Technical note
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Accurate SNP analysis using the IntelliQube and duplex BHQplus genotyping assays with a fast PCR protocol

A. Kolb and L. Linz
LGC, Alexandria, Minnesota, USA

Introduction

Improvements to next generation sequencing technologies have enabled extensive discovery of single nucleotide polymorphisms (SNPs) in numerous organisms. Due to their large numbers and genome-wide distribution, SNPs are the molecular marker of choice in plant, animal, and human genetic research. As the discovery of SNP markers continues to expand, there is a need for a more efficient method for routine genetic analysis. The IntelliQube® real-time qPCR instrument (Figure 1) in conjunction with BHQplus® probe-based SNP genotyping assays provides an effective solution to address this need. BHQplus probes incorporate duplex stabilizers allowing enhanced binding stability and enabling compact probe sequences with excellent mismatch discrimination. Utilizing Array Tape® technology, the IntelliQube integrates liquid handling and thermal cycling with qPCR analysis in miniaturized reaction volumes. In this study, we assess the performance of custom BHQplus genotyping assays run in a duplex fashion using fast thermal cycling protocols. Accuracy and reproducibility of this platform were assessed using purified gDNA samples from cell lines obtained from the NIGMS Human Genetic Cell Repository at the Coriell Institute for Medical Research. The resulting genotypes were compared to previously published literature. The results demonstrate that the IntelliQube, when used in conjunction with BHQplus assays, provides an accurate and streamlined real-time PCR-based method for genetic analysis.

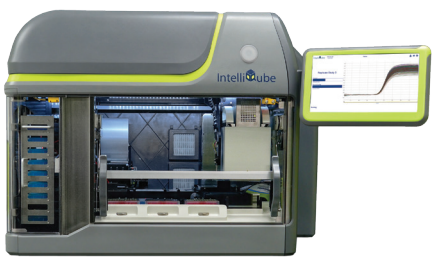

Figure 1: The IntelliQube fully integrates liquid handling, thermal cycling, detection, and data analysis into one real-time quantitative PCR instrument optimized for use with miniaturized reactions in 384- or 768-well Array Tape.

Methods

Purified gDNA from 56 cell lines was obtained from the NIGMS Human Genetic Cell Repository at the Coriell Institute for Medical Research. Custom BHQplus probe-based genotyping assays were designed and synthesized by LGC Biosearch Technologies™. Duplex BHQplus SNP genotyping assays were tested using different thermal cycling protocols (Table 1) on the IntelliQube using a commercially available PCR master mix. The primer and probe sequence information for each of the assays can be found in Table 2. Total thermal cycling time was recorded at 35, 40, and 45 cycles for each protocol.

The IntelliQube from LGC Douglas Scientific® was used for the automated assembly of 1.6 µL reactions in Array Tape consisting of 800 nL of gDNA (6.25 ng/µL) and 800 nL of master mix containing the primers and probes. Real-time fluorescence was captured and end-point cluster plot analysis was performed using IntelliScore® software.

Table 1. Thermal cycling protocols.

	Initial activation		PCR cycling		
	Cycles	Time	Cycles	Denature	Annealing/ extension
Standard	1	95 °C, 5 min	35-45	95 °C, 15 s	60 °C, 60 s
Fast	1	95 °C, 30 s	35-45	95 °C, 1 s	60 °C, 15 s

Table 2. SNP target information.

rs#	Gene	FAM probe	CAL Fluor® Orange 560 probe	Forward primer	Reverse primer
rs15524	CYP3A5	ACCAAAGTAGAAGTCCTTA	AGACCAAAGTAGAAATCCTTA	AAGTTGAAATCTCTGGTGTTCTGG	GGAACCCTAAGTGGAGAATGAGT
rs776746	CYP3A5	CAAACAGGGAAGAGATATTGAAAG	CAGGGAAGAGATACTGAAAGAC	CAGCAAGAGTCTCACACAGGA	CCCAGCTTAACGAATGCTCTAC
rs2740574	CYP3A4	TCGCCTCTCTCTTGC	CGCCTCTCTCCTGC	TCAGAAACTCAAGTGGAGCCATT	TCTGGGTTTGGAAGGATGTGTAG
rs1045642	ABCB1	CACAGGAAGAGATCGTGAGG	CACAGGAAGAGATTGTGAGGG	CTGCAGCATTGCTGAGAACATTG	GCCAGAGAGGCTGCCA
rs3892097	CYP2D6	CCCCAAGACGCCC	ACCCCCAGGACGCC	AGCGGGAACTGGGAAGG	TCACGGCTTTGTCCAAGAGA
rs#	**Gene**	**CAL Fluor Red 610 probe**	**Quasar® 670 probe**	**Forward primer**	**Reverse primer**
rs1799853	CYP2C9	CATTGAGGACCGTGTTC	CATTGAGGACTGTGTTCA	AGTTTCGTTTCTCTTCCTGTTAGGAATT	GTCCAGTAAGGTCAGTGATATGGAGTAG
rs1057910	CYP2C9	TCCAGAGATACATTGACCT	CCAGAGATACCTTGACCT	ACAGATGCTGTGGTGCACGAG	GGAGAAACAAACTTACCTTGGGAAT
rs2108622	CYP4F2	CCAGCTGTGTGGCC	ACCCAGCTATGTGGCC	GCCTCATCAGTGTTTTCGGAAC	AGGAGCCTTGGAATGGACAA
rs9923231	VKORC1	CATTGGCCGGGTGC	CATTGGCCAGGTGCG	GTCAAGCAAGAGAAGACCTGAA	CTCCTGACCTCAAGTGATCCA
rs20455	KIF6	CAGCATGAATGGTCCCA	CCAGCATGAACGGTCC	CGAAGCCATTTCTCCAGACATC	ACTCTAACACCTCCGGTGAGTTC

Table 3. Thermal cycling time (min).

Total cycles	Standard time	Fast time
35	66	29
40	75	33
45	84	37

Results

Genomic DNA from human reference samples was accurately genotyped using five duplex BHQplus SNP genotyping assays and fast thermal cycling in 1.6 µL reactions using Array Tape. The scatter plots shown in Figure 2 compare the performance of the duplex assays with standard and fast cycling conditions with varying number of total PCR cycles. The corresponding thermal cycling times are displayed in Table 3. Decreasing the total number of PCR cycles to 35 had a negligible impact on cluster quality for most assays based on Davies-Bouldin and Silhouette Index analyses (data not shown) and did not impact final genotype call. The genotyping results were concordant with available published genotypes from Pratt et al., 2010 (top part of Table 4). Of the 56 samples tested in this study, 19 did not have previously published genotypes for these SNP targets (bottom part of Table 4). Identical allele calls were observed across all conditions tested.

To assess an alternative probe fluorophore, TAMRA dye was substituted for CAL Fluor Red 610. The use of TAMRA as a probe dye in place of CAL Fluor Red 610 allows for the utilization of a ROX passive reference dye in the PCR reaction. Duplex 4 was used to compare performance of the VKORC1 assay designed with either Quasar 670/TAMRA labeled probes or Quasar 670/CAL Fluor Red 610 labeled probes. The VKORC1 assay was paired with the ABCB1 assay (FAM/CAL Fluor Orange 560). A comparison of the cluster plots is shown in Figure 3. Both probe sets produced comparable cluster quality and identical genotype calls.

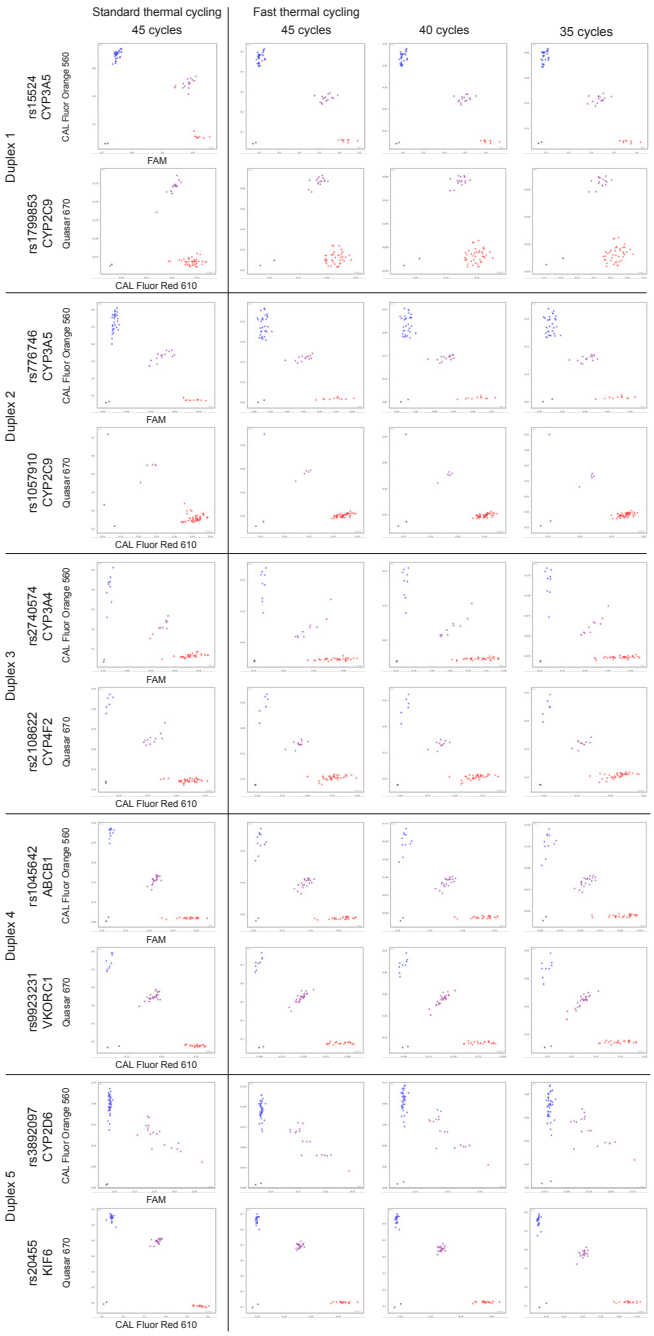

Figure 2: Cluster plot analysis of duplex BHQplus SNP assays on the IntelliQube. In each duplex, assay 1 has FAM signal plotted on the x-axis and CAL Fluor Orange 560 plotted on the y-axis. Assay 2 has CAL Fluor Red 610 signal plotted on the x-axis and Quasar 670 signal plotted on the y-axis. Cluster plots are shown for each duplex using standard or fast thermal cycling conditions for the number of cycles indicated.

Table 4: Genotyping results. Alleles for five duplex assays are reported based on the positive chromosomal strand. The color of the call corresponds to the associated cluster in Figure 2.

	Duplex 1		Duplex 2		Duplex 3		Duplex 4		Duplex 5	
Sample	rs15524 (CYP3A5)	rs1799853 (CYP2C9)	rs776746 (CYP3A5)	rs1057910 (CYP2C9)	rs2740574 (CYP3A4)	rs2108622 (CYP4F2)	rs1045642 (ABCB1)	rs9923231 (VKORC1)	rs3892097 (CYP2D6)	rs20455 (KIF6)
NA02016	GA	CC	TC	AA	TC	CC	GG	CT	CC	GG
NA07439	GG	CC	TT	AA	TT	CC	GG	CC	TC	GG
NA10005	GG	CC	TT	AA	CC	CT	GG	CC	CC	AG
NA12244	AA	CT	CC	AC	TT	CC	AA	CC	CC	AA
NA12273	AA	CT	CC	AA	TT	CT	AA	CC	CC	AG
NA17039	AA	CC	CC	AA	TT	CC	GG	CC	CC	GG
NA17052	GA	CC	TC	AA	TT	CC	GA	TT	CC	AA
NA17057	AA	CC	CC	AA	TT	TT	GG	CT	CC	GG
NA17058	GA	CC	TC	AA	TT	CC	AA	TT	CC	AG
NA17084	AA	CT	CC	AA	TT	TT	GG	CT	CC	AG
NA17114	GA	CC	TC	AA	TC	CC	GG	CC	CC	AG
NA17115	GA	CC	TC	AA	TC	CC	GG	CC	CC	GG
NA17119	GG	CC	TT	AA	CC	CC	GG	CC	CC	GG
NA17130	GA	CC	TC	AC	CC	CC	GG	CC	CC	GG
NA17203	AA	CC	CC	AA	TT	CC	AA	CT	TC	AA
NA17204	AA	CC	CC	AC	TT	CC	GA	TT	CC	AA
NA17209	AA	CT	CC	AA	TT	CT	AA	CC	TC	AG
NA17210	AA	CT	CC	AA	TT	TT	GA	TT	TC	AG
NA17221	AA	CT	CC	AC	TT	CT	GG	CT	CC	AA
NA17226	AA	CT	CC	AA	TT	CT	GA	CT	TT	AA
NA17227	AA	CT	CC	AA	TT	CT	GG	CT	CC	AA
NA17232	AA	CC	CC	AA	TT	CT	GG	CT	CC	AG
NA17235	AA	CC	CC	AA	TT	CC	AA	CC	CC	AG
NA17240	AA	CC	CC	AA	TT	CC	GA	TT	CC	AG
NA17246	AA	CT	CC	AA	TT	CC	AA	CT	TC	AG
NA17247	GA	CC	TC	CC	TC	TT	GA	CT	CC	AA
NA17248	AA	CC	CC	AA	TT	CT	GA	TT	TC	AA
NA17252	AA	CT	CC	AC	TT	CC	GG	CT	TC	GG
NA17272	AA	CC	CC	AA	TT	TT	GA	TT	TC	AA
NA17276	AA	CC	CC	AA	TT	CC	GA	CC	CC	AA
NA17280	AA	CT	CC	AA	TT	CC	GA	CC	CC	AA
NA17281	AA	CC	CC	AA	TT	CC	AA	CT	CC	GG
NA17289	GA	CC	TC	AA	TT	CT	GG	TT	TC	AA
NA17293	AA	CT	CC	AA	TT	CC	AA	CT	CC	AG
NA17296	AA	CC	CC	AA	TT	TT	GG	CT	CC	AA
NA17298	GA	CC	TC	AA	TT	CT	AA	CT	CC	AG
NA17300	AA	CC	CC	AA	TT	CT	GA	CT	CC	GG
NA01251	AA	CC	CC	AA	TT	CC	GA	CC	CC	GG
NA08873	GA	CC	TC	AA	CC	CC	GG	CT	CC	AG
NA09912	GA	CC	TC	AA	TT	CC	GG	TT	TC	AG
NA12877	AA	CT	CC	AA	TT	CC	GA	CT	TC	AA
NA12878	AA	CT	CC	AA	TT	CC	GA	CT	TC	AA
NA12891	AA	CC	CC	AA	TT	CC	GA	CC	TC	AA
NA12892	AA	CT	CC	AA	TT	CC	GA	CT	CC	AG
NA17104	GG	CC	TT	AA	CC	CC	GG	CC	CC	GG
NA17105	AA	CC	CC	AA	TC	CC	GG	CC	TC	GG
NA17107	GA	CC	TC	AA	TC	CC	GA	CT	CC	GG
NA17109	GG	CC	TT	AA	CC	CC	GG	CT	CC	GG
NA17113	GA	CC	TC	AA	TC	CC	GG	CT	CC	GG
NA17117	GA	CC	TC	AA	TT	CC	GG	CC	TC	GG
NA17123	GG	CC	TT	AA	TC	CC	GG	CC	TC	AG
NA17129	AA	CT	TT	AA	TC	CC	AA	CC	TC	GG
NA17131	GG	CC	TT	AA	CC	CC	GA	CT	CC	GG
NA17155	GG	CC	TT	AA	CC	CC	GA	CT	CC	GG
NA17194	GA	CC	TT	AA	TC	CC	GA	CC	CC	AG
NA19240	GG	CC	TT	AA	CC	CC	GG	CC	CC	GG

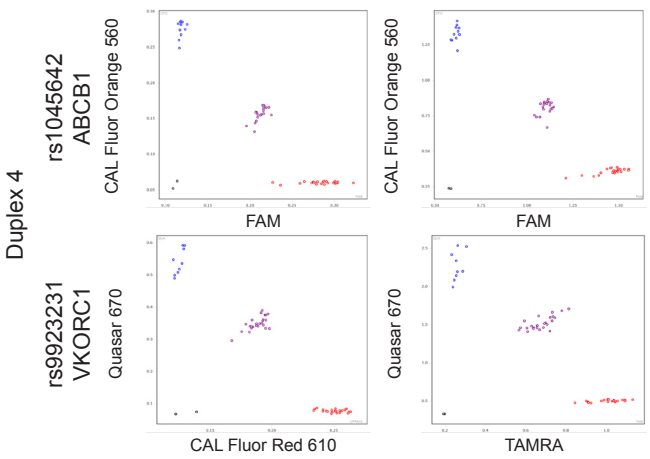

Figure 3: Alternative probe fluorophore comparison. Duplex 4 was used to compare performance of the VKORC1 assay designed with either Quasar 670/TAMRA labeled probes or Quasar 670/CAL Fluor Red 610 labeled probes. The use of TAMRA as a probe dye in place of CAL Fluor Red 610 allows for the utilization of a ROX passive reference dye. The cluster plots for ABCB1 have FAM signal plotted on the x-axis and CAL Fluor Orange 560 plotted on the y-axis. The cluster plots for VKORC1 have CAL Fluor Red 610 or TAMRA signal plotted on the x-axis and Quasar 670 signal plotted on the y-axis.

Conclusions

The IntelliQube, when used in conjunction with BHQplus SNP genotyping assays, provides a rapid and accurate solution for genotyping human DNA samples. The five fluorescence channels allow for duplexing two SNP assays in a single well with flexibility in fluorophore choice based on user needs. These results also demonstrate that fast PCR protocols can decrease the time to result to under 30 minutes depending on the number of cycles required for the assays. All cycling conditions tested in this study produced clusters that could be reliably scored. In addition to providing accurate and reliable genotyping data, the integration of liquid handling, thermal cycling, detection, and analysis within the IntelliQube enables users to benefit from more efficient and economical PCR workflows.

References

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