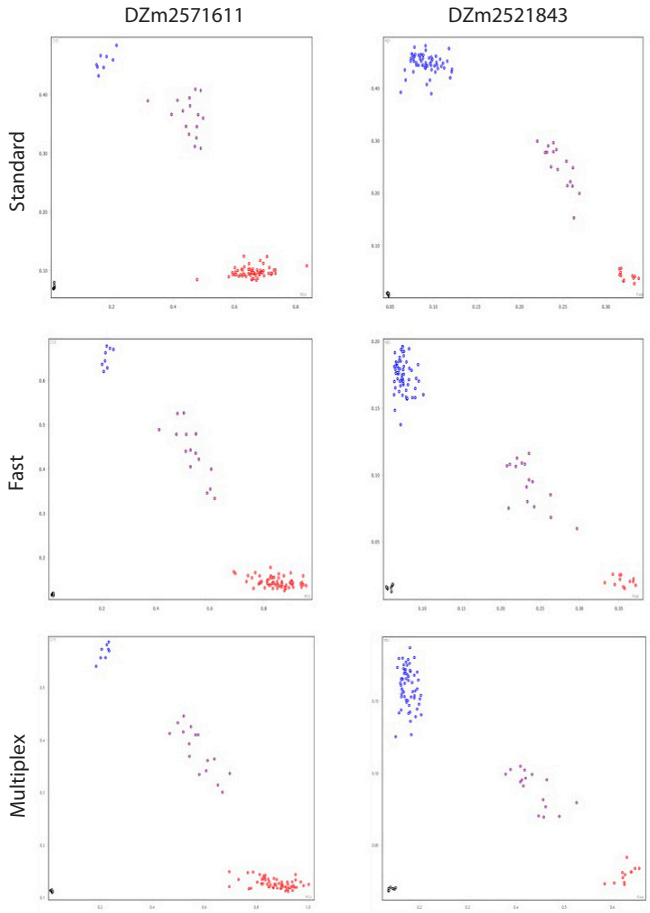


Figure 5: Cluster plot analysis. The SNP genotyping cluster plots generated inline on the IntelliQube using crude corn DNA extracts are shown. For DZm2571611, each sample is plotted with CAL Fluor Red 610 signal along the X-axis and Quasar 670 signal along the Y-axis. For DZm2521843, each sample is plotted with FAM signal along the X-axis and CAL Fluor Orange 560 signal from the HEX channel along the Y-axis. The fast thermal cycling conditions were used for the multiplex run.

CONCLUSIONS

The IntelliQube provides researchers the ability to increase throughput through the use of multiplexed SNP assays and decreased thermal cycling times. This study demonstrates the feasibility of achieving two SNP genotyping results in a single reaction well through the use of BHQplus probes and crude corn samples, while maintaining the miniaturized reaction volumes of Array Tape. The IntelliQube integrates liquid handling, thermal cycling, and detection systems, enabling users to achieve efficient and economical high throughput sample processing in Array Tape for real-time PCR applications. This platform provides significant cost savings in the form of reduced PCR reaction volumes, shorter thermal cycling times, and multiplex reactions, without compromising data quality.

	DZm2571611			DZm2521843		
Corn Seed	Standard	Fast	Multiplex	Standard	Fast	Multiplex
S1	G/G	G/G	G/G	T/T	T/T	T/T
S2	G/G	G/G	G/G	T/T	T/T	T/T
N1	T/T	T/T	T/T	T/T	T/T	T/T
N2	T/T	T/T	T/T	T/T	T/T	T/T
F1	G/G	G/G	G/G	T/T	T/T	T/T
F2	G/G	G/G	G/G	T/T	T/T	T/T
H1	G/T	G/T	G/T	T/T	T/T	T/T
H2	G/G	G/G	G/G	T/T	T/T	T/T
G-A1	G/T	G/T	G/T	T/T	T/T	T/T
G-A2	G/G	G/G	G/G	T/T	T/T	T/T
G-B1	G/G	G/G	G/G	T/T	T/T	T/T
G-B2	G/G	G/G	G/G	T/T	T/T	T/T
G-C1	G/G	G/G	G/G	C/C	C/C	C/C
G-C2	G/G	G/G	G/G	C/T	C/T	C/T
P1	G/T	G/T	G/T	C/T	C/T	C/T
P2	G/T	G/T	G/T	T/T	T/T	T/T
SC-A1	G/G	G/G	G/G	C/T	C/T	C/T
SC-A2	G/G	G/G	G/G	T/T	T/T	T/T
SC-B1	G/G	G/G	G/G	C/T	C/T	C/T
SC-B2	G/G	G/G	G/G	C/C	C/C	C/C
V1	G/G	G/G	G/G	C/C	C/C	C/C
V2	G/G	G/G	G/G	T/T	T/T	T/T

Table 3: Summary of the SNP genotyping calls

Run Format	Approx. Run Time/Array (min)	No. of Assays/Well	No. of Array/Day	No. of Data Points /Array	Data Points/8 Hour Day
Standard	80	1	6	768	4,608
Fast	30	1	15	768	11,520
Fast Multiplex	30	2	15	1,536	23,040

Table 4: Determination of the number of data points achievable in an eight hour day.

REFERENCES

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Lance Honig, USDA Newsroom (http://www.nass.usda.gov/Newsroom/Executive_Briefings/2015/11_10_2015.pdf posted November 10 2015; visited November 17, 2015)

Mammadov, J. A., Chen, W., Ren, R., Pai, R., Marchione, W., Yalcin, F., Witsenboer, H., Greene, T. W., Thompson, S. A., Kumpatla, S. P. Development of highly polymorphic SNP markers from the complexity reduced portion of maize [Zea mays L.] genome for use in marker-assisted breeding. Theor Appl Genet (2010) 121:577-588

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