Introduction of new alternative pipeline using multiplexed fast COLD-PCR together with sequencing approach highlighting pharmacoeconomics by detection of *CYP* variants

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Abstract. In precision medicine, multiple factors are involved in clinical decision-making because of ethnic and racial genetic diversity, family history and other health factors. Although advanced techniques have evolved, there is still an economic obstacle to pharmacogenetic (PGx) implementation in developing countries. The aim of the present study was to provide an alternative pipeline that roughly estimate patient carrier type and prescreen out wild-type samples before sequencing or genotyping to determine genetic status. Fast co-amplification at lower denaturation temperature (COLD)-PCR was used to differentiate genetic variant non-carriers from carriers. The majority of drugs are hepatically cleared by cytochrome P450 (CYP) enzymes and genes encoding CYP enzymes are highly variable. Of all the *CYPs*, CYP2 family of *CYP2C9*, *CYP2C19*, and *CYP2D6* isoforms have clinically significant impact on drugs of PGx testing. Therefore, five variants associated with these *CYPs* were selected for preliminary testing with this novel pipeline. For fast COLD-PCR, the optimal annealing temperature and critical denaturation temperature were determined and evaluated via Sanger sequencing of 27 randomly collected samples. According to precise Tc, to perform in a single-reaction is difficult. However, in this study, this issue was resolved by combination of precise Tc using 10+10+20 cycles. The results showed 100% sensitivity and specificity, with perfect agreement (κ=1.0) compared with Sanger sequencing. The present study provides a prescreening platform by introducing multiplex fast COLD-PCR as a pharmacoeconomic implementation. Our study just present in five variants which are not enough to describe patient metabolic status. Therefore, other actional genetic variants are still needed to cover the actual patient's genotypes. Nevertheless, the proposed method can well-present its efficiency and reliability for serving as a PGx budget platform in the future.

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Abbreviations: CYP, cytochrome P450; COLD-PCR, co-amplification at lower denaturation temperature polymerase chain reaction; IM, intermediate metabolizer; PM, poor metabolizer; UM, ultra-rapid metabolizer; Ta, annealing temperature; Tm, melting temperature; Tc, critical denaturation temperature; BLAST, Basic Local Alignment Search Tool; SNP, single nucleotide polymorphism; WT, wild-type; MT, mutant; bp, base pair

Key words: pharmacogenetics, cytochrome P450, fast co-amplification at lower denaturation temperature, genetic variant, sequencing

Introduction

Drug-metabolizing cytochrome P450 (CYP) phase I bioactivation system affects drug responses. Among >50 CYPs, genetic variations of CYP2C9, CYP2C19 and CYP2D6 enzymes potentially affect drug efficacy and toxicity. *CYP2C9*, *CYP2C19* and *CYP2D6* polymorphisms comprise the most frequent enzyme variations because nearly 80% of drugs used in today are metabolized by these enzymes. Accordingly, these *CYP* genetic mutations lead to different phenotypes of metabolism status, such as ultra-rapid (UMs), normal, intermediate (IMs) and poor metabolizers (PMs). In UMs, individuals metabolize drugs very rapidly, resulting in lack of response and subtherapeutic plasma concentrations at normal doses whereas in IMs or PMs, these lead to altered risk for adverse drug reactions (1-3). For example, altered CYP2D6 activity affects antidepressant treatment (4) and *CYP2C19*17* leading to UM phenotype causes risk of therapeutic failure in drug treatment (5). Therefore, annotations of these *CYP* genes and pharmacogenomics (PGx)-based drug-dosing guidelines are being constantly updated to make dose adjustment to avoid toxicity and increase drug efficacy (6,7). This may

maximize drug efficacy and minimize toxicity for individuals from drugs, thereby improving patient compliance and safety. Thus, PGx testing has been implemented in these three *CYP* polymorphisms (*CYP2C9*, *CYP2C19* and *CYP2D6*) to achieve optimal quality use of medicines (8). Various approaches have been conducted, such as allele-specific PCR, invader assay, pyrosequencing and oligonucleotide microarray (9). A variety of testing kits for *CYP450* genotyping have also been approved by the U.S. FDA, including Amplicon Chip CYP450 GeneChip®, TaqMan real-time PCR and Luminex *CYP2D6* and *CY2C19* xTAG detection kits (10).

Nevertheless, use of PGx testing as a routine practice is still challenging due to an underestimation of clinical importance, lack of health information and high cost in developing countries (11). Even though physicians are educated on healthcare, optimistic attitudes to PGx are still demanding because of lack of participation in controlled trials and clinical validity. One example of variations between pharmacogenetic clinical guidelines and recommendations was found in clopidogrel in which clinical guidelines and FDA demonstrated different recommendations upon the interpretation of PGx testing (12). The key role of PGx is to divide drug responders from non-responders for physicians.

The aim of this study was to qualitatively evaluate pharmacoeconomic characteristics. The objective of this study was to introduce an alternative pipeline to detect mutation before traditional genotyping for PGx. Co-amplification at lower denaturation temperature (COLD)-PCR technology is considered to be a better qualitative detection method in minority allele detection than conventional PCR because of its feasibility, simplicity, time-efficiency and cost-effectiveness with preferential denaturation on mismatch-forming variants (13). There are several forms of COLD-PCR, among them, fast COLD-PCR is cheapest and easiest. Fast COLD-PCR is a modified form of conventional PCR involving an additional parameter, the critical denaturation temperature (Tc), primarily suitable for Tm-reducing mutations (for example, G:C>A:T or G:C>T:A) (14). Following this selective denaturation, only mutant (MT) A/T-containing alleles are obtained and wild-type (WT) G/C alleles are left double-stranded (ds). This can result in increased sensitivity in the detection of low-abundance variants over conventional PCR (15). It not only enables robust enrichment but is also easily accessible with high reproducibility. As a consequence of this, COLD-PCR has been widely applied to detect cancer mutations (16-23).

To date, there are only a few reports of pharmacogenomics (PGx) studies (2,7,12) in which fast COLD-PCR has not yet been applied. Therefore, the present study introduce an affordable methodology to determine whether the patient carries a variant without requiring heterozygous or homozygous variant typing. Accordingly, this can decrease unnecessary expensive direct genotyping in uncharacterized patients. Moreover, the present study aimed to demonstrate how to multiplex fast COLD-PCR based on precise Tc values, which has previously been difficult because the critical denaturation temperature of COLD-PCR must be controlled precisely (within ±0.2˚C). Therefore, it is critical to set up a thermocycler with precise temperature. The novel assay panel used selected gene-associated single nucleotide polymorphisms (SNPs) specific to Asian populations published in previous studies (24-28). The present method may promote use of more pharmacogenetic-associated SNPs. To assess the efficiency, the testing results were compared with those from Sanger sequencing, which is the reliable available gold-standard method (29) to determine heterozygous or homozygous patients.

Materials and methods

Database for searching for sequences of CYP genes. The reference sequences of *CYP2C9* and *CYP2C19* on chromosome 10 (accession no. NC_000010.11) and *CYP2D6* on chromosome 22 (accession no NC_000022.11) from Homo sapiens genome assembly, GHCh38.p13, were downloaded from the National Center for Biotechnology Information (ncbi.nlm.nih.gov, accession date 22 June 2022) to perform target gene analysis. Genetic polymorphisms of cytochrome related pharmacogenomic studies focusing on Asian populations were selected (24-28). Respective reference SNP (rs) numbers were obtained from the Human *CYP* Allele Nomenclature Database and shown in Table I (pharmvar.org/htdocs/archive/index_original.htm, accession date 22 June 2022). SNPs on reference sequences of these genes were mapped on the reference chromosome sequences and identified using NCBI BLAST tool (blast.ncbi.nlm.nih.gov/Blast.cgi).

Selection of SNPs, new primers and synthetic oligonucleotide designs. The novel primers were designed to develop a gene panel focusing on *CYP2C9*, *CYP2C19*, and *CYP2D6* variants. Homology to function and evolution with other gene families were assessed using Basic Local Alignment Search Tool (BLAST) against the GHCh38.p13 assembly (ncbi.nlm.nih.gov/tools/primer-blast/, 22 June 2022). DNA melting temperature (Tm) of WT and MT variants was predicted using the web-based tool uMelt version 3.6.2, developed by the Wittwer lab (dna-utah.org/, 22 June 2022). Synthetic ds DNA fragments (gBlocks®, Integrated DNA Technologies) of 250-500 bp for each variant were used for quality control, as previously described (30). Position of synthetic ds DNA and primer sequences are shown in Table I.

Ethical considerations. All participants were recruited from unrelated Thai volunteers with the following inclusion criteria: i) Age 18-60 years old, ii) no history of drug ADRs and SCARs and iii) have history of drug ADRs or SCARs (but at the time of recruiting participants, have no symptom of ADRs). A total of 27 volunteers including 13 males and 14 females was recruited between January to March 2022 at Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, Thailand. All participants provided written consent to participate in the study before collecting blood samples from vein. The study was approved by Research Ethics Committee, Faculty of Associated Medical Sciences, Chiang Mai University, Thailand (approval no. AMSEC-64EX-130; date of approval: 28 December 2021).

DNA extraction. A total of 6 ml of blood samples were collected from vein and stored in EDTA tube and DNA extraction was performed using the PureLink™ Genomic DNA mini kit (Invitrogen; Thermo Fisher Scientific, Inc.), following the manufacturer's instructions. At least 200 μl of buffy coat from

Table I. Gene panel selection of *CYP2C9*, *CYP2C19* and *CYP2D6* variants.

Allele	Mutation	Rs number	Position of synthetic oligonucleotide sequence (length)	Primer sequence, 5'→3'
*CYP2C9*2*	430C>T	rs1799853	94942072 *G*AA---------CGT---------- TT*C* 94942561 (490 bp)	F: GAAATGGAAGGAGATCCGGC R: GATATGGAGTAGGGTCACCC
*CYP2C19*2*	681G>A	rs4244285	94781615 *C*AT---------GGG--------- GA*C* 94782055 (441 bp)	F: CGCCAACCAGAGCTTGGCAT R: CGGGCCATCGATTCTTGGTG
*CYP2C19*3*	636G>A	rs4986893	94780502 *C*AC---------GAT---------- TG*C* 94780955 (454 bp)	F: GGCCGCCAGAAACGTTTCGA R: CGGTACTTCAGGGCTTGGTC
*CYP2D6*10*	100C>T	rs1065852	42130444 *C*AG--------GGT--------- CG*C* 42130890 (447 bp)	F: GGAAGTCCACATGCAGCAGG R: GCAGGTATGGGGCTAGAAGC
*CYP2D6*41*	2988G>A	rs28371725	42127601 *C*CT---------CCT---------- GT*C* 42128090 (490 bp)	F: GGTCAAGCCTGTGCTTGGAG R: CCTACATCCGGATGTGCAGC

Italics indicate start and end synthetic sequence position. Underline indicates location of single nucleotide polymorphisms. F, forward; R, reverse; CYP, cytochrome 450; rs, reference SNPs.

EDTA blood was used to a final elution volume of 100 μl extracted DNA. The quality of extracted DNA was determined using an Epoch Microplate Spectrophotometer (BioTek Instruments, Inc.) with a 260/280 absorbance ratio of 1.65-1.80. The DNA concentration was normalized to 50 ng/μl.

Optimization of annealing temperature (Ta) and critical denaturation temperature (Tc) of fast COLD-PCR. Optimization of Ta was performed by conventional PCR for primer annealing to a target sequence. A total of 50 ng genomic DNA template was used in a total reaction volume of 12.5 μl. PCR reaction was performed using 1X Quick Taq™ HS Dye Mix (Toyobo Life Science) with 0.2 μM all primers (Table I) according to the manufacturer's instructions. The conditions for PCR cycling were 94˚C for 2 min followed by 30 cycles at 94˚C for 30 sec, 50-65˚C with gradient PCR for 30 sec, 68˚C for 1 min/kb and 68˚C for 7 min.

Selective denaturation stage (Tc) is vital for fast COLD-PCR to precisely denature the mutated sequence (14,23). First, 10 rounds of conventional PCR were performed to amplify and generate a sufficient template for COLD-PCR. Afterwards, the precise Tc was determined by gradual reduction of the denaturation temperature (Tm). The amplified PCR products were analyzed by 2% agarose gel electrophoresis in 10x Tris-Borate-EDTA (TBE) buffer for 40 min. As a result, only MT PCR products were observed in comparison with WT and MT synthetic DNA templates. The reaction mixture and total volume of fast COLD-PCR were the same as those of conventional PCR. A total of 10 cycles of conventional PCR and fast COLD-PCR conditions were optimized as follows: 30 cycles of precise Tc (gradually decreasing Tm until only MT DNA was enriched) for 30 sec, 65˚C for 30 sec, 68˚C for 12 sec and final extension of 68˚C for 7 min.

Tc combination of fast COLD-PCR assay evaluation. To multiplex *CYP2C9*, *CYP2C19* and *CYP2D6*, combined-Tc fast COLD-PCR was performed, starting from the lowest to highest Tc by sequentially adding 10+10+20 cycles. The optimization condition was the as the precise Tc determination. An initial denaturation of 94˚C for 2 min was followed by 10 cycles of conventional PCR, as aforementioned. Next, 10 cycles of Tc1 (75.0˚C) for 30 sec with annealing and extension steps were performed as aforementioned. Another 10 cycles at Tc2 (87.0˚C) for 30 sec, followed by 20 cycles of Tc3 (90.5˚C) for 30 sec with annealing and extension were performed, as shown in Fig. 1. Afterwards, the resulting assay was tested and evaluated on 27 samples in comparison with Sanger sequencing.

Sanger sequencing. After screening 27 samples for all variants with fast COLD-PCR, the resulting positive MT samples were determined by traditional Sanger sequencing to determine homozygous or heterozygous status. To evaluate the efficiency of fast COLD-PCR screening, all 27 samples were subjected to Sanger sequencing. To perform Sanger sequencing, DNA samples were amplified using the aforementioned conventional PCR. The obtained PCR amplicons were sequenced by the Sanger reference method at Macrogen, Inc. (31). Sanger sequence assemblies were analyzed using SeqMan Ultra DNASTAR Bioinformatics Software version 17.2 (dnastar.com/software/lasergene/seqman-ultra/, 22 June 2022).

Statistical analysis. As data of COLD-PCR and Sanger sequencing were examined each clinical sample for variants to determine whether they will be found to match. Data will be converted from category data into quantitative data (data 0-1 means negative-positive). The Cohen's Kappa (κ) agreement between two assays was calculated using SPSS software v. 22.0. Moreover, we have also calculated the allelic frequency of five variants found in this study.

Results

Determination of Ta and Tc for initial fast COLD-PCR screening test. Total five variants were selected from *CYP2C9*, *CYP2C19*, and *CYP2D6* genes and these five variants were optimized at the Ta, 50-65˚C. Following this, 65˚C was selected as the optimal Ta as all five genetic variants were shown the same annealing reaction at 65˚C. No amplification in WT and a

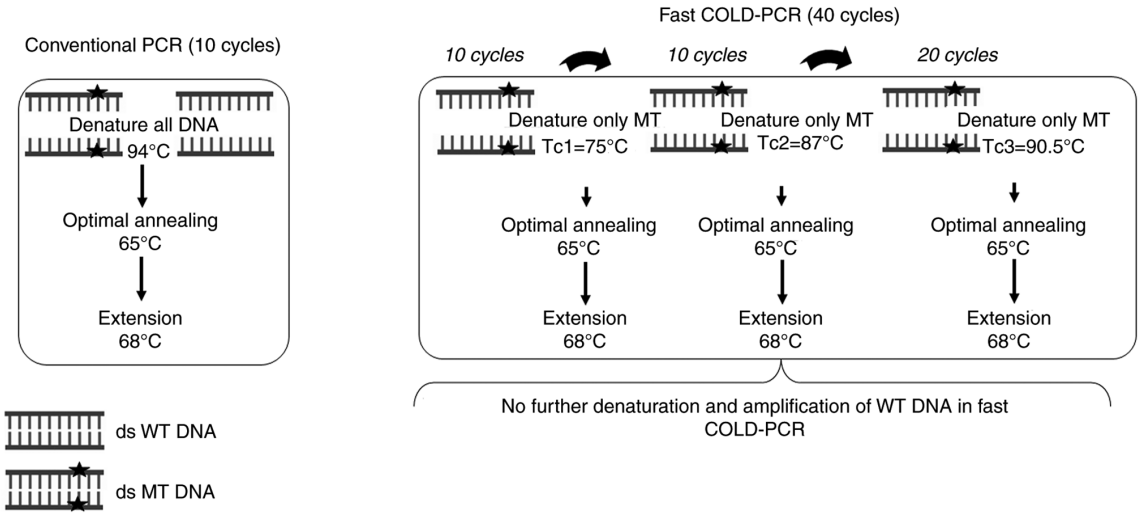

Figure 1. Overall workflow of fast COLD-PCR. Ten cycles of regular PCR were performed for the target amplicons. Subsequently, fast COLD-PCR was applied as follows: Tc1=75.0˚C (10 cycles), Tc2=87.0˚C (10 cycles) and Tc3=90.5˚C (20 cycles). The final products of five variants were successfully enriched by preferential amplification using multiplexed fast COLD-PCR. The bold text is optimal Ta at 65˚C and stars indicate the SNP position on the mutant sequence. COLD, co-amplification at lower denaturation temperature; WT, wild-type; MT, mutant; ds, double-stranded.

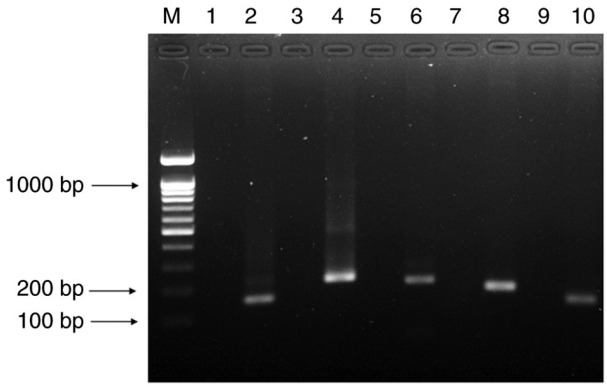

Figure 2. Precise Tc validation of five variants obtained by fast COLD-PCR. Lane M, DNA ladder 100 bp; lanes 1 and 2, synthetic WT and MT of *CYP2C9*2* (150 bp); lanes 3 and 4, synthetic WT and MT of *CYP2C19*2* (206 bp); lanes 5 and 6, synthetic WT and MT of *CYP2C19*3* (192 bp); lanes 7 and 8, synthetic WT and MT of *CYP2D6*10* (160 bp) and lanes 9 and 10, synthetic WT and MT of *CYP2D6*41* (124 bp). COLD, co-amplification at lower denaturation temperature; WT, wild-type; MT, mutant; CYP, cytochrome P450.

positive band in MT variants were obtained with three different Tc values for the five targets: Tc=75.0˚C for *CYP2C9*2* (150 bp), *19*2* (206 bp) and *19*3* (192 bp); Tc=87.0˚C for *CYP2D6*41* (124 bp) and Tc=90.5˚C for *CYP2D6*10* (160 bp). These Tc results were validated using synthetic WT and MT oligonucleotide templates at a concentration of 50 ng/µl with 10 cycles of conventional PCR at Ta=65˚C (Fig. 2). Accordingly, optimal Ta at 65˚C and three Tcs (75.0˚C, 87.0˚C and 90.5˚C) for fast COLD-PCR screening test were obtained.

Establishment and evaluation of combined fast COLD-PCR. The proposed assay was modified to discriminate all variants within a single reaction through multiplex fast COLD-PCR by combining three Tc values (75.0, 87.0 and 90.5˚C) with 10+10+20 cycles. A total of 27 randomly collected samples were tested by multiplex performance with control samples (Fig. 3). For the detection of *CYP2C9*2*, only one sample (sample 20) showed a positive band (Fig. 3A). For *CYP2C19*2*, positive bands are observed for 15 samples (samples 1, 2, 4, 5, 9, 10, 12-14, 16, 21-23, 25 and 27; Fig. 3B). For *CYP2C19*3*, a positive band is observed for one sample (sample 11; Fig. 3C). A total of 21 samples (samples 1, 2, 4, 5, 7-13, 15, 17, 19-25 and 27) was *CYP2D6*10*-positive (Fig. 3D). A total of four samples (samples 3, 10, 17 and 24) was *CYP2D6*41*-positive (Fig. 3E). In comparison of fast COLD-PCR with Sanger sequencing, the results show 100% consistency (κ=1.0) for all variants (Table II). The results of Sanger sequencing in five variants are shown in Fig. 4. Fast COLD-PCR correctly identified heterozygous or homozygous SNPs variant in Sanger results as 'Positive' and WT sample of Sanger sequencing results as 'Negative'. The percentage of five variants present in 27 samples as follows: 3.7% of *CYP2C9*2* and *CYP2C19*3*, 55.5% of *CYP2C19*2*, 77.7% of *CYP2D6*10*, and 14.8% of *CYP2D6*41*.

Discussion

Genetic DNA variations of *CYP* genes alter pharmacokinetics and responses to certain drugs. In The Human CYP Allele Nomenclature Database (32), CYP2 family is primarily involved in drug 'physiology', 'toxicology' and 'diverse regulatory mechanisms' (33). Of CYP2 family members, *CYP2D6* presents highly polymorphic and complex structural variations (34) and sequence similarities >90% are seen in *CYP2C9* and *CYP2C19* (35). In previous studies, *CYP* gene copy numbers (*CYP2D6*) have been determined by pyrosequencing (36), loop-mediated isothermal amplification, electrochemical DNA chip (37) and real-time PCR detection (38). Commercial kits and advanced genotyping techniques have also been developed for clinical implementation, including AmpliChip CYP450, TaqMan assays, Luminex xTAG, next-generation sequencing platforms and MassARRAY as systematic algorithms (39).

CYP genetic testing is used to monitor patients effectively for therapeutic indication. However, the greater challenge of PGx testing is economic (40). Therefore, the present aimed to introduce a new basic platform to be able to use before traditional genotyping methods in unknown patient samples. Although the present data cannot demonstrate the extent of genotype predictable phenotype, it may serve as the fundamental consideration whether patient has genetic variants. Using fast COLD-PCR as initial screening test and combination with other genotyping testing for positive results may provide a more affordable approach in precision medicine. Therefore, the present study aimed to evaluate fast COLD-PCR as prescreening strategy to monitor patient safety. In addition, fast COLD-PCR is also simple, easy, and cheap enough to be widely used in routine lab work.

The present study observed a positive MT band with no WT band on 2% agarose gel on the precise Tc of each SNP. Previously, different PCR protocols for each reaction were required for Tc per amplicon and multiplexing in fast COLD-PCR was problematic due to precise Tc which only denatures mutant sequence at its specific temperature. However, the present study combined multiplexed fast COLD-PCR using 10+10+20 cycles with three different Tc values of five variants (from low to high Tc). The performance evaluation showed notable results in this single system. The following estimated frequencies were obtained in our Thai-population-focused study: 3.7% in *CYP2C9*2* and *CYP2C19*3*, 55.5% in *CYP2C19*2*, 77.7% in *CYP2D6*10*, and 14.8% in *CYP2D6*41*. In previous studies, frequencies of only 0.08% for *CYP2C9*2*, 25.6% for *CYP2C19*2* and 2.5% for *CYP2C19*3* alleles were found in a Thai population (26,41). For *CYP2D6* in Thai population, the decreased-function allele *CYP2D6*10* is the most common allele found in patients treated with risperidone, at 51.8%, followed by *CYP2D6*41* at 6.8% (42). Although the present allele representation frequency was higher than previous studies (26,41,42), it may be due to small sample size. Nevertheless, the present COLD-PCR results showed 100% agreement with Sanger sequencing results. Therefore, the present method may be applicable as an initial test for unknown samples before haplotyping. Furthermore, the cost (not including DNA extraction) of fast COLD-PCR in our routine pharmacogenetic laboratory service is Thai baht (THB) 125/test (USD $3.6), as opposed to THB 1,200/test (USD $34.59) for Sanger sequencing. Our study introduces an easily applicable prescreening methodology in PGx settings.

However, the present methodology had limitations. One constraint is in enriching only Tm-reducing variations. *CYP2C9*3* (1075A>C), a Tm-increasing mutant, was not included and further COLD-PCR technique, such as full COLD-PCR, is required to detect all types of mutations (Tm-increase, Tm-equivalent, Tm-decrease). The present study used only 27 samples; thus, larger sample size is required to identify further SNPs. The present method is a qualitative screening; for patients with positive results, additional methods should be used to distinguish homozygous or heterozygous genotypes. Nevertheless, the aim of this study is to consider PGx testing in a cost-effective way. Currently, the plurality of commercial assay is available, however, our main objective is to view PGx testing by applying only conventional PCR machine before sequencing or genetic testing.

Table II. Evaluation of fast COLD-PCR showing 100% consistency with Sanger sequencing on 27 collected samples.

	*CYP2C9*2*		*CYP2C19*2*		*CYP2C19*3*		*CYP2D6*10*		*CYP2D6*41*	
Result	Fast COLD-PCR	Sanger sequencing	Fast COLD-PCR	Sanger sequencing	Fast COLD-PCR	Sanger sequencing	Fast COLD-PCR	Sanger sequencing	Fast COLD-PCR	Sanger sequencing
Positive	1	1	15	15	1	1	21	21	4	4
Negative	26	26	12	12	26	26	6	6	23	23
κ value	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

COLD, co-amplification at lower denaturation temperature; CYP, cytochrome P450. Positive, the respective variant/SNP was detected in the sample; Negative, the respective variant/SNP was not detected in the sample.

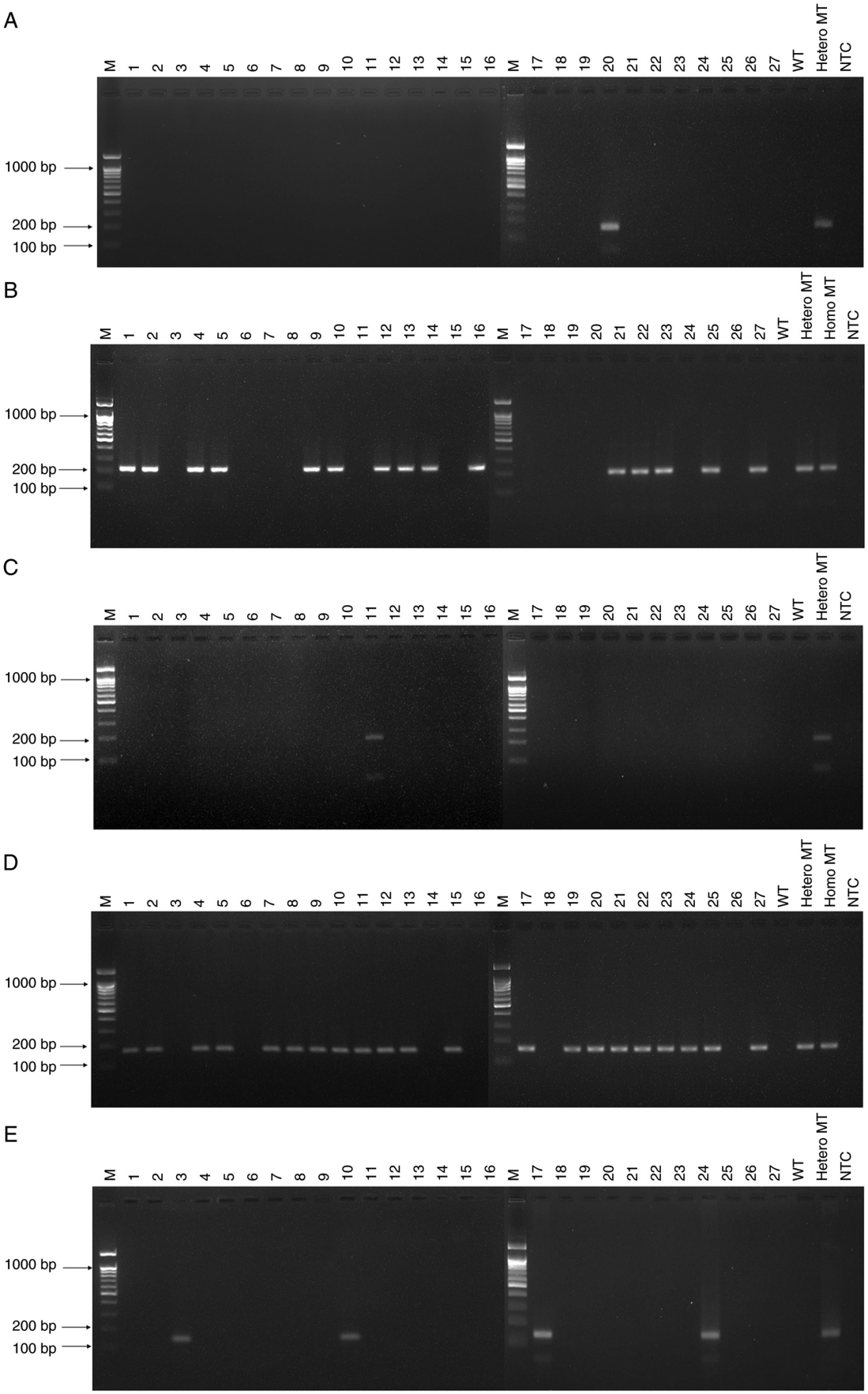

Figure 3. Analysis of 27 samples by combined fast co-amplification at lower denaturation temperature-PCR using control samples. (A) *CYP2C9*2* (150 bp) showed a positive band in 1 sample using a hetero MT control. (B) *CYP2C19*2* (206 bp) showed positive bands in 15 samples using both hetero and homo MT controls. (C) *CYP2C19*3* (192 bp) showed a positive band in 1 sample using a hetero MT control. (D) *CYP2D6*10* (160 bp) showed positive bands in 21 samples using both hetero and homo MT controls. (E) *CYP2D*41* (124 bp) showed positive bands in 4 samples using a hetero MT control. WT, wild-type; MT, mutant; CYP, cytochrome P450; hetero, heterozygous; homo, homozygous; NTC, no template control.

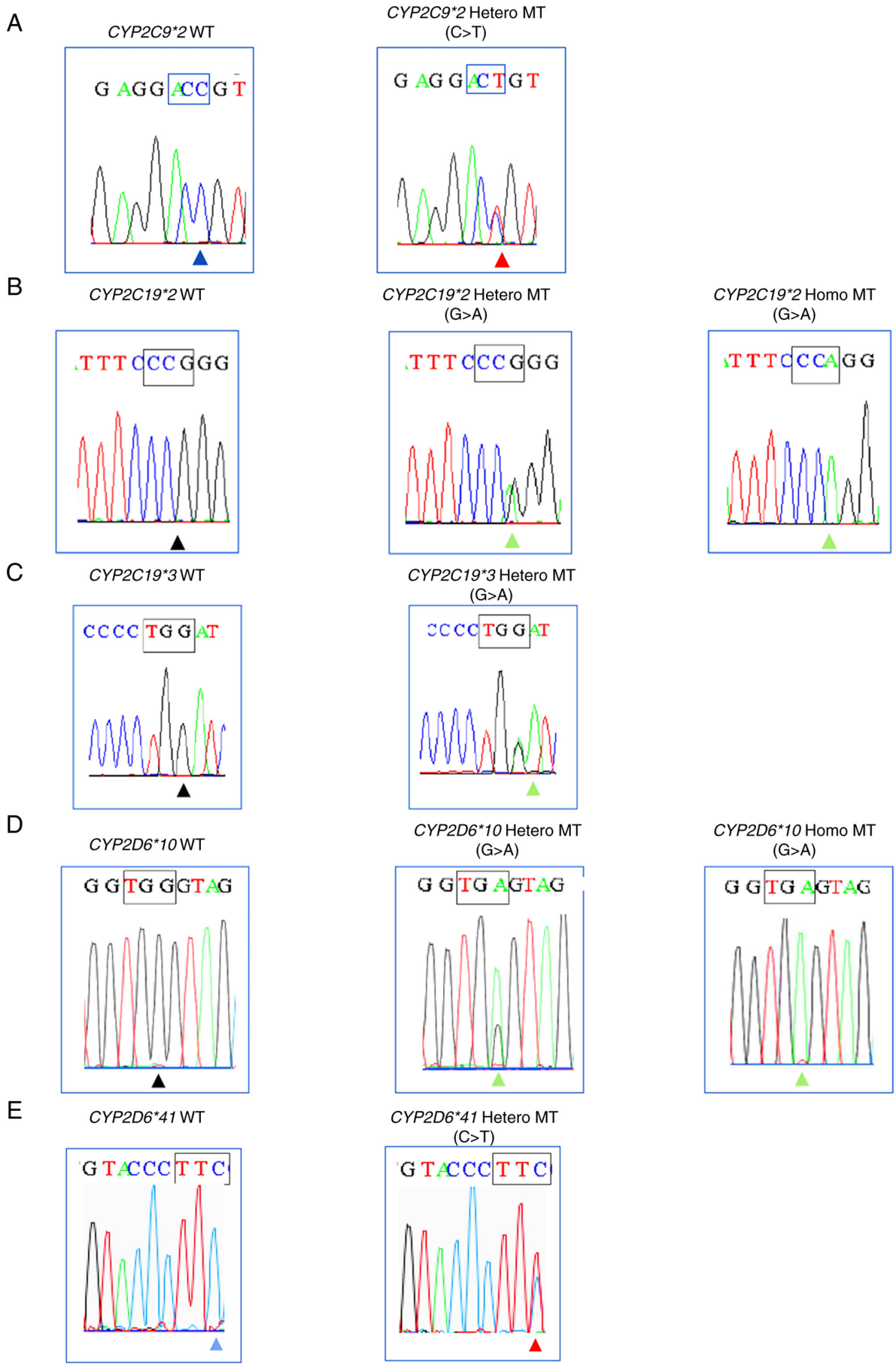

Figure 4. Sanger sequence profiles with chromatogram on five variants. (A) *CYP2C9*2* in WT and hetero MT; (B) *CYP2C19*2* in WT, hetero MT and homo MT; (C) *CYP2C19*3* in WT and hetero MT; (D) *CYP2D6*10* in WT, hetero MT and homo MT; (E) *CYP2D6*41* in WT and hetero MT. CYP, cytochrome P450; WT, wild-type; MT, mutant; hetero, heterozygous; homo, homozygous.

The present study approached the first prescreening pipeline in mutation detection before traditional genotyping methods. Here, fast COLD-PCR methodology correctly identified all WT samples. Only MT samples only need traditional genotyping to distinguish homozygous or heterozygous type. For example, for *CYP2C9*2* and *CYP2C19*3*, 26 out of 27 samples were WT. Therefore, sequencing or genetic testing for genotype (heterozygous or homozygous) would not be needed in 26 samples. Therefore, cost for the whole pipeline would be decreased and WT samples reported faster with the present screening PCR test. This method may decrease unnecessary costs of expensive genotyping in patients.

To the best of our knowledge, the present study is the first to propose fast COLD-PCR as a solution to the economic barrier of PGx implementation. This method only needs conventional PCR machine to perform and it shows the results as positive/negative. Therefore, it can screen out WT samples and only positive MT bands need traditional genetic testing. This method can be used to assess genetic variants as an easy cost-effective strategy.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YMN, SD, CS, AL, PP and NA conceptualized the study. YMN, SD and NA designed the experiments. YMN and NA analyzed data. YMN performed the experiments and visualized data. SD and CS confirm the authenticity of all the raw data. Resources from CS. YMN, AL, PP, SD, CS and NA drafted and edited the manuscript. NA supervised the study. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Research Ethics Committee, Faculty of Associated Medical Sciences, Chiang Mai University, Thailand (approval no. AMSEC-64EX-130; date of approval: 28 December 2021). Written informed consent was obtained from all subjects.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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