Nanopore sequencing of the pharmacogene CYP2D6 allows simultaneous haplotyping and detection of duplications

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Abstract

Background: The accurate genotyping of *CYP2D6* is hindered by the very polymorphic nature of the gene, high homology with its pseudogene *CYP2D7*, and the occurrence of structural variations. Long read sequencing offers the promise of overcoming some of these challenges, along with the advantage of straightforward variant phasing. We have established methods for sequencing and analysis of DNA amplicons containing the whole *CYP2D6* gene, using the GridION nanopore sequencer. **Materials and methods:** Seven reference and 25 clinical samples covering various haplotypes including gene duplication were barcoded and sequenced over two sequencing runs. Sequenced raw reads were analyzed using a pipeline of bioinformatics tools including two mapping tools and two variant calling tools. **Results:** Using minimap2 and nanopolish (mapping and variant calling tools respectively) resulted in the most accurate variant detection. Haplotypes of 52 alleles could be matched accurately to known alleles or subvariants, while the remaining 12 alleles being assigned as novel star (*) allele of novel subvariants of known alleles in the PharmVar *CYP2D6* haplotype database. Allele duplication could be detected by analyzing the allelic balance between the sample haplotypes. **Conclusion:** Nanopore sequencing of *CYP2D6* offers a high throughput method for genotyping, accurate haplotyping, and detection of new variants and duplicated alleles.

Keywords: nanopore sequencing, pharmacogenetics, CYP2D6, haplotype, gene duplication, GridION

Introduction

CYP2D6 is one of the most important and widely studied genes in pharmacogenetics. It codes for the cytochrome P450 2D6 enzyme which is responsible for the metabolism or bioactivation of ~25% of all available drugs, including antidepressants such as selective serotonin reuptake inhibitors (SSRIs), analgesics such as codeine, and anti-cancer drugs such as tamoxifen [1]. *CYP2D6* is also one of the most polymorphic human genes, leading to considerable variability in enzyme activity between individuals with the need for accurate genotype information for clinical purposes [2].

CYP2D6 is a relatively small gene (4.4 kilobases from start to stop codon) located on the long arm of chromosome 22. However, the complexities of this gene make accurate genotyping challenging [3-5]. The first challenge lies in the number of variants in the gene. PharmVar (https://www.pharmvar.org/gene/CYP2D6) has compiled more than 100 allelic variations and more than 200 genetic variants [6] in this gene. Moreover, the gnomAD database of more than 140K individuals suggests there may be over 1000 variants in this gene, including more than 400 missense and 30 loss-of-function variants [7].

In addition to its polymorphic sequence, *CYP2D6* can be affected by structural variations including gene deletion, gene duplication, hybrid gene conversion or tandem rearrangement with its upstream pseudogene, *CYP2D7*. Since *CYP2D6* and *CYP2D7* share more than 90% sequence similarity and occur in tandem on the chromosome, care must be taken to selectively target CYP2D6 to obtain accurate genotype information for clinical utility [8, 9].

Various methods have been developed to genotype *CYP2D6,* from the basic restriction fragment length polymorphism to the more sophisticated real time polymerase chain reaction (PCR), microarray, mass spectrometry, or next generation sequencing [10-12]. Most methods are limited by the ability to detect only a subset of pre-selected variants. While next generation sequencing (NGS) methods have the ability to screen all *CYP2D6* variants, these methods are hampered by the risk of misalignment of *CYP2D6* and *CYP2D7* due to the short-reads generated. Most of these methods also do not provide information for variant phasing to confidently determine haplotypes. In practice, this is often carried out using allele specific primers to isolate one or both alleles, followed by Sanger sequencing of the amplification products [13]. This is labour intensive, requires a greater investment of time, and can be technically demanding.

Long read sequencing is envisaged to resolve challenges faced by previous short read technology. For *CYP2D6* genotyping, long amplicon sequencing not only permits unequivocal variant detection without interference from the homologous pseudogene, it also means that variant phasing can be carried out in a reliable and straightforward fashion. A preliminary report

described *CYP2D6* sequencing of a single sample using the Oxford Nanopore Technologies (ONT) MinION with an earlier, more error-prone version of the technology [14]. In this paper, we explore the application of current ONT long read sequencing chemistry to simultaneously analyze *CYP2D6* from multiple reference and clinical samples. We performed the sequencing on the GridION platform, a compact nanopore-based instrument from ONT (Oxford, UK) which enables sequencing and data computation in the same module in a real time manner.

Materials and Methods

Samples

Thirty-two samples were genotyped over two sequencing runs, with 12 samples in the first and 24 samples in the second run. Four samples from the first run were repeated in the second run as control samples. Seven *CYP2D6* genotyped samples [15] were obtained from Coriell Institute (Camden, NJ, USA) and 25 unrelated clinical samples were collected from two adverse drug reaction cohorts established in our laboratory, Understanding Adverse Drug Reactions Using Genomic Sequencing (UDRUGS) and Genetics of Antidepressants study (GO-A) [16]. These studies received ethical approval respectively from Southern Health and Disability Ethics Committee (URA/11/11/065) and Northern B Health and Disability Ethics Committee (18/NT/21), Ministry of Health, New Zealand. The clinical samples consisted of 14 blood samples and 11 saliva samples. DNA from blood samples was extracted using a modified salting-out method [17] followed by a phenol-chloroform purification step (Supplementary File 1). Saliva samples were collected via an Oragene DNA (OG-500) kit (DNA Genotek Inc., ON, Canada) and DNA was extracted according to the manufacturer's protocol.

CYP2D6 Long PCR

The 6.6 kilobases (kb) long PCR amplifying the whole *CYP2D6* gene, including upstream and downstream non-coding regions, was performed according to Gaedigk, 2007 [18]. Specific sequences from ONT were added to the 5' end of forward and reverse primers to enable sample barcoding in subsequent steps. 250 ng of genomic DNA was amplified in the presence of 1x Kapa LongRange Buffer, 0.3 mM dNTPs, 1.75 mM $MgCl_2$, 0.7 μM of each primer, 1 M of betaine, and 1.25 units of Kapa LongRange Taq Polymerase (Sigma-Aldrich) in a 50 μL reaction. PCR was run as follows: 94°C for 3 minutes, 30 cycles of 94°C for 25 seconds, 68°C for 10 seconds, 68°C for 7 minutes, and a final extension at 68°C for 7 minutes.

Deletion and multiplication of the *CYP2D6* gene can be identified by amplifying a 3.5 kb fragment with CYP-13 and CYP-24 primers [19] for deletion and Frag B primers [18] for duplication in a

duplex PCR together with the 6.6kb primers. This PCR was carried out as above using 0.4 μM of the 6.6 kb primers and 0.3 μM of the deletion or duplication primers.

In samples where a gene duplication was detected, the duplicated allele was amplified with tailed FragD primers [18] which produced 8.6 kb fragments, and these were subsequently barcoded and sequenced on the GridION. This primer set recognizes the REP-DUP region, a unique combination of *CYP2D6* and *CYP2D7* downstream regions commonly found in duplications of the CYP2D6 gene. This PCR was similar to that for *CYP2D6* described above, except 0.4 μM primers and extension for 10 minutes instead of 7 minutes were used. The sequences of all primers used in this study are listed in Table 5.

Barcoding PCR

Specific barcodes were assigned to the 6.6 kb PCR products of each sample and 8.6 kb PCR products (where applicable) using the PCR Barcoding Expansion 1-96 kit (EXP-PBC096) (ONT). Barcodes were added using a second-round PCR to 0.5 nM of CYP2D6 6.6 kb or 8.6 kb purified PCR product in the presence of 1x LongAMP PCR buffer, 2 mM of $MgCl_2$, 0.3 mM of dNTPs, and 5 units of LongAmp Taq DNA polymerase in 50 μL reaction. PCR was performed as follows: 95˚C for 3 minutes, 15 cycles of 95˚C for 15 secs, 62˚C for 15 seconds, 65˚C for 7 minutes (6.6 kb products) or 10 minutes (8.6 kb products), with a final extension of 65˚C for 7 minutes or 10 minutes.

Modified Beads Purification

Both initial and barcoding PCR products were purified using magnetic beads (HighPrep™ PCR, MagBio, Gaithersburg, MA, USA). In the first sequencing run, we observed that many short (~1 kb), off-target DNA fragments were sequenced. Therefore, for the second run, to remove any DNA fragments shorter than 3-4 kb, modified beads were made based on the protocol of Ramawater and Schwessinger [20]. In brief, Magbio beads (e.g. 5 mL) were pelleted and re-dissolved in buffer containing 10 mM Tris-HCL, 1 mM EDTA pH 8, 1.6 M NaCl, 11% (w/v) PEG 8000, 0.20% (v/v) Tween-20, and Millipore water to make 5 mL bead solution. For each sample, 0.8x volume of beads was used.

Nanopore Sequencing Library Preparation

Each DNA library was prepared according to the 1D PCR barcoding (96) amplicons ONT protocol, SQK-LSK109. Briefly, after pooling the barcoded amplicons, 200 fmoles of the DNA pool were subjected to end-repair and ONT adapter ligation. After purification, approximately 350 ng (~80 fmoles) were introduced to the R9.4 flow cell to be sequenced for 48 hours on the GridION.

Nanopore Sequencing Data analysis

DNA sequences were extracted from fast5 files using Guppy v2.2.2 with flip-flop basecalling (ONT, Oxford, UK). Only pass reads (qscore > 7) were used for subsequent genotyping analysis as outlined in Figure 1. Guppy basecaller did not accommodate demultiplexing or length filtering when we performed this analysis, and these were done subsequently using Porechop (https://github.com/rrwick/Porechop) and NanoFilt [21]. Filtered fastq files obtained from NanoFilt were then mapped to reference sequence (NG_008376.3, 8593 bps) using minimap2 [22] or NGMLR [23], and variants were called from the mapped Binary Alignment MAP(BAM) files to a Variant Calling Format (VCF) file using nanopolish [24] or Clairvoyante [25]. Threshold for calling variants was set at 0.2 for nanopolish and 0.25 for Clairvoyante as recommended. A trained model for ONT was also used when calling variants using Clairvoyante. Variant phasing in samples containing more than one heterozygote variant was carried out using WhatsHap [26] with maximum coverage of 25X, resulting in phased VCF and phased BAM files. Quality control metrics were obtained using NanoOK [27] and MinIONQC [28].

Variant nomenclature follows the numbering based on NG_008376.3 counting from the sequence start. Haplotype information for each allele was determined by comparing the variants in each allele to the PharmVar haplotype table. Duplicated alleles were identified by examining the ratio of allele frequency between variants from the two alleles of each sample. The presence of duplicated alleles was indicated by a skewed allele balance from 1:1 to 2:1, or more in the case of multiple copies.

Genotype information for reference samples was available through the published Get-RM dataset [15]. Genotype information for the clinical samples was determined using Sanger Sequencing based on key variants (Table 6).

Sanger sequencing

Nested PCRs covering different regions on the 1:1000 diluted 6.6 kb *CYP2D6* PCR product were performed and used for Sanger sequencing on an AB3130XL Genetic Analyzer (Thermo Fisher Scientific) (Table 5). Nested PCRs were carried out in 20 μL reactions containing 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM of each primer, 0.5 unit of TAQ-TI Heat-Activated DNA Polymerase (Fisher Biotec, Wembley, WA, Australia) and 2 uL of diluted PCR product. DNA was amplified in a touchdown PCR consisting of 15 cycles of 94°C for 15 seconds, 70°C for 15 seconds, and 72°C for 1 minute with annealing temperature decrease of 1°C /cycle followed by 20 cycles of 94°C for 15 seconds, 55°C for 15 seconds, and 72°C for 1 minute and a final extension at 72°C for 5 minutes. Sanger sequencing was performed using 1 μL product of nested PCR, 1 μL of primer, 1x sequencing buffer, and 0.5 μl of Big Dye Terminator (ThermoFisher Scientific, Carlsbad, USA) in

10 μL reaction. Dye incorporation was performed in a thermal cycler with 25 cycles of 96˚C for 10 seconds, 50˚C for 5 seconds, and 60˚C for 4 minutes, and products were subsequently purified using Sephadex G-50 (Sigma-Aldrich, Saint Louis, USA).

Results

Both sequencing runs resulted in similar yield (~3.6 gigabases), but different numbers of reads. The first sequencing run resulted in more than 90% reads having length less than 6 kb, possibly due to unspecific amplification during barcode PCR. In the second run, short reads were significantly reduced and target sequences of 6 kb were enriched (~55% of total reads) with the use of modified beads during PCR product purification (Figure 2). Although the first run resulted in more reads in total, there were more barcoded reads with with >6 kb length in the second run (35% compared to 4%). Despite the differences in number of reads between runs and between samples, all samples had adequate coverage for subsequent analysis and genotyping, with the lowest sample coverage at 235x (Table 1).

Comparison of Mapping and Variant Calling Tools

Amplicon data analysis was performed by comparing two mapping tools, minimap2 [22] and NGMLR [23], and two variant calling tools, nanopolish [24] and Clairvoyante [25], resulting in a total of four different analyses. These comparisons were carried out with seven reference samples from the first sequencing run. False positive and negative variants were confirmed by visually inspecting the BAM file on Integrative Genomics Viewer (IGV, Broad Institute, Boston, MA) [29]. The minimum negative predictive value was 99.96% (NGMLR – nanopolish) and the minimum accuracy was 99.68% (NGMLR – Clairvoyante). The calculation for these two measurements was dominated by the relatively high number of true negative variants (6514 – 6561 variants). Clairvoyante generally generated more false positive variants compared with nanopolish, resulting in higher False Discovery Rate values (Table 2). In addition, we observed a misalignment event in NGMLR in the intron 1 CYP2D7 conversion region (seven SNPSs, 4414-4445) (Figure 4). Due to this misalignment, the combination of NGMLR and nanopolish inaccurately called 4423C>G and 4427T>C as 4423insG and 4427delT (Figure 4). Based on our analyses, the combination of minimap2 and nanopolish for mapping and variant calling resulted in the highest sensitivity and positive predictive value and lowest false discovery rate compared to the other tool combinations, and hence was used to analyze the entire sample cohort.

CYP2D6 Genotyping Results

Together, the reference and clinical samples cover a total of 16 different star (*) alleles (*1, *2, *3, *4, *6, *7, *9, *10, *15, *17, *28, *29, *33, *35A, *41, and *71), as well as one sample with gene deletion (*5) and four samples with gene duplication. The only sample with a gene deletion (S022) was heterozygous, so only one allele was available to be sequenced. This allele contains a hemizygous variant (6684G>T), which corresponds to *33. This variant was confirmed on Sanger sequencing and the sample was assigned as *5/*33.

The genotyping results for all samples are shown in Table 3. For the 64 alleles represented by the 32 samples, 52 of them were assigned to known subvariant catalogue entries on PharmVar. For the remaining 12 alleles in 11 samples, they either had key variants matched to certain alleles but not to the existing subvariants, or contained variants that did not match any known allele pattern on PharmVar. These, upon submission to PharmVar, were assigned as novel allele or novel subvariants of known alleles. These findings are described below.

One sample (S003) had a 6750delA variant in one allele which was called as *3. In the other allele, there are nine SNPs and one insertion variant, which taken together, cannot be matched to any star (*) allele in PharmVar (4470C>T, 7436A>G, 7455T>C, 7466G>A, 7585A>C, 7679A>G, 7685G>A, 7746insT, 7753C>T, 7783A>G). From the 10 variants found in this allele, three of them are in the exonic regions, one of which (7436A>G) is a missense variant, classified as moderate by Variant Effect Predictor (VEP, Ensembl) [30] and is one of the key variants associated with *108. However, the *108 allele has another key variant also in exon 7, 7427A>G (rs61736517), which was not present in our sample. 7466G>A, also a missense variant and predicted to be deleterious by VEP, has been associated with *4.021, but this sample lacks other key variants of *4. This was assigned as a new allele by PharmVar, *127.

One sample (S016) contained only one variant (6504C>T, rs79738337) which is associated only with *60, but lacks the key variant of *60 (6088insTA). As 6504C>T is an intronic variant, this sample was assigned as a new subvariant of *1, *1.026. The other allele of S016 matched *10.002 except for the presence of 4945C>G in this sample. This allele was assigned as *10.003.

Another sample (S007) had two variants (4325G>A and 5695T>C), associated with *71 but lacked the 2617C>G variant and therefore cannot be matched to either *71.001 or *71.002 as per the PharmVar catalogue. This was therefore assigned as a new subvariant, *71.003.

We found five variants not yet catalogued in PharmVar. Two of the Coriell samples (NA18518 and NA23297) with *17 allele had an additional intronic variant (4853C>T, rs376217512). This is a rare variant with allele frequency (AF) of ~1%. This allele was assigned as *17.003 by PharmVar.

One sample with variants matching to *41 allele (S013) contained two non-catalogued variants, 5578C>G (rs143170489) in intron 2 and 5814C>T (rs61736504) in exon 3. Both are rare with AF ~0.1%. The exonic variant is predicted to be benign when annotated using VEP. This sample was assigned as a new subvariant of *41, *41.004. Another sample with *4 allele (S023) also had a rare (AF: 0.001) intron 2 variant, 5604C>T, rs557722765, and was assigned as a new subvariant, *4.026.

The fifth variant not catalogued in PharmVar was an upstream variant (3836G>A, rs1080992) which was found in four samples, and was the only variant in the particular allele of each sample. This rare variant with an AF of less than 1% [7] has been reported in a non CEU population [31]. No functional information is available for this variant. This allele was assigned as *1.025.

Detection of duplicated alleles

The presence of a duplicated allele in samples was determined by interrogating the ratio of coverage between alleles. Samples without a duplication were expected to have a 1:1 ratio between alleles, while samples with a duplication were expected have a skewed ratio of ~2:1 (Figure 3). All four samples which indicated gene duplication during the initial PCR screening showed an uneven allele coverage ratio, and the duplicated alleles could be determined from the over-represented allele. In addition, we sequenced the 8-kb fragments in three out of the four samples with duplication. The 8-kb fragments originated only from the duplicated allele, and aligned to the NG_008376.3 reference sequence from position 3860 to 8593. Sequencing of these fragments revealed the same variants as the duplicated alleles identified from the 6 kb fragments and thus confirmed the duplicated allele in all three samples (NA07439, NA23297, S018) (Table 4).

Validation of duplicated allele and variant phasing using the 8-kb fragment.

Interrogating the variants in the 8 kb fragments served two purposes. First, this information identified the duplicated allele as explained in the previous section. Second, comparison of the variants found in the 8-kb amplicon to those found in the 6.6kb amplicon allowed validation of variant phasing performed in the 6.6 kb amplicon. All variants found in the 8-kb amplicons of the three samples matched completely those found on the over-represented (duplicated) allele on the 6.6 kb fragments, thus validated the accuracy of variant phasing of the 6.6kb amplicons. In addition, all phased variants in all samples could be matched to a particular allele or subvariant, confirming the accuracy of variant phasing.

Identification and Filtering of False Positive Variants

Nanopore sequencing still fails to accurately basecall homopolymer strings. When aligned to the reference, this usually leads to a drop in coverage and variant calling tools report this as a deletion. Two to 13 false positive deletion/insertion events were found in each sample, and all were in regions with three or more sequential identical bases. In addition to errors due to homopolymers, false positive transition variants also occurred at two or three sites per sample. These false positive variants were non-random, always transitions (A↔G or C↔T) and were called only in specific positions: 2381A>G, 2446T>C, 5207C>T, 6440T>C, 8046C>T, 8047C>T, or 8723G>A. These false positive variants have lower quality in the nanopolish produced VCF files compared to the true variants. We applied a previously reported strategy [32] by using quality/coverage ratio to differentiate true and false variants. Most of the true variants have quality/coverage ratio of >1, while most of the false variants have ratio < 0.5. For variants with ratio value between 0.5-1, the trueness of the variant was determined visually in the BAM files using IGV.

Although all samples could be genotyped unequivocally, several variants failed to be detected and were not reported in the VCF file (Table 3). One variant that was present but not reported in many samples was 5042T>G. This SNP sits between four Gs in the upstream and another G downstream, and when the alternate G exists, it created a string of six G's and the read depth dropped significantly at that site, presumably due to the inability of nanopore sequencing to detect homopolymers correctly. As a result, this SNP was often called incorrectly as a T-deletion instead of a SNP. However, this variant is quite common and is shared by many alleles including the wildtype *1, *2, *4, *10, *41 and many others, and the absence did not interfere with correct haplotyping of any sample.

Discussion

We performed long amplicon nanopore sequencing of the whole *CYP2D6* gene (including upstream and downstream regions) on the GridION nanopore sequencing platform. To our knowledge, this is the first extensive analysis of *CYP2D6* on a nanopore sequencing platform across multiple star (*) alleles, including assessment of structural variation. An earlier report of *CYP2D6* nanopore sequencing only used one reference sample and was done on the earlier version of ONT chemistry and flow cell. High error rates were associated with that version, and unresolved ambiguity was reported in the sample diplotype, although variant phasing was performed accurately [14]. The initial relatively high error rate associated with nanopore sequencing has improved as the technology continues to evolve. This is mainly attributed to

improvements in the pore, sequencing chemistries and bioinformatic algorithms available for analysis. The accuracy of ONT nanopore sequencing has improved from less than 80% with the R7.3 flow cell [33] used by Ammar et. al, to more than 90% with the current R9.4 version [34].

We used the R9.4 flow cell with 1D sequencing chemistry, and generated DNA sequences using the guppy basecaller from ONT. Two different mapping and variant calling tools were then used to analyze the reference samples to select the best bioinformatic workflow for our dataset. We observed that a combination of minimap2 as a mapping tool and nanopolish as a variant calling tool generated the least number of false positive and false negative variants compared to other workflows. The comparison results between nanopolish and Clairvoyante in this study reflected the findings in another study [25], where Clairvoyante reported more false positive variants, and less false negative variants. However, in our study, although Clairvoyante reported some variants missed by nanopolish, it still failed to call several other variants, especially those in the intron 1 CYP2D7 conversion region, resulting in comparable false negative variants between the two tools. Moreover, the quality scores in nanopolish-generated VCF files were more indicative of the trueness of the variants, and false positive variants were more easily filtered.

The advantages of long amplicon sequencing of *CYP2D6* have been recently reported by two papers, using the PacBio single molecule real-time (SMRT) sequencing platform [35, 36]. Although both have the advantages of long read sequencing, the ONT nanopore sequencing platform offers the advantage of very low capital costs and presents a unique opportunity to deliver results in real-time settings such as in the field [24, 37] or at point of care.

One of the issues reported with nanopore sequencing is the limitation in reliable detection of small insertion and deletion (indel) events [32, 38], and we encountered several false positive indels in homopolymer regions. However, true indels outside the homopolymer regions could be detected accurately, including key indel variants associated with common *CYP2D6* star (*) alleles such as 6750delA (*3), 5098delT (*6), 6816delAAG (*9), and 4337insT (*15). All samples with these haplotypes could be identified accurately. In addition, other indel variants were also detected including 4933delC, 7746insA, and 8857delACA which are associated with several star (*) alleles including *2, *41, and *35.

The challenge that homopolymer regions present to the current nanopore sequencing also means that this technology remains limited for investigating the two long homoplymer strings of A's in the upstream region of the *CYP2D6* gene (2943-2964 and 3098-3107). The regions in between these two strings are notoriously difficult to sequence, resulting in a gap in most PharmVar catalogued alleles or subvariants. Variation in the number of A's within 2943-2964 has been reported previously [36], however this region has not been included as part of the haplotype definition [6]. Although nanopore sequencing was not able to reliably detect variation in the poly

A string, the regions adjacent to the strings were sequenced accurately in this study, including the detection of variants such as 2966A>G, which are still ambiguous in many star (*) alleles.

Sample barcoding permits efficient use of the flow cell and reduction in per sample assay costs. For high throughput laboratories or for research studies on larger cohorts, sample number could potentially be multiplexed up to 96 using the ONT barcode kit. Our 24-plex sequencing run resulted in a minimum read depth of ~500x, which is more than adequate for accurate variant calling and phasing, while some samples had tens of thousands of reads. The lowest read depth in our sample cohort was ~200x, and we observed no difference in accuracy compared to samples with higher read depth. Using nanopore sequencing for a long amplicon from the *GBA* gene, Leija-Salazar et al reported that coverage >100x is sufficient for accurate variant calling [32].

The main advantage of long read sequencing is enabling straightforward variant phasing. In this study, all variants could be phased accurately (provided more than one heterozygote variant is present), resulting in highly confident haplotyping to the subvariant level. In addition, duplicated alleles when present, were also identified. Using the 8-kb PCR amplicon derived from duplicated alleles as a confirmation sequence, we showed that duplicated alleles could be detected from the 6kb *CYP2D6* amplicon by the skewed allelic balance of reads. The allele ratio could be observed from the VCF file or by visualizing the phased BAM file on IGV. All our duplication samples have an allele ratio of ~2:1, indicating two copies of the duplicated gene. Due to limitation of the samples used in this cohort, it remains inconclusive whether this method could determine the exact number of gene copies accurately in cases with more than two copies.

Other challenges remain. The whole gene deletion allele (*5) still requires detection by a separate duplex PCR, and despite the accurate determination of haplotypes and duplication, the methods described here have not been extended to the detection of other CYP2D6 structural variations such as the CYP2D6-CYP2D7 hybrids or tandem rearrangements.

Conclusion

In summary, nanopore sequencing of the *CYP2D6* gene allows accurate detection and phasing of variants and determination of sample haplotypes to the subvariant level. New variants not yet catalogued on the PharmVar CYP2D6 page (rs1080992, rs376217512, rs143170489, rs61736504, rs557722765) were also reported in several samples, and key indel variants could be reliably detected. In total this study contributed to one novel allele (*127) and seven novel subvariants of known alleles (*1.025, *1.026, *4.026, *10.004, *17.003, *41.004, *71.003). The ability of nanopore sequencing to detect duplicated alleles when present provides better

prediction of CYP2D6 phenotype. In addition, sample barcoding permits sequencing and analysis of multiple samples simultaneously, improving cost and time efficiency.

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Table 1. Metrics of first and second CYP2D6 sequencing runs

No.	Parameters	1st run	2nd run
1.	Total yield	3.24M reads (3.63 Gbases)	748K reads (3.62 Gbases)
2.	Read length	N50: 2955 bps Median: 307 bps	N50: 6741 bps Median: 6437 bps
3.	Number of barcodes	14	26
4.	Number of barcoded reads	690K reads (47.6% of total pass reads)	242K reads (46% of total pass reads)
6.	Number of barcoded reads with length > 6kb	57K reads (3.96% of total pass reads)	186K reads (35.4% of total pass reads)
7.	Number of reads per barcode (length > 6 kb and qscore > 7)	235-11832	555-21853
8.	Mean qscore[a]	6.1	8.1
9.	Error rates (subs – del – ins)[b]	6.53% - 4.56% - 5.13%	4.99% - 3.97% - 3.78%

[a] Mean qscores were obtained from MinION QC
[b] Error rates were obtained from NanoOK

Table 2. Comparison between mapping and variant calling tools

Mapping tool	Variant Calling Tool	TP Variants (median[range])	FN Variants (median[range])	FP Variants (median[range])	PPV (%)	FDR (%)	Sensitivity (%)
minimap2	nanopolish	27 [1-32]	1 [0-4]	6 [5-7]	79.12	20.87	96.43
minimap2	Clairvoyante	23 [1-32]	2 [0-5]	17 [10-19]	57.50	42.50	92.0
NGMLR	nanopolish	25 [1-30]	3 [0-6]	11 [6-13]	66.52	33.48	89.29
NGMLR	Clairvoyante	26 [1-30]	2 [0-4]	19 [12-21]	54.55	45.45	92.86

Abbreviation: TP: True Positive, FN: False Negative, FP: False Positive, PPV: Positive Predictive Value, FDR: False Discovery Rate. PPV, FDR, and Sensitivity were calculated based on median values.

Table 3 Genotyping Results

No.	Sample	Sequencing run	Reference Genotype (Get-RM or Sanger)	Allele	Genotype (GridION)	No. of Variants		
						subs	del	ins
Reference Samples								
1	NA07439	run 1	*4XN/*41	hap1	*4.004XN	15	0	0
				hap2	*41.003	23[a]	1	0
		run 2	*4XN/*41	hap1	*4.004XN	14[b]	0	0
				hap2	*41.003	22[a,b]	1	0
2	NA23348	run 1	*7/*35	hap1	*35.001	23[b]	1	0
				hap2	*7.001	1	0	0
		run 2	*7/*35	hap1	*7.001	1	0	0
				hap2	*35.001	23[b]	1	0
3	NA18518	run 1	*17/*29	hap1	*17.003#	20[b]	0	0
				hap2	*29.001	17[c]	1	0
4	NA21781	run 1	*2/*4(XN)	hap1	*2.001XN	22[b]	1	0
				hap2	*4.001	14[b,d]	0	0
5	NA12878	run 1	*3/*4	hap1	*3.001	0	1	0
				hap2	*4.001	17[b]	0	0
6	NA23405	run 1	*1/*7	hap1	*7.001	1	0	0
				hap2	*1.001	0	0	0
7	NA23297	run 1	*10(XN) / *17	hap1	*17.003#	20[b]	0	0
				hap2	*10.002XN	13[b]	0	0
Clinical Samples								
8	S001	run 1	*1/*1	hap1	*1.001	0	0	0
				hap2	*1.025#	1	0	0
		run 2	*1/*1	hap 1	*1.025#	1	0	0
				hap 2	*1.001	0	0	0
9	S002	run 1	*6/*4	hap1	*6.005	2	1	0
				hap2	*4.001	17[b]	0	0
		run 2	*6/*4	hap 1	*6.005	2	1	0
				hap 2	*4.001	17[b]	0	0
10	S003	run 1	*1/*3	hap1	*127.001#	9	0	1
				hap2	*3.001	0	1	0
11	S004	run 1	*4/*9	hap1	*4.001	17[b]	0	0
				hap2	*9.001	0	1	0
12	S005	run 1	*2/*10	hap1	*2.001	22[b]	1	0
				hap2	*10.002	13[b]	0	0
13	S006	run 2	*2/*9	hap 1	*2.001	22[b]	1	0
				hap 2	*9.001	0	1	0
14	S007	run 2	*71/*41	hap 1	*71.003#	2	0	0
				hap 2	*41.001	22[b]	1	0

15	S008	run 2	*15/*2	hap 1	*15.001	1	0	1
				hap 2	*2.001	22[b]	1	0
16	S009	run 2	*1/*6	hap 1	*6.005	2	1	0
				hap 2	*1.025[#]	1	0	0
17	S010	run 2	*4/*10	hap 1	*10.002	13[b]	0	0
				hap 2	*4.015	18[b]	0	0
18	S011	run 2	*2/*2	hap 1	*2.001	22[b]	1	0
				hap 2	*2.001	22[b]	1	0
19	S012	run 2	*4/*4	hap 1	*4.001	17[b]	0	0
				hap 2	*4.001	17[b]	0	0
20	S013	run 2	*2/*41	hap 1	*41.004[#]	23	1	0
				hap 2	*2.001	23	1	0
21	S014	run 2	*4/*28	hap 1	*28.001	25	1	0
				hap 2	*4.015	19	0	0
22	S015	run 2	*4/*35	hap 1	*4.001	18	0	0
				hap 2	*35.001	24	1	0
23	S016	run 2	*10/*1	hap1	*1.026[#]	1	0	0
				hap2	*10.003[#]	14	0	0
24	S017	run 2	*35/*41	hap1	*41.001	22[b]	1	0
				hap2	*35.001	23[b]	1	0
25	S018	run 2	*1/*1	hap1	*1.016XN	1[b]	0	0
				hap2	*1.012	2	0	0
26	S019	run 2	*1/*1	hap1	*1.012	1	0	0
				hap2	*1.001	0	0	0
27	S020	run 2	*1/*1	hap1	*1.025[#]	1	0	0
				hap2	*1.001	0	0	0
28	S021	run 2	*1/*1	hap1	*1.025[#]	1	0	0
				hap2	*1.001	0	0	0
29	S022	run 2	*5/*33	hap1	*5.001			
				hap2	*33.001	1	0	0
30	S023	run 2	*4/*4	hap1	*4.001	17[b]	0	0
				hap2	*4.026[#]	18[b]	0	0
31	S024	run 2	*3/*4	hap1	*3.001	0	1	0
				hap2	*4.001	17[b]	0	0
32	S025	run 2	*4/*41	hap1	*41.001	22[b]	1	0
				hap2	*4.001	16[e]	0	0

New allele/subvariant

a. Variant 7189G>A was not called in the VCF file but visually confirmed on BAM file.

b. Variant 5042T>G was incorrectly called as delT

c. Variant 6416A>G was not called in the VCF file but visually confirmed on BAM file.

d. Variants 2775C>T, 3201G>A and 4300C>T were not called in the VCF file but visually confirmed on BAM file.

e. Variant 2775C>T was not called in the VCF file but visually confirmed on BAM file.

Table 4. Variants in duplicated allele in 6.6kb and 8kb fragments.

No.	Sample	Variants found in duplicated allele in 6.6 kb amplicon (from position 3860 – 8593)	Variants found in 8.6 kb amplicon (from position 3860 – 8593)
1.	NA07439 (*4XN) (run 1)	4300C>T, 4510G>T, 5042T>G, 5238C>T, 5862G>C, 6047G>A, 6298A>G, 7585A>C, 7783A>G, 8381G>C, 8602C>T, 8923T>G	4300C>T, 4510G>T, 5238C>T, 5862G>C, 6047G>A, 6298A>G, 7585A>C, 7783A>G, 8381G>C, 8602C>T, 8923T>G *
2.	NA23297 (*10XN)	4300C>T, 4510G>T, 5238C>T, 5862G>C, 6298A>G, 7585A>C, 7783A>G, 8381G>C, 8602C>T, 8923T>G*	4300C>T, 4510G>T, 5238C>T, 5862G>C, 6298A>G, 7585A>C, 7783A>G, 8381G>C, 8602C>T, 8923T>G*
3.	NA07439 (*4XN) (run 2)	4300C>T, 4510G>T, 5238C>T, 5862G>C, 6047G>A, 6298A>G, 7585A>C, 7783A>G, 8381G>C, 8602C>T, 8923T>G *	4300C>T, 4510G>T, 5238C>T, 5862G>C, 6047G>A, 6298A>G, 7585A>C, 7783A>G, 8381G>C, 8602C>T, 8923T>G
4.	S018 (*1XN)	5369G>A*	5369G>A*

*5042T>G missed by the variant calling tool

Table 5 Primer sequences

No.	Primer name	Sequence (5'-3')	Reference
Primers for long PCR			
1.	6.6kbF	tttctgttggtgctgatattgcATG GCA GCT GCC ATA CAA TCC ACC TG	[18]
2.	6.6kbR	acttgcctgtcgctctatcttcCGA CTG AGC CCT GGG AGG TAG GTA G	[18]
3.	FragD_8.6kbF	tttctgttggtgctgatattgcCCAGAAGGCTTTGCAGGCTTCAG	[18]
4.	FragD_8.6kbR	acttgcctgtcgctctatcttcCGGCAGTGGTCAGCTAATGAC	[18]
5.	CYP-13	ACC GGG CAC CTG TAC TCC TCA	[19]
6.	CYP-24	GCA TGA GCT AAG GCA CCC AGA C	[19]
7.	FragB_Dup_F	CCA TGG AAG CCC AGG ACT GAG C	[18]
8.	FragB_Dup_R	CGG CAG TGG TCA GCT AAT GAC	[18]
Primers for Sanger Sequencing			
1.	2D6_5UTRF	GCAGCTGCCATACAATCCAC	
2.	2D6_5UTRF	TGCTTTCTGGCCTCCATGTT	
3.	2D6Prom_R	TCCTCCATAACGTTCCCACCAGAT	[39]
4.	2D6Prom_F	CCATACCTGCCTCACTACCAAATG	[39]
5.	2D6Ex1F	TCTGGAGCAGCCCATACCCG	[39]
6.	2D6Ex1SR	CCCCAGACTACAGGTCCTAGTCCTATTTG	[12]
7.	2D6Ex2F	TCCTCCTTCCACCTGCTCAC	[39]
8.	2D6Ex2SR	CTTTGCCCCACCTCGTCTCT	[39]
9.	2D6Ex34F	AGCTGGAATCCGGTGTCGAA	[39]
10.	2D6Ex34SR	AGCCATCTCCAGGTAGACCCAG	[39]
11.	2D6Ex56F	ACAGGCAGGCCCTGGGTCTA	[12]
12.	2D6Ex56SR	CCTGGTCACCCATCTCTGGTC	[39]
13.	2D6Ex7F	CCAACATAGGAGGCAAGAAG	[39]
14.	2D6Ex7R	ACTGGACTCTAGGATGCTGG	[39]
15.	2D6Ex8F	CAGAATGTTGGAGGACCCAA	[39]
16.	2D6Ex8R	AGGAAAGCAAAGACACCATG	[39]
17.	2D6Ex9F	TGAAGGATGAGGCCGTCTGG	[39]
18.	2D6-R	ACTGAGCCCTGGGAGGTAGGTAG	[39]

Table 6. Sequence variants to detect star (*) alleles in Sanger Sequencing

Star (*) Alleles	**Variants**	
	RSID	Position based on NG_008376.3 (1=sequence start)
***2**	rs16947	7051C>T
***3**	rs35742686	6750delA
***4**	rs1065852, rs3892097	4300C>T, 6047G>A
***6**	rs5030655	5098delT
***7**	rs5030867	7136A>C
***9**	rs5030656	6816delAAG
***10**	rs1065852	4300C>T
***15**	rs774671100, rs1081004	4337insT, 5369G>A
***17**	rs28371706	5222C>A/T
***28**	rs72549358, rs78482768	4219G>A, 5905C>G
***29**	rs61736512, rs59421388	5860G>A, 7384G>A
***33**	rs28371717	6684G>T
***35**	rs769258	4231G>A
***41**	rs28371725	7189G>A
***71**	rs118203758	4325G>A

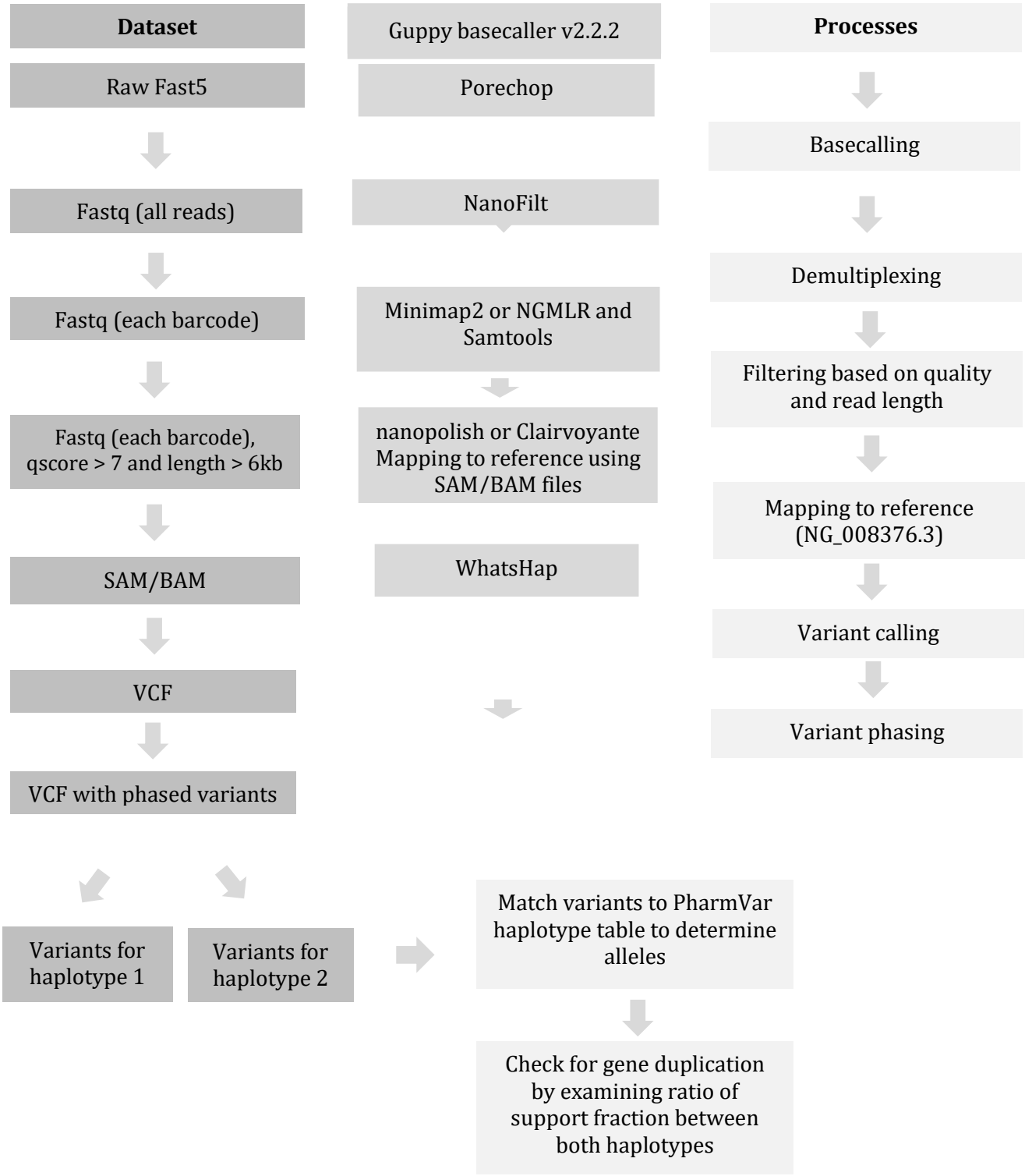


Figure 1. Bioinformatics workflow for *CYP2D6* nanopore sequencing

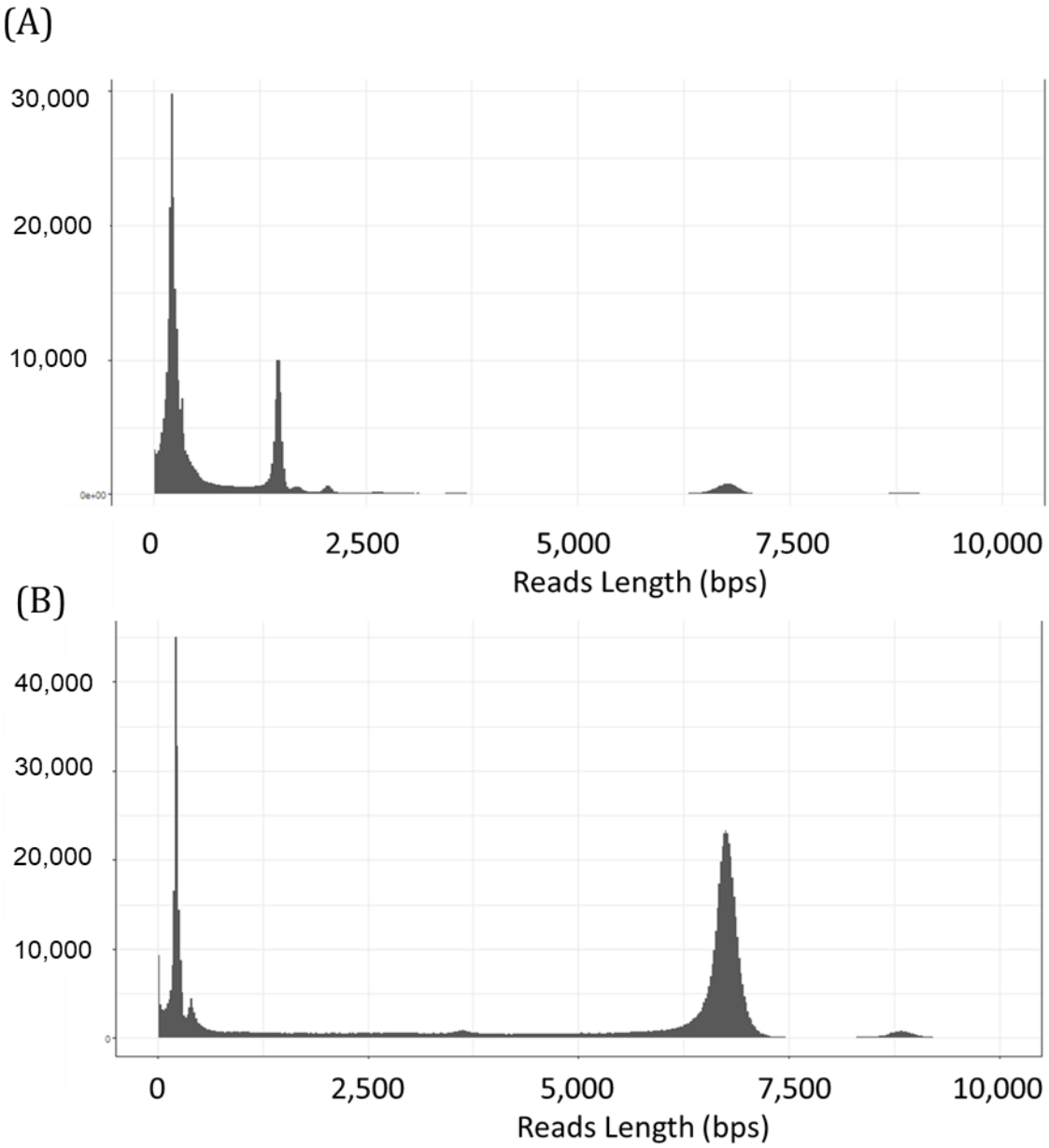
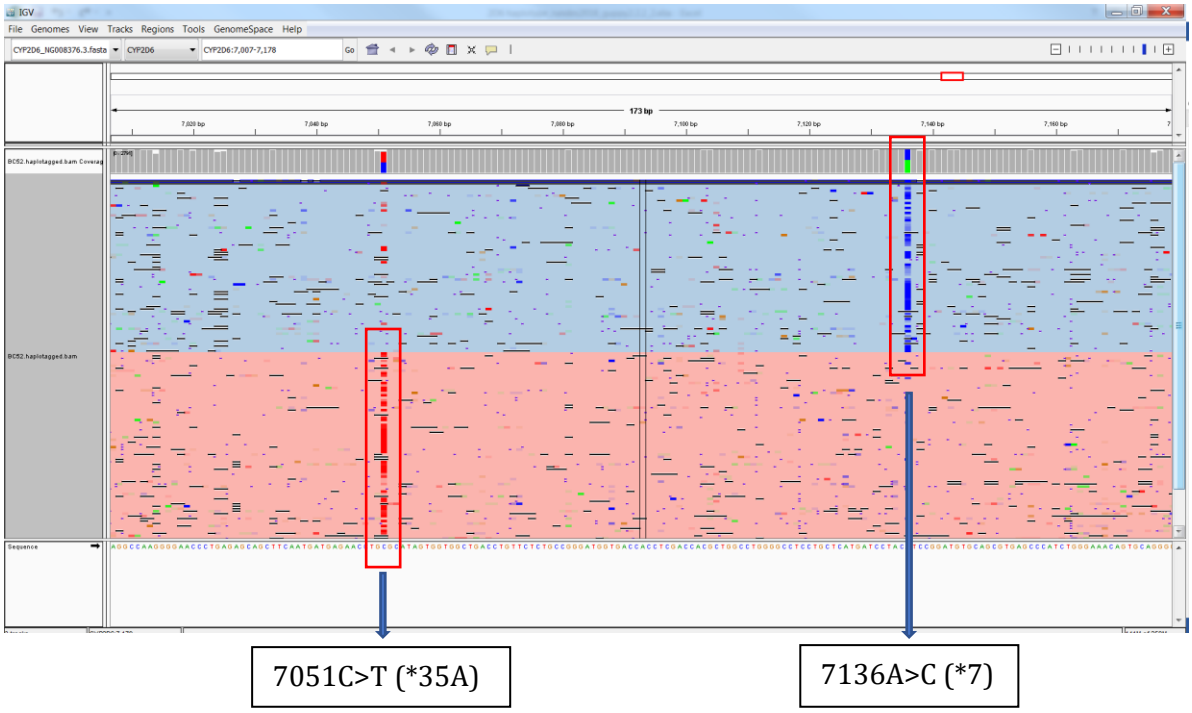


Figure 2. Read Length distribution of (A) the first run, and (B) the second run of *CYP2D6* sequencing

(A)



(B)

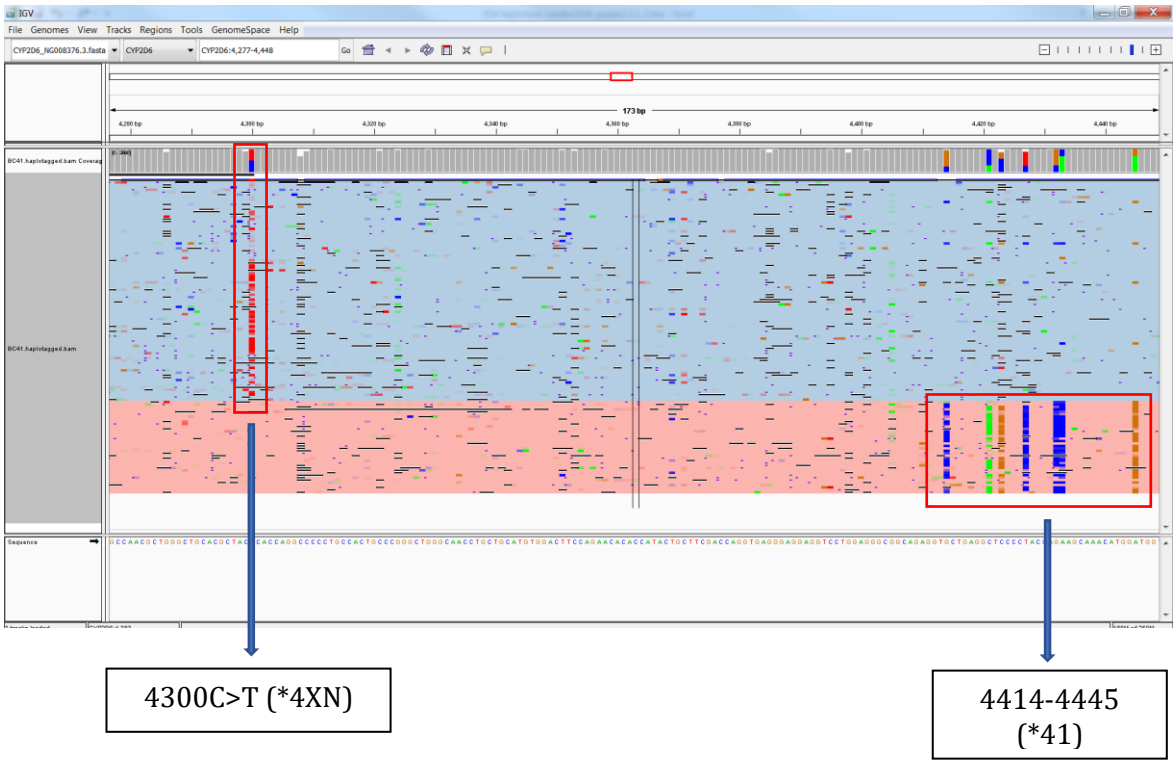


Figure 3. Phased variants for (A) sample with no duplicated allele (NA23348, *7/*35A) and (B) sample with duplicated allele (NA07439, *4XN/*41). Colours represent each of the phased alleles of the samples.

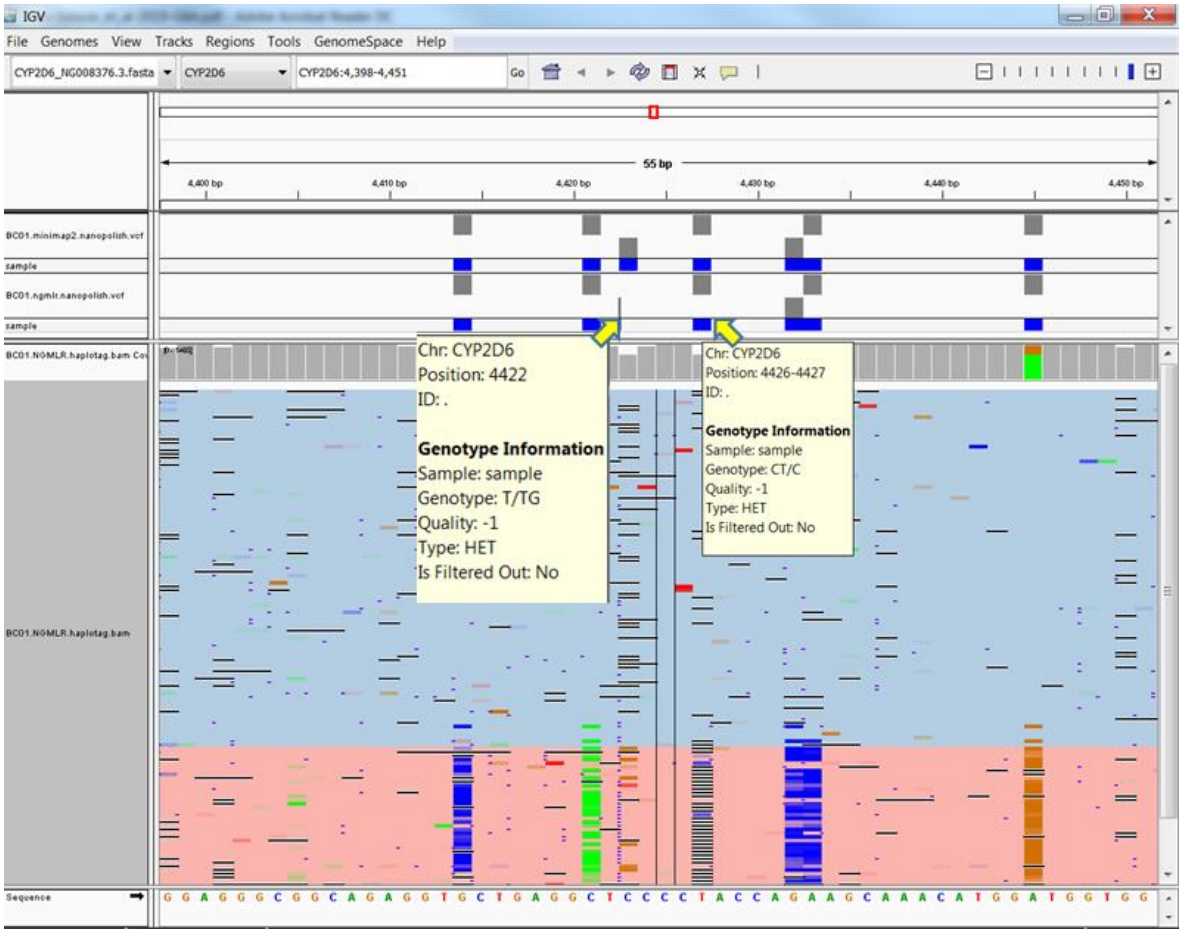


Figure 4. Misdetection of two out of the seven variants of intron 1 CYP2D7 conversion due to misalignment by NGMLR. The two variants were called incorrectly as insertion and deletion (4423insG and 4427delT) instead of the two variants of 4423C>G and 4427T>C.

Supplementary File 1

Protocol for DNA extraction from blood samples

For each patient sample, 4 mL of peripheral blood was mixed with 6 mL of red blood cell lysis buffer (1 mM EDTA) and spun at 2000 rcf for 10 minutes at room temperature. The supernatant was carefully discarded and the pellet resuspended and the initial step repeated until a layer of white buffy cells is clearly visible. The supernatant was then discarded and 1 mL of 1 M NaCl solution added. This solution was then repeatedly vortexed to eliminate any clumping. Once the solution was clear of clumps, 6 mL of white cell lysis solution (10 mM Tris HCl, 26 mM EDTA, 17.3 mM (0.5%) SDS) and 50 μL of RNase A solution was added and incubated at 37°C for at least 1 hour.

After incubation 1mL of 3M sodium acetate solution was added and mixed. The resulting solution was then added to a 15 mL tube containing 2.5 gr of high vacuum grease (Dow Corning Corporation, USA) and then 1 ml of phenol:chloroform:isoamyl alcohol 25:24:1 was added. The tubes were capped and thoroughly mixed before centrifuging for 5 minutes at 1500 rcf. Five mL of the resultant supernatant was then transferred to a new tube containing 5mL of 100% isopropanol. The tubes were gently inverted until DNA precipitates. The tubes containing DNA were then centrifuged at 2000 rcf for 5 minutes to form a DNA pellet at the bottom of the tube. The isopropanol was carefully discarded and then washed with 10 mL of 70% ethanol (10 mL) and centrifuged at 2000 rcf for 3 minutes. After discarding the ethanol, the DNA pellet was dissolved in 500 mL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) at 37°C for at least 8 hours. The DNA solution was quantified using a spectrophotometer at 260 nm and diluted to 50 ng/mL.

EDTA: Ethylenediaminetetraacetic acid

TRIS HCL: tris(hydroxymethyl)aminomethane hydrochloric acid

SDS: Sodium dodecyl sulfate

TE buffer: Tris-EDTA buffer

NaCl: Sodium chloride