Multiplex Genotyping of Human Reference DNA Samples with Pharmacologically Important Single Nucleotide Polymorphisms using the IntelliQube® from Douglas Scientific®

ABSTRACT

Genetic analysis of Single Nucleotide Polymorphisms (SNPs) must be accurate, reliable, and economical to be a viable part of human genetic research, drug discovery, and diagnostics. 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®. In addition to decreased reaction volumes, the IntelliQube offers users the ability to multiplex SNP assays and shorten thermal cycling times leading to profound reagent and time savings. The study described here demonstrates the efficacy, flexibility, and economic advantages of the IntelliQube for genetic testing. Custom BHQplus® probe-based SNP genotyping assays were used to analyze human reference DNA samples for two pharmacologically significant SNPs. Standard and fast thermal cycling conditions were compared in parallel to test the ability to examine two SNP targets in a single well. The results produced using the IntelliQube matched those previously published for all SNPs and samples included in the study.

INTRODUCTION

Single Nucleotide Polymorphisms (SNPs) are powerful tools for genetic analysis. They are used in many fields, ranging from plant breeding to human diagnostics. Certain SNPs in human genomic DNA are associated with significant variation in drug metabolism or response to drug treatment between individuals. Therefore, development of accurate and economical methods for SNP genotype analysis is of utmost importance to customizing healthcare delivery. While there are several methods and instruments for this purpose on the market, there remains an unmet need for a fully automated method of SNP analysis that gives laboratories the flexibility and scalability necessary to economically expand access and availability of genetic data.

The IntelliQube from Douglas Scientific is designed to address this need by producing accurate and reliable results while providing cost savings through automation and reduced reagent expenditures. The IntelliQube is a fully integrated laboratory instrument that combines liquid handling with real-time quantitative PCR (qPCR) analysis in miniaturized reaction volumes. The system 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 polypropylene consumable that, in combination with miniature reaction volumes (1.6 µL), enables both outstanding PCR performance and profound reagent savings. The IntelliQube also offers the capability to run multiplex reactions, which allows for even more reagent and time savings.

Research suggests that metabolism and dosage requirements of the commonly prescribed anticoagulant drug warfarin can vary up to 20-fold in Caucasian populations. Takeuchi, et al. demonstrated the link between warfarin dosage and SNP alleles within the VKORC1 and CYP2C9 genes. These SNPs have been demonstrated to significantly impact patient health and outcomes when diseases are subsequently treated with warfarin. As a result, these markers have made their way into assays targeting these SNPs as well as a variety of research uses.

In this study, we analyzed two pharmacologically important SNPs (CYP2C9*2 and VKORC1) using custom BHQplus SNP assays from LGC Biosearch Technologies™, Genotyping ToughMix® from Quanta BioSciences, and the IntelliQube from Douglas Scientific. Reaction setup, thermal cycling, and real-time fluorescence detection were all performed inline on the IntelliQube. This study included analysis of 38 highly characterized human genomic DNA reference samples

purchased from the Coriell Institute for Medical Research. In addition to comparing the consensus genotypes documented by Coriell, we were also able to directly compare the genotyping results generated in Array Tape to those previously published by Pratt, et al.

MATERIALS AND METHODS

Samples and Supplies: Purified genomic DNA samples were obtained from the NIGMS Human Genetic Cell Repository at the Coriell Institute for Medical Research. DNA from 38 individual cell lines (Set 1 described by Pratt, et al.) were used in this study. DNA samples were diluted to 5 ng/µL in molecular grade water before use. Genomic information about the SNPs analyzed in this study can be found in Table 1. AccuStart™ Genotyping ToughMix (Quanta BioSciences) was used to genotype all samples. The master mix was provided at 2X concentration and used according to the manufacturer's instructions. An addition of 400 µM TAMRA™ reference dye was combined with 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 SNP assays CYP2C9*2 and VKORC1 were all designed with FAM and CAL Fluor® Orange 560 labeled probes. Alternatively, the VKORC1 assay was also designed using probes labeled with CAL Fluor Red 610 and Quasar® 670 in order to perform two SNP assays in a single well utilizing all five detection channels on the IntelliQube. The fluorescence probes and the associated detection channels on the instrument are outlined in Table 2. The assays were added at 2X concentration to the 2X master mixes (400 nM and 1.8 µM, respectively) to achieve a final concentration in the PCR reaction of 200 nM probes (per assay), 900 nM primers (per assay), 200 nM TAMRA reference dye, and 1X master mix.

Instrumentation: The IntelliQube (shown in Figure 2) was used for all sample and master mix dispensing, thermal cycling, and real-time fluorescence detection for SNP genotyping in Array Tape. DNA samples (800 nL) were dispensed into 768 reaction wells per array with the multi-channel, Pipette Head from CyBi® Product Line. Master mix containing 2X BHQplus probe-based assay (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. Real-time amplification curves and end-point cluster plots were generated with the IntelliScore® Software.

SNP Name	dbSNP rs#	Chromosomal Location	Pharmacological Significance
CYP2C9*2	rs1799853	Chr10 (q23.33)	Genetic variability impacts metabolism and dosage of warfarin
VKORC1	rs9923231	Chr16 (p11.2)	Genetic variability impacts metabolism and dosage of warfarin

Table 1: SNP information

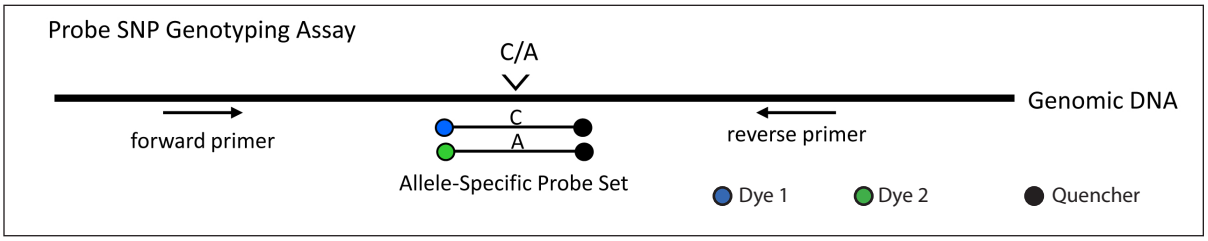


Figure 1: Assay design for probe SNP genotyping reactions. Probe-based SNP genotyping assays utilize forward and reverse primers to amplify a segment of genomic DNA surrounding a SNP. Two fluorogenic probes differentially bind to their allele-specific complement. Fluorescence signal is produced during 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	1	480/20	510/20
CAL Fluor Orange 560 (CFO 560)	2	530/30	565/20
CAL Fluor Red 610 (CFR 610)	3	580/25	625/30
Quasar 670 (Q670)	4	620/60	705/72

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

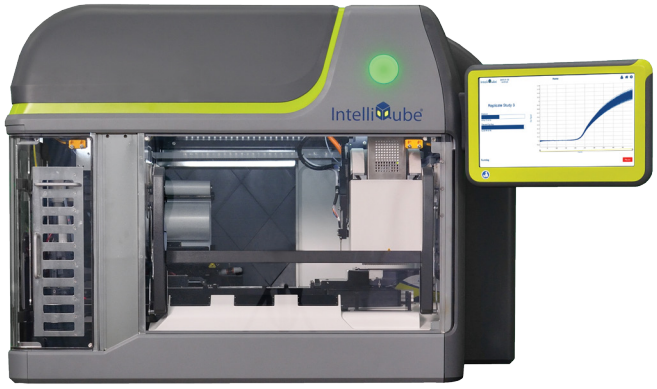


Figure 2: The IntelliQube is a fully integrated liquid handling and real-time quantitative PCR instrument optimized for use with miniaturized reactions in 384- or 768-well Array Tape.

RESULTS

Genomic DNA from 38 individual cell lines was successfully genotyped with the four BHQplus SNP genotyping assays using miniaturized (1.6 µL) reactions in the IntelliQube. Concordance was observed between the results generated using all of the experimental conditions tested. The real-time curves for CYP2C9*2 and VKORC1 are shown in Figures 3 and 4. The cluster plots generated for CYP2C9*2 and VKORC1 are shown in Figure 5. Observed and expected SNP allele calls for each cell line and SNP assay are given in Table 4, showing 100% concordance. Expected calls listed in Table 4 are from previously published results by Pratt, et al. The cluster plots of each assay from the IntelliQube contain all 38 cell lines in duplicate, for a total of 80 data points—76 samples and four no template controls. Calculation estimates in Table 5 show data points achievable in one eight-hour day were 4,608 for standard PCR run format, 10,752 for fast PCR run format and 21,504 for fast multiplex PCR format.

A	Step	Time	Temp
	Enzyme Activation	3 min.	95 ˚C
45 Cycles	Denaturation	15 sec.	95 ˚C
	Annealing/Extension	60 sec.	60 ˚C

B	Step	Time	Temp
	Enzyme Activation	3 min.	95 ˚C
45 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).

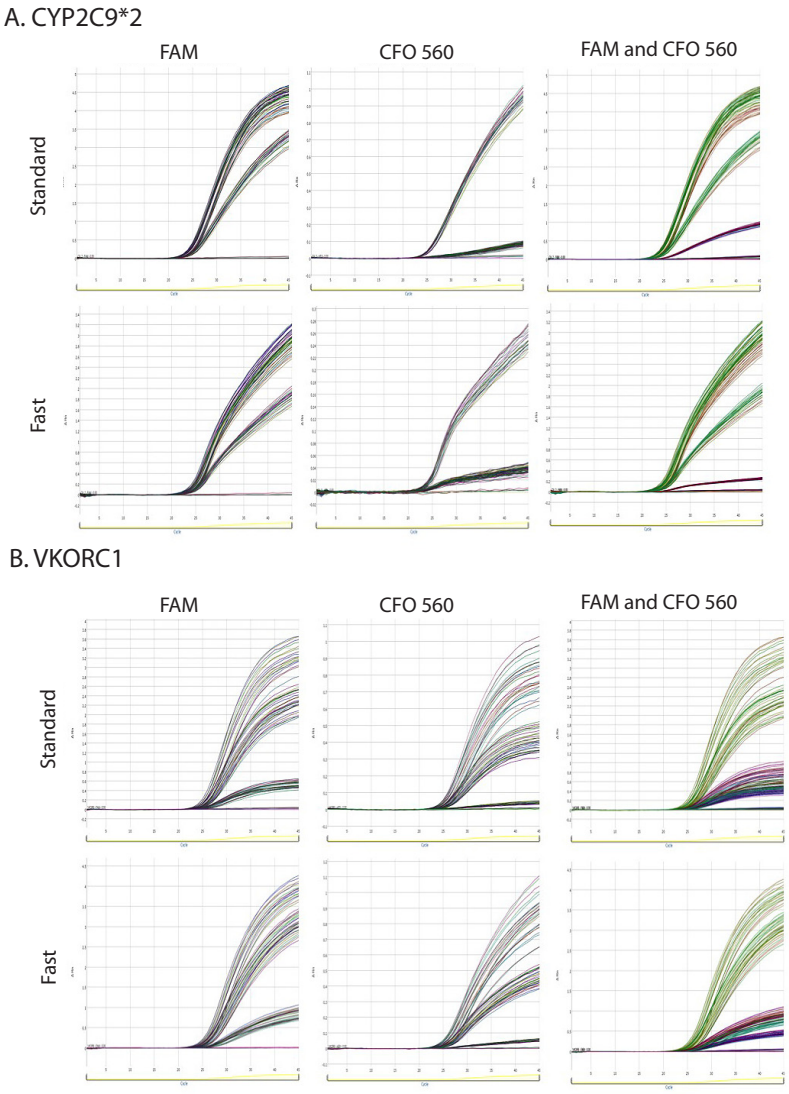


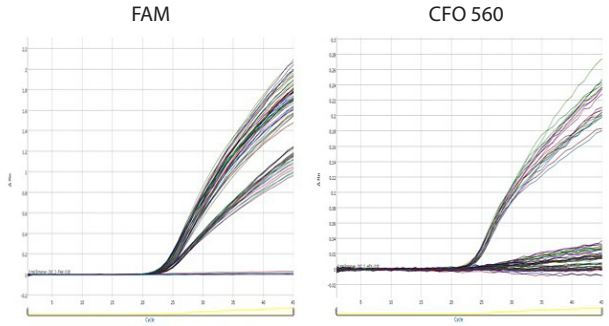
Figure 3: Real time amplification curves. The real time PCR curves for CYP2C9*2 (A) and VKORC1 (B) are shown for both standard and fast protocols. The amplification curves are shown for the FAM and HEX channels for both CYP2C9*2 and VKORC1. In both sets the curves are shown for each channel individually and combined.

CONCLUSIONS

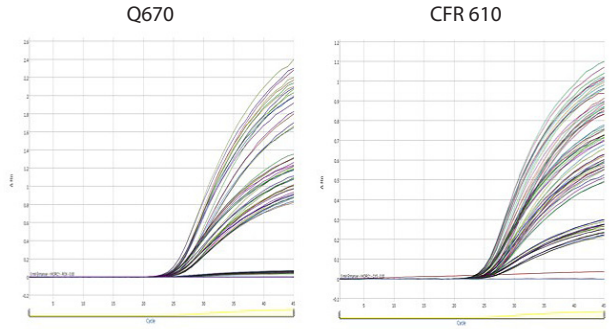
This study demonstrates the ability of the IntelliQube along with Array Tape, to successfully multiplex SNP assays in a single well and decrease thermal cycling times without compromising data quality. The combination of achieving two results per reaction well and shortened cycling times dramatically increases overall throughput. The genomic DNA samples used in this study were successfully genotyped for pharmacologically significant markers and were found to have 100% concordance with previously published alleles. In addition to automating the SNP genotyping process, this platform provides significant cost savings in the form of reduced PCR reaction volumes, shorter thermal cycling times, and multiplex reactions. The integration of liquid handling, thermal cycling, and detection systems, in the

IntelliQube enables users to achieve efficient and economical high throughput sample processing in Array Tape for end-point genotyping applications.

A. CYP2C9*2



B. VKORC1



C. CYP2C9*2 and VKORC1

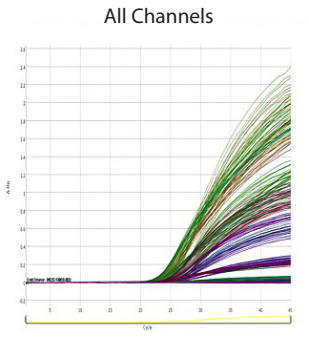


Figure 4: Real-time amplification curves. The real-time multiplex PCR amplification curves for CYP2C9*2 (A), VKORC1 (B), and overlaid assays (C). The curves are displayed either as a single channel for their respective probe or as a combination of four channels required for multiplexing. The standard thermal cycling conditions were used for this run.

	CYP2C9*2			VKORC1		
CELL LINE	Expected	Fast	Multiplex	Expected	Fast	Multiplex
1251*	Unknown	C/C	C/C	Unknown	G/G	G/G
2016	C/C	C/C	C/C	A/G	A/G	A/G
7439	C/C	C/C	C/C	G/G	G/G	G/G
8873*	Unknown	C/C	C/C	Unknown	A/G	A/G
9912*	Unknown	C/C	C/C	Unknown	A/A	A/A
10005	C/C	C/C	C/C	G/G	G/G	G/G
12244	C/T	C/T	C/T	G/G	G/G	G/G
12273	C/T	C/T	C/T	G/G	G/G	G/G
17039	C/C	C/C	C/C	G/G	G/G	G/G
17052	C/C	C/C	C/C	A/A	A/A	A/A
17057	C/C	C/C	C/C	A/G	A/G	A/G
17058	C/C	C/C	C/C	A/A	A/A	A/A
17084	C/T	C/T	C/T	A/G	A/G	A/G
17114	C/C	C/C	C/C	G/G	G/G	G/G
17115	C/C	C/C	C/C	G/G	G/G	G/G
17119	C/C	C/C	C/C	G/G	G/G	G/G
17129	C/T	C/T	C/T	G/G	G/G	G/G
17130	C/C	C/C	C/C	G/G	G/G	G/G
17203	C/C	C/C	C/C	A/G	A/G	A/G
17204	C/C	C/C	C/C	A/A	A/A	A/A
17210	C/T	C/T	C/T	A/A	A/A	A/A
17221	C/T	C/T	C/T	A/G	A/G	A/G
17227	C/T	C/T	C/T	A/G	A/G	A/G
17235	C/C	C/C	C/C	G/G	G/G	G/G
17240	C/C	C/C	C/C	A/A	A/A	A/A
17246	C/T	C/T	C/T	A/G	A/G	A/G
17247	C/C	C/C	C/C	A/G	A/G	A/G
17248	C/C	C/C	C/C	A/A	A/A	A/A
17252	C/T	C/T	C/T	A/G	A/G	A/G
17272	C/C	C/C	C/C	A/A	A/A	A/A
17276	C/C	C/C	C/C	G/G	G/G	G/G
17280	C/T	C/T	C/T	G/G	G/G	G/G
17281	C/C	C/C	C/C	A/G	A/G	A/G
17289	C/C	C/C	C/C	A/A	A/A	A/A
17293	C/T	C/T	C/T	A/G	A/G	A/G
17296	C/C	C/C	C/C	A/G	A/G	A/G
17298	C/C	C/C	C/C	A/G	A/G	A/G
17300	C/C	C/C	C/C	A/G	A/G	A/G

Table 4: Expected and observed SNP genotype alleles for genomic DNA from each cell line in this study. Expected alleles are the consensus allele calls published by Pratt, et al.

*No existing reference calls are included in published literature. Therefore, expected call is listed as unkown.

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	35	1	14	768	10,752
Fast Multiplex	35	2	14	1,536	21,504

Table 5: Determination of the number of data points achievable in an eight-hour day.

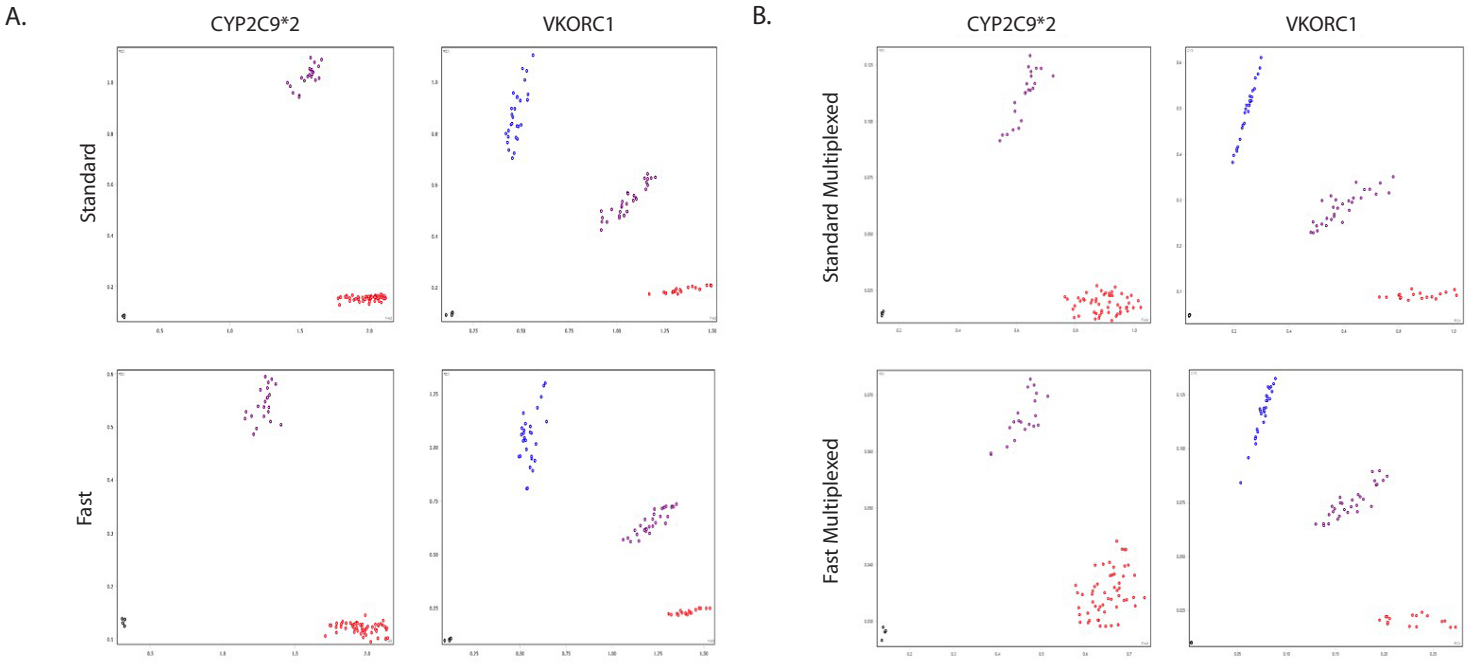


Figure 5: Cluster plot analysis. The SNP genotyping cluster plots generated inline on the IntelliQube using human genomic DNA are shown. For the standard and fast runs (A), each sample is plotted with FAM signal along the X-axis and CAL Fluor Orange 560 signal along the Y-axis. For the standard multiplex and fast multiplex runs (B), CYP2C9*2 is plotted with FAM signal along the X-axis and CAL Fluor Orange 560 signal along the Y-axis. VKORC1 is plotted with CAL Fluor Red 610 signal along the X-axis and Quasar 670 signal along the Y-axis.

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