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Fast, simple SNP genotyping with multiplex qPCR

SNP genotyping

Genotyping from crude lysates: fewer steps, dependable results

Summary

- Multiplexing Applied Biosystems™ TaqMan™ SNP Genotyping Assays can increase testing throughput and reduce the amount of reagents and plastics used
- Time-consuming and costly sample extraction steps can be omitted by using crude lysates
- In this technical note, we observed 100% concordance between purified DNA and crude lysates using multiplex SNP genotyping assays targeting *CYP2D6, COMT, DPYD, G6PD,* and *AOC1*

Keywords

real-time PCR (qPCR), single nucleotide polymorphism (SNP), crude lysate, multiplexing, genotyping, drug metabolism enzyme

Introduction

Single nucleotide polymorphisms (SNPs) in genes play a profound role in human, animal, and plant traits, health, and disease. Notable examples of germline SNPs include the E6V substitution in *HBB,* known as sickle cell trait, and C61G in *BRCA1,* which confers elevated cancer risk. Agricultural breeding programs routinely screen for SNPs that confer desirable attributes, such as disease resistance, drought tolerance, and food quality [1]. More recently, pharmacogenomics (PGx) research has benefited from the identification of SNPs that influence drug metabolism, toxicity, and efficacy, which can be used to guide drug choice and dosing [2,3]. Finally, SNPs can serve as biomarkers for research into specific diseases [4,5].

Uncharacterized SNPs are typically discovered using sequencing. However, once a SNP of interest has been identified, real-time PCR (qPCR) is well suited for screening large numbers of samples quickly. Applied Biosystems TaqMan SNP Genotyping Assays identify SNPs in a single well using a common set of primers and two probes, one that detects the major variant and one that detects the minor or alternate variant. Compared with sequencing, which can take hours or days, SNP genotyping by qPCR can be completed in as little as an hour, particularly when crude lysates in lieu of purified samples are run (Figure 1).

The option to identify multiple SNPs in a single well by multiplexing offers several advantages for SNP genotyping (Figure 1B). First, less sample and master mix are

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required compared with running multiple SNP genotyping assays in separate wells. Multiplexing also reduces per-sample hands-on time and permits increased throughput by freeing up valuable space on a plate for additional samples. Finally, splitting a single sample across multiple wells to test with several assays in parallel increases the risk of sample or assay mix-up, so multiplexing can also reduce errors and retests.

Historically, nucleic acid extraction (purification) from biological samples was required for downstream use in molecular techniques due to the presence of enzyme inhibitors such as hemoglobin, anticoagulants, lipids, bile salts, and other biomolecules, as well as the reagents used for lysis [6]. Sample extraction involves first lysing the cells or tissues followed by binding of nucleic acids to a filter or paramagnetic beads (Figure 1C). Then, multiple wash

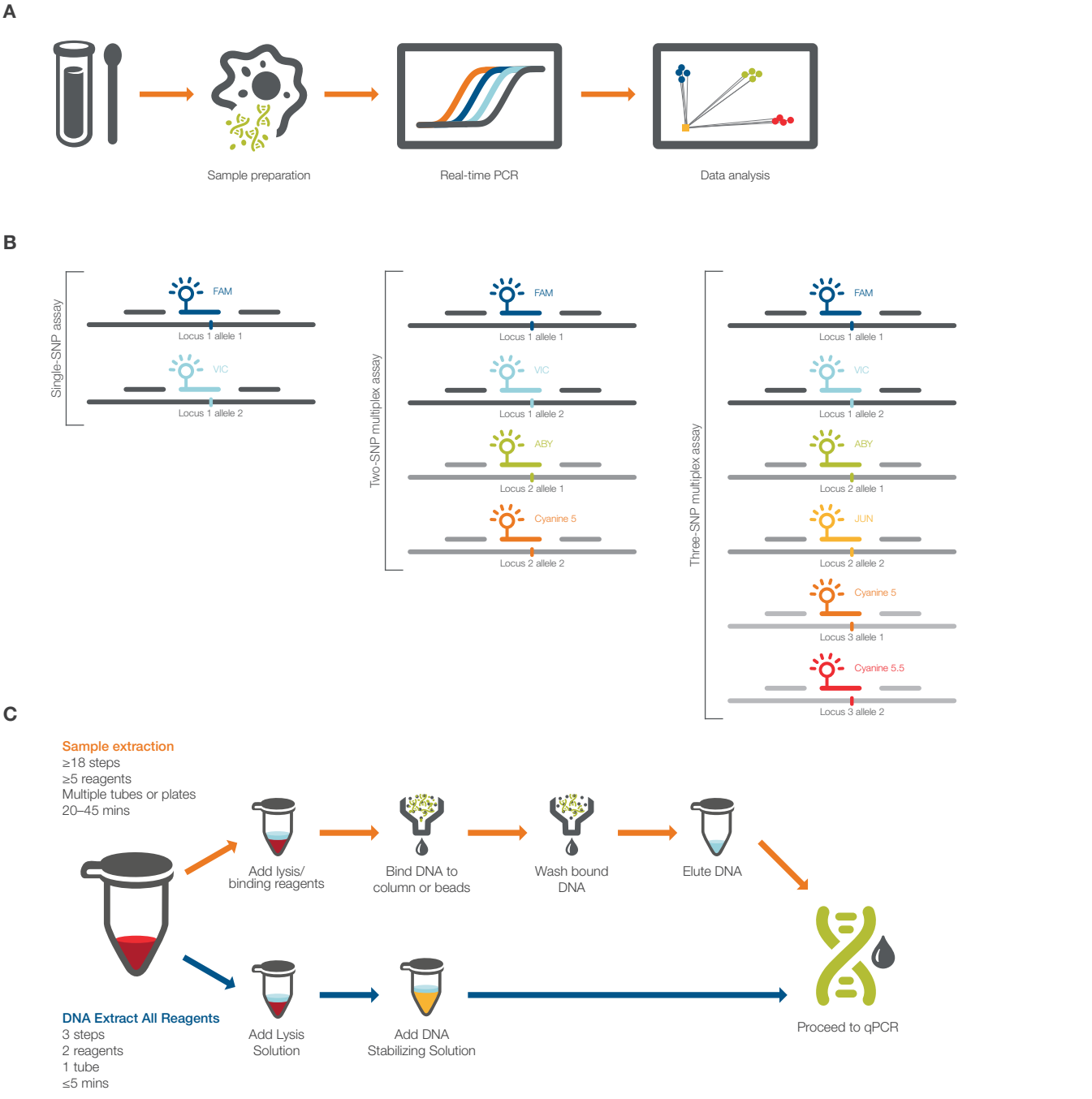

Figure 1. SNP genotyping workflow. (A) High-level workflow. **(B)** Structure of single-SNP and multiplex SNP assays. Single-SNP assays query two variants at a single locus using allele-specific probes containing two different dyes. Multiplex SNP assays query multiple loci simultaneously using four or more dyes. Dyes shown are examples only. **(C)** Comparison of hands-on time, turnaround time and consumables used for sample extraction and crude lysate preparation. Estimated times and consumables are per sample and depend on the number of samples processed.

steps are performed to remove proteins, lipids, salts, chaotropic agents, detergents, and other potential PCR inhibitors. Finally, the now-purified bound DNA and/or RNA is eluted in a low-ionic-strength buffer, often in a smaller volume than the original starting sample. Whereas sample extraction produces clean, more concentrated samples for downstream analysis, it also increases turnaround time, sample handling, and per-sample cost.

Compared with sample extraction, the use of crude lysates reduces both cost and handling but requires robust reagents to overcome inhibition and maintain performance. Recent advances in enzymology and PCR additives have enabled the use of crude lysates for numerous biological sample types, including whole blood and buccal swabs, without loss of amplification efficiency. Compared with the multiple steps, reagents, plastics, and waste entailed in sample extraction, crude lysates can be prepared in two steps or fewer, often in a single tube (Figure 1C).

In this technical note, we evaluate the performance of multiplexed SNP genotyping assays compared with the same assays run in separate wells. Assays for three drug metabolism enzyme (DME) targets and two non-DME targets were tested individually (single SNP) and in multiplex on both the Applied Biosystems™ QuantStudio™ 5 Dx Real-Time PCR System and the Applied Biosystems™ QuantStudio™ 5 384-well Real-Time PCR System. Furthermore, we demonstrate successful multiplexing with both crude lysates and purified DNA from whole blood and buccal swabs. Multiplexing, skipping lengthy sample extraction steps, and the robust built-in genotype calling tools in Applied Biosystems software enable significant improvements to the overall SNP genotyping workflow for your research.

Materials and methods

Assays

Crude lysates and purified (extracted) DNA were tested in duplicate qPCR reactions with each single-SNP or multiplex SNP assay. Two-SNP multiplex assay testing was performed using the Applied Biosystems™ TaqPath™ ProAmp™ Master Mix, and three-SNP multiplex assay testing was performed using a custom modification of the TaqPath ProAmp Master Mix without ROX passive reference dye. To assess concordance, the samples were tested with each single-SNP assay in parallel. Assay IDs and dyes are provided in Table 1. Custom versions of inventoried assays were ordered with Applied Biosystems™ FAM™-MGB, Applied Biosystems™ VIC™-MGB, Applied Biosystems™ ABY™-MGB, Applied Biosystems™ JUN™-MGB, cyanine 5-MGB, or cyanine 5.5-QSY2 probes as indicated in Table 1. The probe for the *CYP2D6*10* assay was redesigned for a QSY2 quencher.

Samples

DNA was purified from 10 whole blood samples preserved in sodium heparin and nine buccal swab samples using a commercially available magnetic bead-based DNA extraction kit. Crude lysates from the same donors were prepared in parallel using the Applied Biosystems™ DNA Extract All Reagents Kit. Both crude lysates and purified (extracted) DNA were quantified by qPCR using the Applied Biosystems™ TaqMan™ Copy Number Reference Assay, human, TERT and a standard curve generated with Applied Biosystems™ TaqMan™ Control Genomic DNA (human). In accordance with the Extract All Reagents user guide, 2 µL of crude lysate was added to each SNP genotyping reaction in a final reaction volume of 10 µL. Purified DNA was diluted as needed so that a similar quantity

Table 1. Composition of multiplex SNP assays

Multiplex ID	Catalog assay ID	dbSNP rs ID	Assay target	Genotype	Dyes
COMT multiplex	C__25746809_50	rs4680	*COMT* locus 1	G>A	VIC/FAM
	C___2538747_20	rs4633	*COMT* locus 2	C>T	Cyanine 5/ABY
DPYD multiplex	C__30633851_20	rs3918290	*DPYD* locus 1	C>T	VIC/FAM
	C__27530948_10	rs67376798	*DPYD* locus 2	T>A	Cyanine 5/ABY
G6PD multiplex	C___2228694_20	rs1050829	*G6PD* locus 1	T>C	VIC/FAM
	C___2228686_20	rs1050828	*G6PD* locus 2	C>T	Cyanine 5/ABY
AOC1 multiplex	C__11630976_1_	rs2052129	*AOC1* locus 1	G>T	VIC/FAM
	C___2658581_10	rs2268999	*AOC1* locus 2	A>T	Cyanine 5/ABY
CYP2D6 multiplex	C__27102425_50	rs16947	*CYP2D6*2*	G>A	VIC/FAM
	C__27102431_D0	rs3892097	*CYP2D6*4*	C>T	JUN/ABY
	C__11484460_40†	rs1065852	*CYP2D6*10*	G>A	Cyanine 5.5/cyanine 5

†Probe conjugated to cyanine 5.5 was redesigned for QSY2 quencher and thus, differed in sequence from catalog assay; for more information about custom assay design including for QSY quenchers, please visit thermofisher.com/multiplex-SNP

of DNA was added to each SNP genotyping reaction (between 2 ng and 33.4 ng per reaction for purified buccal swab DNA; between 4.2 ng and 45.2 ng per reaction for buccal swab crude lysates; between 3 ng and 9 ng per reaction for purified whole blood DNA; and between 2.4 ng and 8 ng per reaction for whole blood crude lysates). Positive controls consisted of the indicated samples from the Coriell Institute for Medical Research.

qPCR and data analysis

qPCR was performed on the QuantStudio 5 Dx system or the QuantStudio 5 384-well system as indicated. *COMT, DPYD,* and *CYP2D6* assays were tested using the standard thermal protocol recommended for the TaqPath ProAmp Master Mix, with a 90-second anneal/extend cycle. The *G6PD* and *AOC1* assays were tested using the fast cycling conditions recommended for the TaqPath ProAmp Master Mix. Reactions were formulated to a 1X final concentration for each individual SNP genotyping assay, and the final reaction volume was 10 µL for both 96-well and 384-well plates. Data from the QuantStudio 5 Dx system were analyzed using Applied Biosystems™ QuantStudio™ 5 Development Software v1.2.1 Genotyping Analysis module with the Analyze Real-Time dRn Data setting. Data from the QuantStudio 5 384-well system were analyzed using Applied Biosystems™ Diomni™ Design and Analysis (RUO) Software v3.0.1 Genotyping Analysis module with the Real-time data Genotyping Analysis setting. Overall concordance was calculated from wells that obtained a genotyping call. Manual calls were made (after data export) where noted. For ease of interpretation, Tables 2–4 are color coded by genotyping call, with blue indicating homozygous Allele 1/Allele 1, green indicating heterozygous Allele 1/Allele 2, and orange indicating homozygous Allele 2/Allele 2. Due to differences in sample background fluorescence, whole blood crude lysates were analyzed independently of purified whole blood samples.

Results

Multiplex SNP assays demonstrate 100% concordance with single-SNP assays

The ability to multiplex is a well-known benefit of TaqMan chemistry. To assess the performance of multiplexed assays, purified (extracted) DNA from whole blood and buccal swabs was tested with both single-SNP and two-SNP multiplex *AOC1* assays in parallel. As shown in Figure 2 and Table 2, concordance in genotype calls was 100% between single-SNP and two-SNP multiplex *AOC1* assays for whole blood and buccal swab samples.

Similarly, a three-SNP multiplex assay for three different *CYP2D6* alleles was tested on purified DNA from buccal swabs and

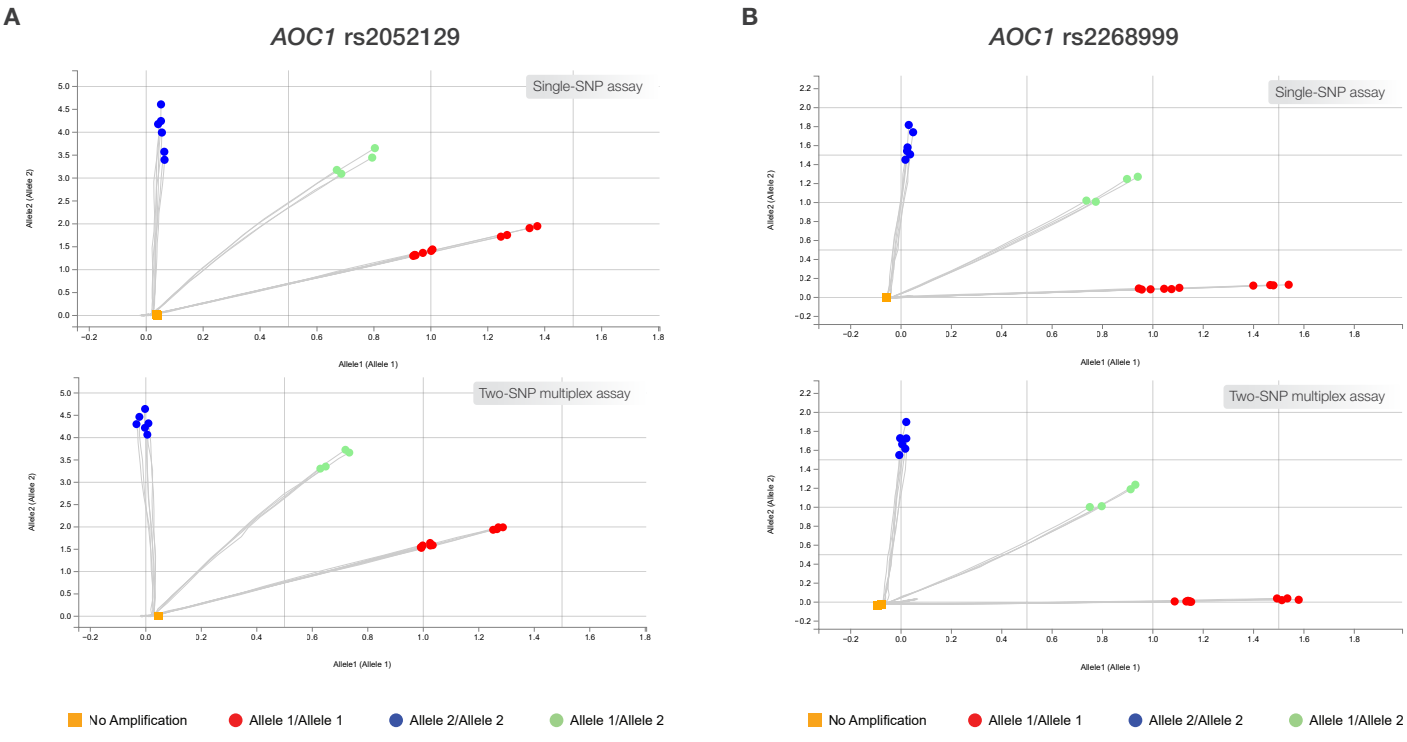

Figure 2. Two-SNP multiplex *AOC1* assay demonstrated 100% concordance with single-SNP assays. *AOC1* rs2052129 and rs2268999 assays were tested with purified DNA from buccal swabs on the QuantStudio 5 384-well system. Note that the software plots each SNP separately even when assays are run in multiplex. **(A)** Allelic discrimination plots for rs2052129 when tested as single-SNP assay (top) and multiplex assay (bottom). **(B)** Allelic discrimination plots for rs2268999 when tested as single-SNP assay (top) and multiplex assay (bottom).

Table 2. Genotyping results for *AOC1*

Sample ID	rs2052129				rs2268999			
	Crude lysate		Purified		Crude lysate		Purified	
	Single SNP	Multiplex	Single SNP	Multiplex	Single SNP	Multiplex	Single SNP	Multiplex
Whole blood 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2
Whole blood 2	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Whole blood 3	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2
Whole blood 4	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Whole blood 5	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Whole blood 6	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2
Whole blood 7	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2
Whole blood 8	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Whole blood 9	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2
Whole blood 10	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2
Buccal 1	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2
Buccal 2	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Buccal 3	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Buccal 4	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Buccal 5	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2
Buccal 6	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2
Buccal 7	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2
Buccal 8	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2
Buccal 9	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
NA17108	Not tested	Not tested	Allele 1/Allele 2	Allele 1/Allele 2	Not tested	Not tested	Allele 2/Allele 2	Allele 2/Allele 2
NA17116	Not tested	Not tested	Allele 1/Allele 1	Allele 1/Allele 1	Not tested	Not tested	Allele 1/Allele 2	Allele 1/Allele 2
NA17129	Not tested	Not tested	Allele 1/Allele 2	Allele 1/Allele 2	Not tested	Not tested	Allele 1/Allele 2	Allele 1/Allele 2

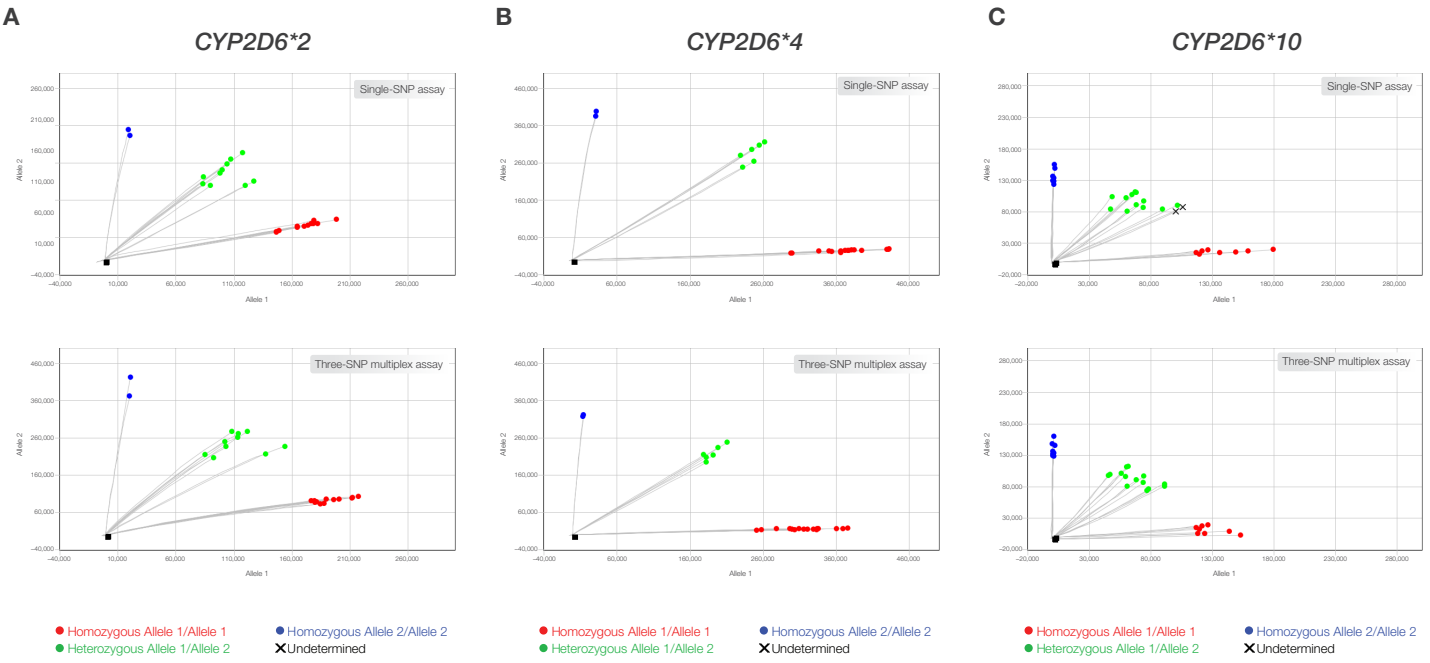

Figure 3. Three-SNP multiplex *CYP2D6* assay demonstrated 100% concordance with single-SNP assays. *CYP2D6*2*, *CYP2D6*4*, and *CYP2D6*10* assays were tested with purified DNA from buccal swabs and the control samples listed in Table 3 on the QuantStudio 5 Dx system. Note that the software plots each SNP separately even when assays are run in multiplex. **(A)** Allelic discrimination plots for *CYP2D6*2* when tested as single-SNP assay (top) and multiplex assay (bottom). **(B)** Allelic discrimination plots for *CYP2D6*4* when tested as single-SNP assay (top) and multiplex assay (bottom). **(C)** Allelic discrimination plots for *CYP2D6*10* when tested as single-SNP assay (top) and multiplex assay (bottom).

Table 3. Genotyping results for *CYP2D6*

Sample ID	*CYP2D6*2*				*CYP2D6*4*				*CYP2D6*10*			
	Crude lysate		Purified		Crude lysate		Purified		Crude lysate		Purified	
	Single SNP	Multiplex	Single SNP	Multiplex	Single SNP	Multiplex	Single SNP	Multiplex	Single SNP	Multiplex	Single SNP	Multiplex
Buccal 1	Allele 1/ Allele 2	Allele 1/ Allele 2	Allele 1/ Allele 2	Allele 1/ Allele 2	Allele 1/ Allele 2	Allele 1/ Allele 2	Allele 1/ Allele 2	Allele 1/ Allele 2	Allele 1/ Allele 2	Allele 1/ Allele 2	Allele 1/ Allele 2	Allele 1/ Allele 2
Buccal 2	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 2/ Allele 2	Allele 2/ Allele 2	Allele 2/ Allele 2	Allele 2/ Allele 2
Buccal 3	Allele 1/ Allele 2	Allele 1/ Allele 2	Allele 1/ Allele 2	Allele 1/ Allele 2	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 1/ Allele 2	Allele 1/ Allele 2	Allele 1/ Allele 2	Allele 1/ Allele 2
Buccal 4	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 1/ Allele 2	Allele 1/ Allele 2	Allele 1/ Allele 2	Allele 1/ Allele 2
Buccal 5	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 1/ Allele 2	Allele 1/ Allele 2	Allele 1/ Allele 2	Allele 1/ Allele 2	Allele 1/ Allele 2*	Allele 1/ Allele 2*	Allele 1/ Allele 2*	Allele 1/ Allele 2*
Buccal 6	Allele 1/ Allele 2	Allele 1/ Allele 2	Allele 1/ Allele 2	Allele 1/ Allele 2	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 1/ Allele 2*	Allele 1/ Allele 2*	Allele 1/ Allele 2*	Allele 1/ Allele 2*
Buccal 7	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 2/ Allele 2	Allele 2/ Allele 2	Allele 2/ Allele 2	Allele 2/ Allele 2
Buccal 8	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 1/ Allele 1
Buccal 9	Allele 1/ Allele 2	Allele 1/ Allele 2	Allele 1/ Allele 2	Allele 1/ Allele 2	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 1/ Allele 1	Allele 1/ Allele 1
HG01190	Not tested	Not tested	Allele 1/ Allele 1	Allele 1/ Allele 1	Not tested	Not tested	Allele 2/ Allele 2	Allele 2/ Allele 2	Not tested	Not tested	Allele 2/ Allele 2	Allele 2/ Allele 2
NA12750	Not tested	Not tested	Allele 2/ Allele 2	Allele 2/ Allele 2	Not tested	Not tested	Allele 1/ Allele 1	Allele 1/ Allele 1	Not tested	Not tested	Allele 1/ Allele 1	Allele 1/ Allele 1
NA18637	Not tested	Not tested	Allele 1/ Allele 2	Allele 1/ Allele 2	Not tested	Not tested	Allele 1/ Allele 2	Allele 1/ Allele 2	Not tested	Not tested	Allele 1/ Allele 2	Allele 1/ Allele 2

*One or more replicates produced an automatic call of undetermined genotype using default analysis parameters; result shown includes manual calling for affected replicates

compared with the same single-SNP assays run separately. Like the *AOC1* assays, the multiplex *CYP2D6* assay showed 100% concordance with the single-SNP assays (Figure 3 and Table 3).

Finally, the remaining SNP assays were tested with purified DNA from whole blood and buccal swabs. As shown in Tables 2–5, the multiplex SNP assays tested demonstrated 100% concordance with the single-SNP assays run in parallel.

Crude lysates demonstrate 100% concordance with purified DNA using multiplex assays

Using crude lysates instead of purified (extracted) DNA for SNP genotyping can reduce per-sample cost, hands-on time, turnaround time, and the chemical and plastic waste generated (Figure 1C). To demonstrate the efficacy of using crude lysates for multiplex SNP genotyping, we compared the performance of crude lysates and purified DNA from whole blood and buccal swab samples using the two-SNP multiplex *COMT* assay. As shown in Figure 4 and Table 4, concordance in genotype calls was 100% between crude lysates and purified DNA for the multiplex *COMT* assay. Results for single-SNP *COMT* assay testing with crude lysates are also shown in Table 4.

Next, the performance of the three-SNP multiplex *CYP2D6* assay on both crude lysates and purified DNA was compared. As shown in Figure 5 and Table 3, 100% concordance in *CYP2D6* genotyping calls was achieved when comparing results from crude lysates with those from purified DNA. Results for single-SNP *CYP2D6* assay testing with crude lysates are also shown in Table 3.

Finally, across all SNP assays tested, 100% concordance was achieved between purified DNA and crude lysates with both single-SNP and multiplex assays (Figure 6 and Tables 2–5). These results demonstrate robust performance, even under a challenging combination of conditions.

Conclusion

In this technical note, we demonstrated successful multiplexing of SNP genotyping assays with 100% concordance to the same single-SNP assays run in parallel. Furthermore, we showed 100% concordance using crude lysates from buccal swabs and whole blood compared with samples that have undergone nucleic acid extraction. Together, the Extract All Reagents Kit and the TaqPath ProAmp Master Mix were able to overcome inhibition from two potent PCR inhibitors: heme and heparin. The ability to assign up to three SNP genotypes in a single qPCR reaction and omitting time-consuming and costly sample extraction enables more science with fewer resources.

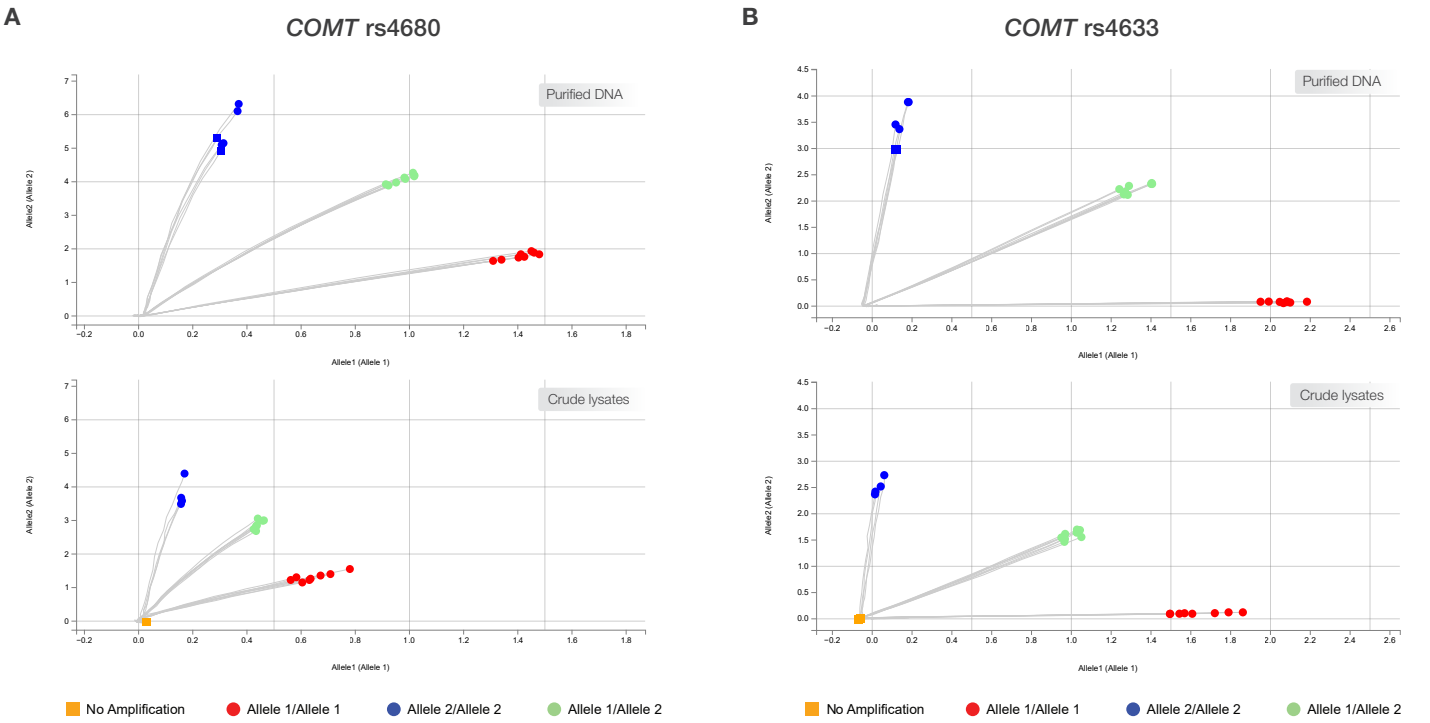

Figure 4. Crude lysates demonstrated 100% concordance with purified DNA for two-SNP multiplex *COMT* assay. The multiplex *COMT* assay was tested with purified DNA and crude lysates from whole blood on the QuantStudio 5 384-well system. Note that software plots each SNP separately even when assays are run in multiplex. Allelic discrimination plots for **(A)** *COMT* rs4680 and **(B)** *COMT* rs4633 for purified DNA (top) and crude lysates (bottom).

Table 4. Genotyping results for *COMT*

Sample ID	rs4680				rs4633			
	Crude lysate		Purified		Crude lysate		Purified	
	Single SNP	Multiplex	Single SNP	Multiplex	Single SNP	Multiplex	Single SNP	Multiplex
Whole blood 1	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2
Whole blood 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2
Whole blood 3	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2
Whole blood 4	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2
Whole blood 5	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2
Whole blood 6	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2
Whole blood 7	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Whole blood 8	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Whole blood 9	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Whole blood 10	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Buccal 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Buccal 2	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Buccal 3	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2
Buccal 4	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Buccal 5	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2
Buccal 6	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Buccal 7	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2	Allele 1/Allele 2
Buccal 8	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Buccal 9	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
HG00332	Not tested	Not tested	Allele 1/Allele 2	Allele 1/Allele 2	Not tested	Not tested	Allele 1/Allele 2	Allele 1/Allele 2
NA12248	Not tested	Not tested	Allele 1/Allele 2	Allele 1/Allele 2	Not tested	Not tested	Allele 1/Allele 2	Allele 1/Allele 2
NA20901	Not tested	Not tested	Allele 2/Allele 2	Allele 2/Allele 2	Not tested	Not tested	Allele 2/Allele 2	Allele 2/Allele 2

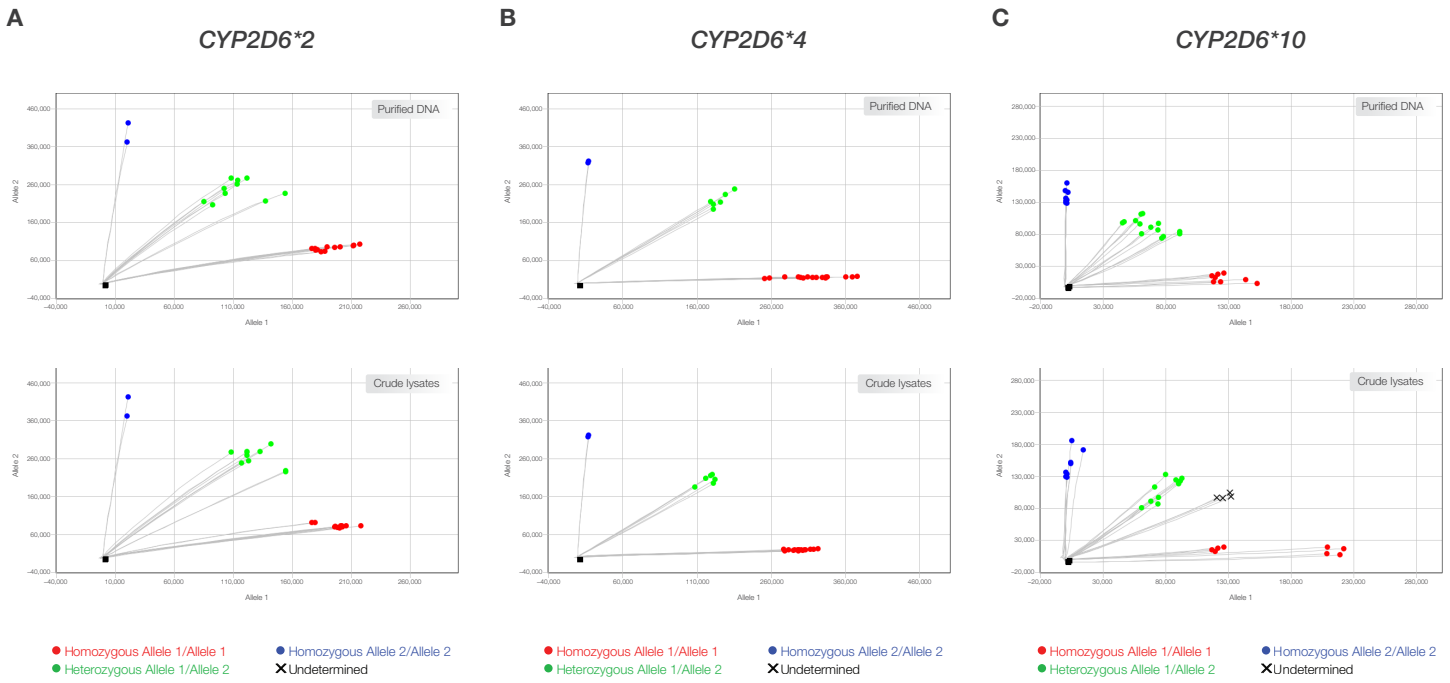

Figure 5. Crude lysates demonstrated 100% concordance with purified DNA for three-SNP multiplex *CYP2D6* assay. The three-SNP multiplex *CYP2D6* assay was tested with purified DNA and crude lysates from buccal swabs and purified DNA from the control samples listed in Table 3 on the QuantStudio 5 Dx system. Note that the software plots each SNP separately even when assays are run in multiplex. **(A)** Allelic discrimination plots for *CYP2D6*2*, **(B)** *CYP2D6*4*, and **(C)** *CYP2D6*10* for purified DNA (top) and crude lysates (bottom).

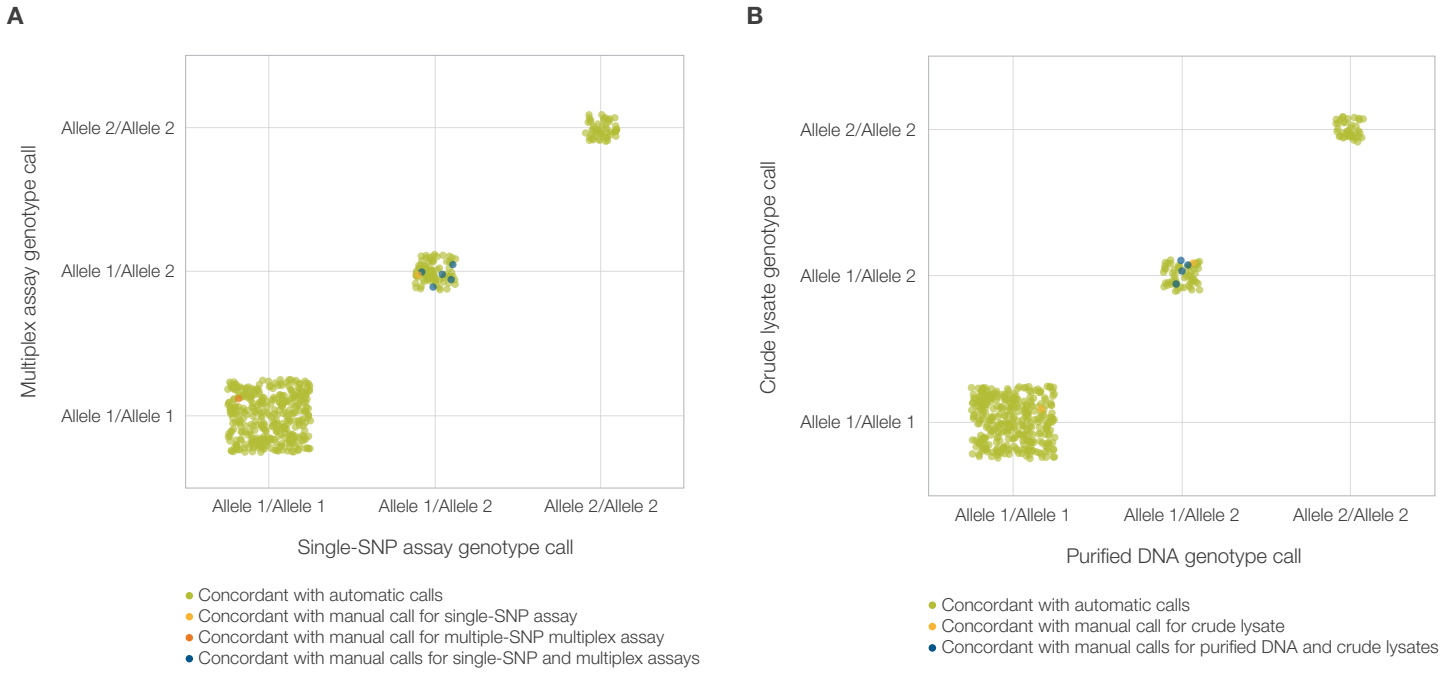

Figure 6. Overall concordance in genotype call between single-SNP and multiplex SNP assays and between purified samples and crude lysates. Details regarding manual calls are provided in Tables 2–4. **(A)** Concordance between single-SNP and multiplex SNP assays across all samples, assays, and sample preparation methods. Green dots, concordant calls between single-SNP and multiplex assays using default analysis settings. Yellow dots, a manual call was made for one or more replicates using the single-SNP assay. Orange dots, a manual call was made for one or more replicates using the multiplex assay. Blue dots, manual calls were made for one or more replicates using both the single-SNP and multiplex assays. **(B)** Concordance between purified DNA and crude lysates across all samples, assays, and assay plexy. Green dots, concordant calls between purified DNA and crude lysates using default analysis settings. Yellow dots, a manual call was made for one or more replicates for crude lysates. Blue dots, a manual call was made for one or more replicates using both purified DNA and crude lysates.

Table 5. Genotyping results for *DPYD* and *G6PD*

Sample ID	Target	Locus 1				Locus 2			
		Crude lysate		Purified		Crude lysate		Purified	
		Single SNP	Multiplex	Single SNP	Multiplex	Single SNP	Multiplex	Single SNP	Multiplex
Whole blood 1	*DPYD*	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Whole blood 2	*DPYD*	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Whole blood 3	*DPYD*	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Whole blood 4	*DPYD*	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Whole blood 5	*DPYD*	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Whole blood 6	*DPYD*	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Whole blood 7	*DPYD*	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Whole blood 8	*DPYD*	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Whole blood 9	*DPYD*	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Whole blood 10	*DPYD*	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Buccal 1	*DPYD*	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Buccal 2	*DPYD*	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Buccal 3	*DPYD*	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Buccal 4	*DPYD*	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Buccal 5	*DPYD*	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Buccal 6	*DPYD*	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Buccal 7	*DPYD*	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Buccal 8	*DPYD*	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Buccal 9	*DPYD*	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
NA07048	*DPYD*	Not tested	Not tested	Allele 1/Allele 1	Allele 1/Allele 1	Not tested	Not tested	Allele 1/Allele 2	Allele 1/Allele 2
NA07055	*DPYD*	Not tested	Not tested	Allele 1/Allele 1	Allele 1/Allele 1	Not tested	Not tested	Allele 1/Allele 2	Allele 1/Allele 2
NA20901	*DPYD*	Not tested	Not tested	Allele 1/Allele 2	Allele 1/Allele 2	Not tested	Not tested	Allele 1/Allele 1	Allele 1/Allele 1
Whole blood 1	*G6PD*	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Whole blood 2	*G6PD*	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Whole blood 3	*G6PD*	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Whole blood 4	*G6PD*	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Whole blood 5	*G6PD*	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2	Allele 2/Allele 2	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Whole blood 6	*G6PD*	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Whole blood 7	*G6PD*	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Whole blood 8	*G6PD*	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Whole blood 9	*G6PD*	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Whole blood 10	*G6PD*	Allele 1/Allele 1*	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Buccal 1	*G6PD*	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Buccal 2	*G6PD*	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Buccal 3	*G6PD*	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Buccal 4	*G6PD*	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Buccal 5	*G6PD*	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Buccal 6	*G6PD*	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Buccal 7	*G6PD*	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Buccal 8	*G6PD*	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
Buccal 9	*G6PD*	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1	Allele 1/Allele 1
NA17108	*G6PD*	Not tested	Not tested	Allele 2/Allele 2	Allele 2/Allele 2	Not tested	Not tested	Allele 2/Allele 2	Allele 2/Allele 2
NA17116	*G6PD*	Not tested	Not tested	Allele 2/Allele 2	Allele 2/Allele 2	Not tested	Not tested	Allele 1/Allele 2	Allele 1/Allele 2
NA17129	*G6PD*	Not tested	Not tested	Allele 1/Allele 2	Allele 1/Allele 2	Not tested	Not tested	Allele 1/Allele 2	Allele 1/Allele 2

*One or more replicates produced an automatic call of undetermined genotype using default analysis parameters; result shown includes manual calling for affected replicates

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