Evaluation of a New Accurate, Automated and Cost-effective Approach for the Analysis of Pharmacologically Important Copy Number Variation

ABSTRACT

Copy number variation (CNV) of the cytochrome P450 2D6 (CYP2D6) gene, including deletions and gene multiplications, can result in reduced or increased metabolism of many clinically relevant drugs. Recent advances within personalized medicine have led medical practitioners to increasingly rely on CYP2D6 CNV status, along with routine SNP genotype data, to guide drug dosage decisions for their patients. Research within this field continues to evolve and quantitative PCR (qPCR) has become a common method for assessing CNV status, with various probe-based assays widely available for analysis of genomic DNA. Moreover, while there are several PCR instruments on the market upon which these assays could be performed, there remains an unmet need for a fully-automated solution that provides laboratories the flexibility and scalability necessary to economically expand the access to the valuable genetic information provided by PCR analysis. The IntelliQube® from Douglas Scientific® is designed to address this need by producing accurate and reliable results for studies such as CNV determination, with walk-away automation that substantially reduces reagent expenditures and labor needs. In this study we assessed the performance of the IntelliQube by analyzing human genomic DNA with commercially available CYP2D6 CNV assays to determine CNV status. Using highly characterized reference samples from Coriell Institute for Medical Research, we compared copy number results from the IntelliQube to those previously published in the literature. An additional comparative data set was produced with the same samples and assays in a 5 µL reaction format using a ViiA™ 7 Real-Time PCR instrument (Thermo Fisher Scientific Inc.). In the study, we found that the IntelliQube demonstrates accurate and reproducible CYP2D6 CNV results consistent with those generated using standard plate-based methods and instruments. Combined with the automated workflow and economic benefits of Array Tape®, the IntelliQube proves to be a useful and powerful platform for high throughput CNV testing.

INTRODUCTION

Copy number variation (CNV) is common throughout the human genome and can significantly impact human health. One area of research where CNV is of particular importance is pharmacogenomics. There are numerous cytochrome P450s that participate in drug metabolism. In particular, the cytochrome P450 2D6 (CYP2D6) gene has been estimated to contribute to the metabolism of 25% of prescribed drugs (Ingelman-Sundberg, 2004). CNV variation within this gene (deletions or duplications) has been shown to greatly affect drug response. Individuals with lower or higher metabolism require different dosages or the use of an alternative drug to prevent side effects and maintain drug efficacy (Hicks et al., 2015). As a result, CYP2D6 CNV assays have increasingly been used in a variety of clinical and research applications. Development of accurate and economical methods for CNV analysis is therefore of the utmost importance to customizing healthcare delivery in the future. While several methods and instruments are on the market for this purpose, there remains an unmet need for a fully-automated method of CNV analysis that gives research 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 with walk-away automation that substantially reduces reagent expenditures and labor requirements. 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 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.

In this study, we purchased three commercially available CYP2D6 CNV assays from Thermo Fisher Scientific for performance testing on the IntelliQube. These three assays target different regions of the CYP2D6 gene

including Intron 2, Intron 6, and Exon 9. The CYP2D6 gene is highly homologous to the CYP2D7 and CYP2D8 psuedogenes, making it prone to homologous recombination. A common recombination event leads to a CYP2D6-CYP2D7 hybrid allele which is non-functional, but can still be detected by the Intron 2 and Intron 6 CNV assays. The Exon 9 assay is typically run in conjunction with the other two assays, as it does not amplify the nonfunctional CYP2D6-CYP2D7 hybrid allele, thus providing more accurate phenotype determinations.

This study included analysis of 51 human genomic DNA reference samples purchased from the Coriell Institute for Medical Research. In addition to comparing the sample genotypes documented by the Pratt et al. and previously published data by Life Technologies, we also generated a comparative data set with the same samples and assays in a 5 µL reaction format using a ViiA 7 Real-Time PCR instrument (Thermo Fisher Scientific Inc.).

MATERIALS AND METHODS

Samples and Reagents: 51 purified genomic DNA samples were obtained from the NIGMS Human Genetic Cell Repository at the Coriell Institute for Medical Research. DNA samples were diluted to 12.5 ng/µL with 1X TE (IDTE) before use. TaqMan probe assays from Thermo Fisher targeting CYP2D6 and RNaseP (endogenous reference) were used to assess copy number variation (Table 1). Assays were supplied at a 20X concentration and diluted in PerfeCTa® qPCR ToughMix®, UNG, Low ROX™ (Quanta BioSciences) to a 2X concentration. The final concentrations in the reactions consisted of 1X assay, 1X master mix, and 6.25 ng/uL gDNA.

Assay Name	Assay Number	Probe
CYP2D6 Intron 2	Hs04083572_cn	FAM™
CYP2D6 Intron 6	Hs04052391_cn	FAM
CYP2D6 Exon 9	Hs00010001_cn	FAM
RNaseP	4403328	VIC®

Table 1: TaqMan® assays used in this study.

Instrumentation and Analysis: The IntelliQube (Figure 1) was used for all sample and master mix dispensing, thermal cycling, and real-time fluorescence detection. DNA samples (800 nL) were dispensed into 768-well Array Tape with the multi-channel Pipette Head from CyBi® product line. Master mix (800 nL) containing 2X CYP2D6 and RNaseP assays was dispensed with the non-contact Dispense Jet to create a total reaction volume of 1.6 µL. For comparison, 5 µL reactions were prepared in a 384-well qPCR microplate by manually dispensing 2.5 µL of DNA sample and 2.5 µL of master mix containing 2X CYP2D6 and RNaseP assays. Thermal cycling was performed on the IntelliQube and ViiA 7 according to the ToughMix thermal cycling protocol recommended by the manufacturer. Real-time amplification curves and Cq values were generated by each instrument and copy number was determined using CopyCaller® Software (Life Technologies) using the DNA sample with the median ΔCq value as the two copy calibrator.

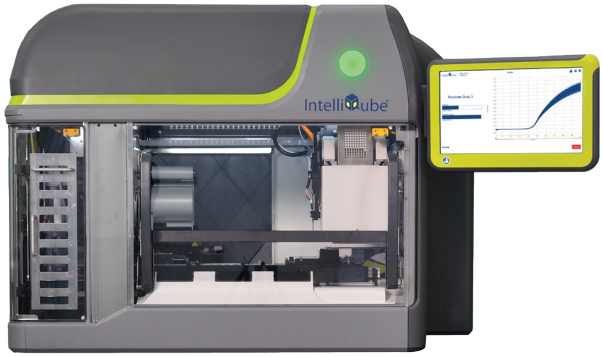

Figure 1: The IntelliQube is a fully integrated liquid handling and real-time quantitative PCR instrument optimized for use with miniaturized reactions in 768-well Array Tape.

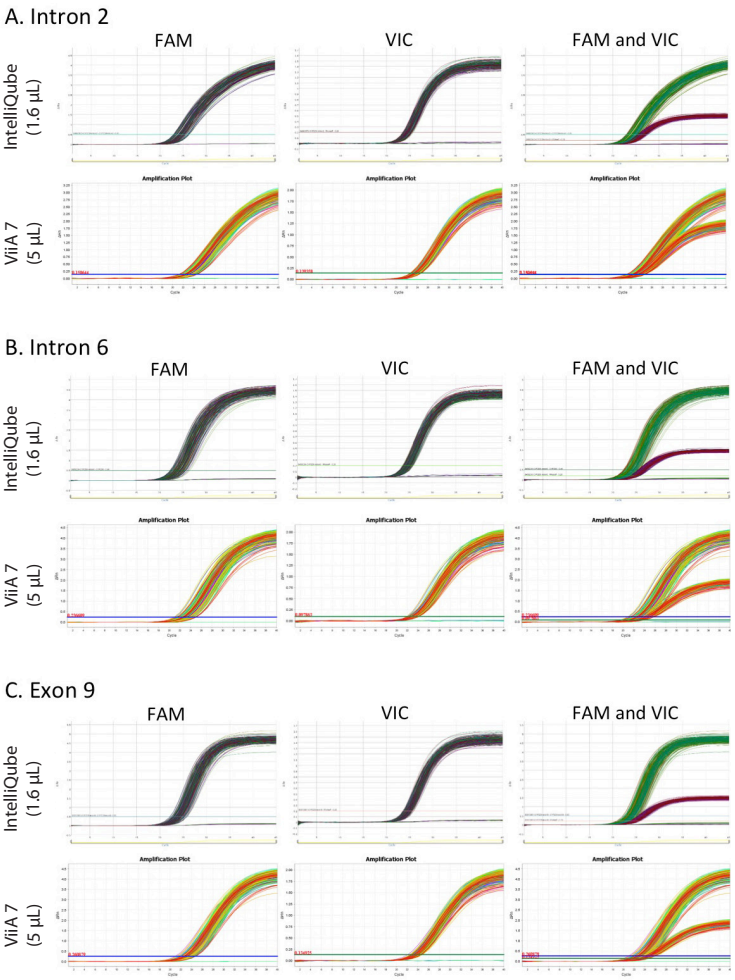

Figure 2: CYP2D6 and RNaseP Amplification Curves: The real time PCR curves for Intron2 (A), Intron6 (B), and Exon9 (C) of CYP2D6 are shown for both the IntelliQube and the ViiA 7. The amplification curves are shown for the FAM™ (CYP2D6) and VIC® (RNaseP) channels individually and combined.

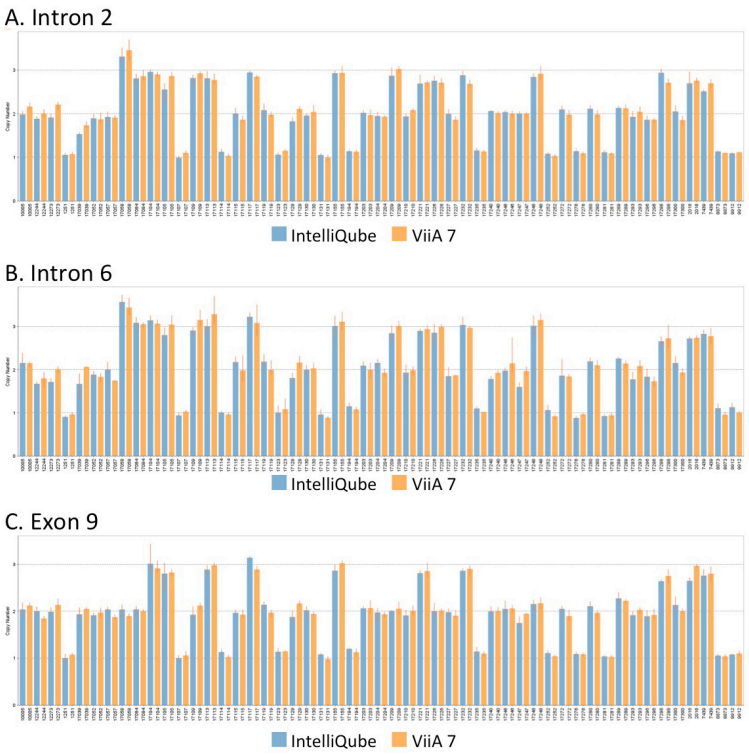

Figure 3: Copy Number Determination: Bar graphs indicating the copy number predicted by CopyCaller for assays Intron2 (A), Intron6 (B), and Exon9 (C) of CYP2D6 are shown for both the IntelliQube (Blue) and the ViiA 7 (Orange). Error bars indicate the range for the calculated copy number of the four replicates for each sample.

RESULTS AND CONCLUSIONS

The CYP2D6 copy number was determined for 51 genomic DNA samples using three TaqMan CNV assays in miniaturized (1.6 µL) reactions on the IntelliQube. A comparison of the real time curves generated on both instruments is shown in Figure 2. The calculated Cq values from both instruments were further analyzed in CopyCaller software using the sample with the median Cq value as the two copy calibrator. The copy number bar graphs generated in CopyCaller are displayed in Figure 3. Observed and expected CYP2D6 copy number for each cell line and CNV assay are given in Table 2. Expected calls listed in Table 2 are from previously published results by Thermo Fisher and sample genotypes reported by Pratt et al., 2010. Genotypes not available in the literature are labeled unknown. The results generated in Array Tape were consistent with those generated in 5 µL reactions on the ViiA 7. There was one exception with sample 17058 and the Intron 6 assay. The calculated copy number value for Intron 6 was 3.57 with the IntelliQube and 3.44 with ViiA 7. However, due to rounding of the CopyCaller software it reported the calls as four and three, respectively. Given the Exon 9 assay produced a clear two copy call for sample 17058 using both instruments, our

		Intron 2					Intron 6					Exon 9				
		Expected	IntelliQube		ViiA 7		Expected	IntelliQube		ViiA 7		Expected	IntelliQube		ViiA 7	
Sample	Star Allelle	Call	Calculated	Call	Calculated	Call	Call	Calculated	Call	Calculated	Call	Call	Calculated	Call	Calculated	Call
1251	*2XN/*17	1	0.98	1	1.07	1	1	0.91	1	0.96	1	1	1	1	1.07	1
2016	*17/2XN	3	2.58	3	2.76	3	3	2.72	3	2.74	3	3	2.65	3	2.97	3
7439	*4XN/*41	3	2.62	3	2.7	3	3	2.83	3	2.78	3	3	2.76	3	2.8	3
8873	*17/*5	1	1.06	1	1.1	1	1	1.11	1	0.96	1	1	1.06	1	1.04	1
9912	*4/*5	1	1.04	1	1.11	1	1	1.13	1	1.01	1	1	1.08	1	1.1	1
10005	*17/*29	2	2.04	2	2.16	2	2	2.16	2	2.15	2	2	2.04	2	2.12	2
12244	*35/*41	2	1.94	2	2.01	2	2	1.67	2	1.8	2	2	2	2	1.85	2
12273	*1/*1	2	1.92	2	2.21	2	2	1.71	2	2.01	2	2	1.99	2	2.13	2
17039	*2/*17	2	1.65	2	1.73	2	2	1.67	2	2.06	2	2	1.94	2	2.05	2
17052	*1/*1	2	1.83	2	1.87	2	2	1.89	2	1.83	2	2	1.91	2	1.97	2
17057	*1/*10	unknown	1.96	2	1.91	2	unknown	2.01	2	1.74	2	2	2.04	2	1.88	2
17058	*10/*10	unknown	3.44	3	3.46	3	unknown	3.57	4	3.44	3	2	2.04	2	1.9	2
17084	*1/*10	unknown	2.8	3	2.86	3	unknown	3.09	3	3.06	3	2	2.04	2	2.01	2
17104	unknown	3	2.97	3	2.9	3	3	3.15	3	3.07	3	3	3.02	3	2.92	3
17105	unknown	3	2.53	3	2.87	3	3	2.8	3	3.05	3	3	2.81	3	2.83	3
17107	unknown	1	1.02	1	1.1	1	1	0.94	1	1.03	1	1	1	1	1.06	1
17109	unknown	3	2.63	3	2.93	3	3	2.91	3	3.15	3	2	1.93	2	2.12	2
17113	unknown	3	2.82	3	2.77	3	3	3.01	3	3.29	3	3	2.89	3	1.98	3
17114	*1/*5	1	1.04	1	1.03	1	1	1.01	1	0.96	1	1	1.13	1	1.02	1
17115	*1/*2	2	1.96	2	1.86	2	2	2.18	2	1.98	2	2	1.97	2	1.93	2
17117	unknown	3	2.99	3	2.85	3	3	3.23	3	3.08	3	3	3.14	3	2.89	3
17119	*1/*2	2	2.02	2	1.98	2	2	2.19	2	2	2	2	2.14	2	1.97	2
17123	unknown	1	1.09	1	1.15	1	1	1.01	1	1.08	1	1	1.14	1	1.15	1
17129	*1/*4	2	1.86	2	2.11	2	2	1.81	2	2.17	2	2	1.88	2	2.16	2
17130	*1/*2	2	1.93	2	2.04	2	2	2	2	2.03	2	2	2.02	2	1.94	2
17131	unknown	1	1.12	1	0.99	1	1	0.96	1	0.89	1	1	1.08	1	0.98	1
17155	unknown	3	2.91	3	2.94	3	3	3.02	3	3.11	3	3	2.87	3	3.03	3
17194	unknown	1	1.19	1	1.13	1	1	1.15	1	1.08	1	1	1.2	1	1.13	1
17203	*4/*35	2	2	2	1.97	2	2	2.09	2	2	2	2	2.06	2	2.07	2
17204	*1/*35	2	2	2	1.93	2	2	2.16	2	1.93	2	2	1.97	2	1.94	2
17209	*1/*4	3	2.89	3	3.03	3	3	2.85	3	3.02	3	2	2.01	2	2.05	2
17210	*1/*4	2	1.91	2	2.08	2	2	1.93	2	1.99	2	2	1.91	2	2.01	2
17221	*1XN/*2	3	2.68	3	2.72	3	3	2.9	3	2.94	3	3	2.81	3	2.86	3
17226	*4/*4	3	2.82	3	2.71	3	3	2.86	3	2.99	3	2	2	2	2.02	2
17227	*1/*9	2	1.98	2	1.86	2	2	1.85	2	1.87	2	2	1.98	2	1.91	2
17232	*2/*2XN	3	2.9	3	2.69	3	3	3.04	3	2.97	3	3	2.86	3	2.9	3
17235	*1/*5	1	1.19	1	1.13	1	1	1.1	1	1.02	1	1	1.14	1	1.1	1
17240	*1/*10	2	2.12	2	2.02	2	2	1.79	2	1.93	2	2	2	2	2	2
17246	*4/*35	unknown	2.02	2	2	2	unknown	1.98	2	2.15	2	2	2.05	2	2.06	2
17247	*1/*4	2	2	2	2	2	2	1.6	2	1.97	2	2	1.75	2	1.94	2
17248	*4/*10	unknown	2.96	3	2.92	3	unknown	3.02	3	3.15	3	2	2.15	2	2.17	2
17252	*4/*5	1	1.12	1	1.03	1	1	1.07	1	0.92	1	1	1.11	1	1.04	1
17272	*4/*10	unknown	2.15	2	1.98	2	unknown	1.86	2	1.84	2	2	2.05	2	1.9	2
17276	*2/*5	1	1.15	1	1.09	1	1	0.88	1	0.96	1	1	1.09	1	1.08	1
17280	*2/*3	2	2.07	2	1.98	2	2	2.19	2	2.1	2	2	2.11	2	1.97	2
17281	*5/*9	2	1.17	1	1.09	1	2	0.92	1	0.94	1	2	1.04	1	1.03	1
17289	*2/*4	2	2.23	2	2.12	2	2	2.26	2	2.14	2	2	2.28	2	2.22	2
17293	*2/*9	2	2	2	2.04	2	2	1.78	2	2.09	2	2	1.92	2	2.03	2
17296	*1/*9	2	2	2	1.86	2	2	1.84	2	1.73	2	2	1.89	2	1.92	2
17298	*1/*1XN	3	2.93	3	2.71	3	3	2.66	3	2.73	3	3	2.64	3	2.75	3
17300	*1/*6	2	2.09	2	1.85	2	2	2.16	2	1.94	2	2	2.13	2	2	2

Table 2: Expected and calculated CYP2D6 copy number for each cell line in this study using Intron 2, Intron 6, and Exon 9 assays from Life Technologies. Expected calls are the consensus genotypes published by Pratt, et al. and results previously published by Life Technologies. Copy number determinations were calculated in CopyCaller Software.

Reagent	Catalog Price/mL (2X)	Cost/1.6 µL Reaction (IntelliQube)	Cost/5 µL Reaction (ViiA 7)
PerfeCTa® qPCR ToughMix®, UNG, ROX™	$81.39	$0.065	$0.20
CYP2D6 Copy Number Assay	$68.00	$0.054	$0.17
RNaseP Reference Assay	$10.30	$0.008	$0.026
Total	$159.69	$0.127	$0.40

Table 3: Reagent cost comparison between 1.6 µL reactions in Array Tape and 5 µL reactions in 384-well PCR plates. Reagent pricing may vary based on order size.

results indicate the presence of a hybrid allele and suggest a *10/*10-*36 genotype not previously reported by Pratt et al. This is likely due to a difference in the assays used between studies.

The copy number results for all other samples examined in this study were 100% concordant between the IntelliQube and ViiA 7, and matched previously published data. When comparing the two methods, the miniaturization of reactions in Array Tape offers a 68% reduction in cost per data point (Table 3), with further cost savings possible through more efficient use of laboratory personnel. Taken together, these results demonstrate that the IntelliQube, when used in conjunction with TaqMan CNV assays, produces not only accurate and reproducible CNV data in Array Tape, but also does so at a substantially lower cost than traditional methods. Therefore, the IntelliQube provides laboratories a compelling new high throughput alternative to traditional PCR-based CNV techniques.

REFERENCES

J. Kevin Hicks, Jeffrey R. Bishop, Katrin Sangkuhl, Daniel J. Müller, Yuan Ji, Susan G. Leckband, J. Steven Leeder, Rebecca L. Graham, Dana L. Chiulli, Adrián Llerena, Todd C. Skaar, Stuart A. Scott, Julia C. Stingl, Teri E. Klein, Kelly E. Caudle, and Andrea Gaedigk. Clinical pharmacogenetics implementation consortium (CPIC) guideline for CYP2D6 and CYP2C19 genotypes and dosing of selective serotonin reuptake inhibitors. Clin Pharmacol Ther 2015 August;98(2):127-134

Victoria M. Pratt, Barbara Zehnbauer, Jean Amos Wilson, Ruth Baak, Nikolina Babic, Maria Bettinotti, Arlene Buller, Ken Butz, Matthew Campbell, Chris Civalier, Abdalla El-Badry, Daniel H. Farkas, Elaine Lyon, Saptarshi Mandal, Jason McKinney, Kasinathan Muralidharan, LeAnne Noll, Tara Sander, Junaid Shabbeer, Chingying Smith, Milhan Telatar, Lorraine Toji, Anand Vairavan, Carlos Vance, Karen E. Weck, Alan H.B. Wu, Kiang-Teck J. Yeo, Markus Zeller, Lisa Kalman. Characterization of 107 genomic DNA reference materials for CYP2D6, CYP2C19, CYP2C9, VKORC1 and UGT1A1: A GeT-RM and Association for Molecular Pathology collaborative project. J Mol Diag 2010 12(6):835-846 Magnus Ingelman-Sundberg. Pharmacogenetics of cytochrome P450 and its application in drug therapy: the past, present, and future. Trends Pharmacol Sci 2004 Apr;25(4):193-200

TaqMan® Copy Number Assays for DME genes. Life Technologies. Jan 25, 2014 https://www.thermofisher.com/content/dam/LifeTech/Documents/XLS/DME_Copy_Number_Assays_Coriell_CNs_25Jan2014.xlsx?v=2

*For research use only. The products of Douglas Scientific, LLC are not FDA-approved for use in human diagnostic procedures.