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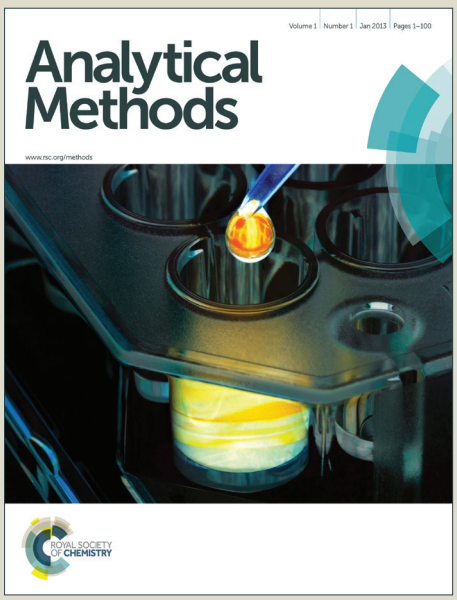

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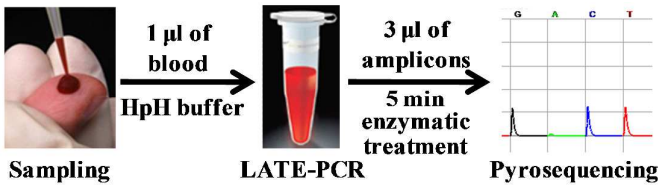
Highlight

Pyrosequencing-based genotyping protocol was simplified by linear-after-the-exponential (LATE)-PCR directly using whole blood as starting material.

Graphical abstract



A simplified pyrosequencing protocol based on linear-after-the-exponential (LATE)-PCR using whole blood as starting material directly

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Abstract

Pyrosequencing has been one of the most commonly used methods for genotyping; however, generally it needs single-stranded DNA (ssDNA) preparation from PCR amplicons as well as purified genomic DNA extraction from whole blood. To simplify the process of a pyrosequencing protocol, we proposed an improved linear-after-the-exponential (LATE)-PCR by employing whole blood as starting material. A successful LATE-PCR was achieved by using a common Taq DNA polymerase in high pH buffer (HpH-buffer). As amplicons from LATE-PCR contain a large amount of ssDNA, pyrosequencing can be performed on the amplicons directly. Since DNA extraction and ssDNA preparation are omitted, the labor, cost and cross-contamination risk is decreased comparing to conventional pyrosequencing-based genotyping protocols. The results for typing three polymorphisms related to personalized medicine of fluorouracil indicate that the proposed whole-blood LATE-PCR can be well coupled with pyrosequencing, thus becoming a potential tool in personalized medicine.

Keywords Pyrosequencing; Whole-blood PCR; Linear-after-the-exponential (LATE)-PCR; genotyping; Fluorouracil

Introduction

Pyrosequencing is a sequencing-by-synthesis method which is based on the bioluminometric detection of inorganic pyrophosphate (PPi) coupled with cascade enzymatic reactions.[1] In addition to its good performance in quantification, no electrophoresis or fluorescence is required; thus various applications of pyrosequencing have been achieved, such as genotyping,[2, 3] methylation detection,[4] resequencing,[5] gene expression analysis,[6-8] micro RNA quantification,[9] disease diagnosis,[10] and microbial typing.[11, 12] For the moment, pyrosequencing has been widely used in clinical detection of genetic biomarkers related to personalized medicine.

However, single-stranded DNA (ssDNA) preparation is needed to separate the immobilized strand before pyrosequencing. This step is tedious in operation, costly in synthesis biotinylated primers, and high in the risk of cross-contamination from amplicons. To overcome this shortcoming, we have proposed a method enabling pyrosequencing directly on double-stranded DNA (dsDNA) digested by nicking endonuclease,[13] in which the recognition sequence of nicking endonuclease was introduced by a PCR primer. After a nicking reaction of the amplicons, pyrosequencing started at the nicked 3' end, and extension reaction occurred when the added dNTP was complementary to the non-nicked strand. However, the strand-displacement activity of Klenow Fragment was limited. Although around ten bases can be accurately sequenced, the quality of pyrograms was no better than that from the template of ssDNA. In addition, nicking endonuclease is expensive.

In contrast to conventional asymmetric PCR with regular PCR primers at unequal concentrations, linear-after-the-exponential (LATE)-PCR could yield a large amount of ssDNA-amplicons directly.[14] The amount of ssDNA is enough for a successful pyrosequencing reaction after a simple pretreatment of amplicons from LATE-PCR.[15] However, a purified genomic DNA is needed for LATE-PCR. Although there are many commercial kits available for DNA extraction, several hundred microliters of blood sample have to be consumed for an extraction conventionally; so it is impossible to use a tiny amount of finger blood for detection. In addition, this labor-intensive step is a possible risk of cross-contamination. Consequently, it is preferable to directly employ a small amount of blood for PCR. Although our previous study showed that the change of PCR buffer could enable a successful PCR using finger blood or paper-dried blood as starting material,[16] it is necessary to investigate whether or not this buffer

condition is suitable to whole-blood LATE-PCR. Hence, three polymorphisms related to personalized medicine of fluorouracil (5-FU) were used as an example. The results indicate that our proposed whole-blood LATE-PCR significantly simplified the pyrosequencing-based genotyping protocol, decreasing the labor, cost and cross-contamination risk.

Experimental

Materials

rTaq DNA Polymerase was purchased from TaKaRa (Dalian, China). AmpliTaq Gold DNA Polymerase was purchased from Applied Biosystems (Foster City, USA). Bovine serum albumin (BSA), D-Luciferin, adenosine 5'-phosphosulfate (APS) and apyrase VII were purchased from Sigma (St. Louis, USA). ATP sulfurylase and Klenow fragment were obtained by gene engineering in our lab. Polyvinylpyrrolidone (PVP) and QuantiLum Recombinant Luciferase were purchased from Promega (Madison, USA). 2'-Deoxyadenosine-5'-O-(1-thiotriphosphate) sodium salt (dATPαS), dGTP, dTTP, and dCTP were purchased from MyChem (San Diego, USA). Streptavidin Sepharose™ Beads were from GE Healthcare (New Jersey, USA). Other chemicals were of a commercially extra-pure grade. All solutions were prepared with deionized and sterilized water.

DNA samples

Purified genomic DNA was extracted from whole blood samples by QIAamp DNA Blood Mini Kit (QIAGEN, Germany). The DNA concentration was determined by a One Drop spectrophotometer (Shanghai, China), and stored at -20 ℃ before use. All of the blood samples were provided by Jinling Hospital (Nanjing, China) with the informed consent form and with ethics committee approval.

Primer design

Three polymorphisms in the methylenetetrahydrofolate reductase (MTHFR) gene and the dihydropyrimidine dehydrogenase (DPYD) gene were investigated: MTHFR C677T, MTHFR A1298C and DPYD IVS14+1G>A. Primer sets were designed to amplify a 201-bp fragment containing the MTHFR C677T site, a 170-bp fragment containing the MTHFR A1298C site and a 192-bp fragment containing the DPYD IVS14+1G>A site according to the principles of LATE-PCR.[15, 17] Briefly, the Tm difference between the limiting primer and the excess primer was

greater than 0 ℃, the Tm difference between amplicon and the excess primer was less than 13 ℃ based on concentration-adjusted values. OligoAnalyzer 3.1 (http://www.idtdna.com/ analyzer/ Applications/ OligoAnalyzer/) was used to calculate the primers' Tm values. PCR primers were listed in Supplementary Table S1.

Whole-blood LATE-PCR

Whole-blood LATE-PCR was performed on Mastercycler PCR system (Eppendorf, Germany). Fifty microliters of PCR contained 1×HpH buffer (100 mM Tris-HCl, 50 mM KCl, pH 9.3-9.5), 2.0 mM $MgCl_2$, 100 μM dNTPs, 1 μM excessive primer, 0.1 μM limited primer, 2.5 U of rTaq DNA polymerase, 0.25 μl of Tween-20, 0.5 μl of whole blood. The PCR program was as follows: 94 ℃ for 3 min; followed by 60 cycles of 90 ℃ for 10 s, 60 ℃ for 10 s, 72 ℃ for 20 s; and finally 72 ℃ for 7 min.

Template preparation for pyrosequencing

The LATE-PCR amplicons contained excessive dNTPs, PPi and incompletely-extended products. These ingredients affected the subsequent pyrosequencing reactions. Therefore, the amplicons had to be treated before pyrosequencing. At first, added 40 μl of self-prepared pyrosequencing mixture[18] as well as 3 μl of APS (3 nmol) into the tube having 3 μl of whole-blood LATE-PCR amplicons, and then incubated the tube at room temperature for 5 min. Then added 10 pmol of sequencing primers annealed at room temperature for 5 min. Sequencing primers were MTHFR C677T (5'-AAA GCT GCG TGA TGA TGA AAT CG-3'), MTHFR A1298C (5'-AAA GAA CGA AGA CTT CAA AGA CAC T-3') and DPYD IVS14+1G>A (5'-AAG GCT GAC TTT CCA GAC AAC-3').

Pyrosequencing

We used a portable bioluminescence analyzer (HITACHI, Ltd., Central Research Laboratory, Japan) for pyrosequencing. This apparatus has a portable size of 140 mm (W)×158 mm (H)×250 mm (D), with an array of 8 photodiodes (Hamamatsu Photonics K.K, Japan) to detect photo signals, and 4 separate capillaries to dispense small amounts of dNTPs into the reaction chamber. Pyrosequencing was carried out by the reported method.[18] The reaction volume was 40-μl, containing 0.1 M tris-acetate (pH 7.7), 2 mM EDTA, 10 mM magnesium acetate, 0.1 mg/ml BSA, 1 mM dithiothreitol, 2 μM APS, 0.4 mg/ml PVP, 0.4 mM D-Luciferin, 2 μM ATP sulfurylase, 5.7×10^8 RLU QuantiLum Recombinant Luciferase, 18 U/ml Klenow Fragment, and 1.6 U/ml

apyrase.

Results and discussion

Development of LATE-PCR system allowing blood to be starting amplification material

The principle of LATE-PCR was shown in Supplementary Fig. S1.[14] As LATE-PCR needs more cycles than conventional PCR, AmpliTaq Gold polymerase, which belongs to a hot-start type with a good thermal-stability, was routinely used for amplification.[14] To investigate whether or not this polymerase is suitable for PCR using blood as starting material, AmpliTaq Gold polymerase-based LATE-PCR using kit-buffer and HpH-buffer (home-made) was carried out on purified genomic DNA and whole blood, respectively. As a proof-of-concept, three SNPs, C677T and A1298C in the MTHFR and DPYD IVS14+1G>A in the DPYD, were employed for the evaluation. These SNPs are related to personalized medicine of 5-FU. As shown in Fig. 1, it is no problem to amplify genomic DNA by LATE-PCR with AmpliTaq Gold polymerase (Fig. 1A); however, it is problematic for AmpliTaq Gold polymerase-based LATE-PCR to directly amplify blood-DNA using either kit-buffer (Fig. 1B) or high pH buffer (HpH-buffer) (Fig. 1C). Therefore the AmpliTaq Gold polymerase which was used in conventional LATE-PCR is not suitable for LATE-PCR directly using blood as starting material.

To enable LATE-PCR with blood directly, we tried to employ a regular Taq polymerase, named rTaq polymerase, for amplifying gDNA at first. As shown in Fig. 2, good pyrosequencing signals were observed from LATE-PCR using either kit-buffer (Fig. 2A) or HpH-buffer (Fig. 2B), suggesting that LATE-PCR based on rTaq polymerase could yield enough amounts of ssDNA amplicons although there are more heating-cooling cycles which may lower the activity of polymerase. Then, blood was employed as starting material directly for LATE-PCR using both buffers. As shown in Fig. 2C and 2D, it is possible to use blood as the starting material of LATE-PCR, but ssDNA yield from kit-buffer-based PCR is much lower than that from HpH-buffer based PCR. Most importantly, the intensities of peaks in the pyrogram from LATE-PCR using rTaq polymerase (Fig. 2D) are much close to those from LATE-PCR using AmpliTaq polymerase (Fig. 1A); thus, HpH-buffer previously developed for conventional blood-PCR is much matchable to LATE-PCR. This enables the escape of the step for extracting gDNA from blood, greatly simplifying the pyrosequencing-based genotyping.

Whole-blood LATE-PCR product cleanup

As residue dNTPs, by-product PPi and incompletely-extended products in whole-blood LATE-PCR amplicons would affect pyrosequencing reactions, it is necessary to perform a cleanup step before pyrosequencing. The incompletely-extended products could be extended by Klenow in the presence of dNTPs; by-product PPi could be converted into ATP by ATP sulfurylase and APS; the residue dNTPs together with the ATP yielded from the by-product PPi could be degraded by apyrase. Therefore, we can directly employ a conventional pyrosequencing mixture to clean up the amplicons. However, it was found that the baseline due to the reaction of APS with luciferase decreased from 0.8 to 0.1 when adding LATE-PCR products, and no subsequent pyrosequencing signals were observed (Fig. 3A) even if dNTP was individually dispensed. We believe that this is resulted from the exhaustion of APS in the mixture after adding LATE-PCR products. To compensate the consumed APS, additional addition of APS is necessary. As shown in Fig. 3B, the baseline kept the same level when adding 3 nmol of APS together with the LATE-PCR products into the pyrosequencing mixture, and an expected pyrogram was observed. The amount of APS for the compensation is not critical, and we found that it is enough to compensate 1 nmol of APS for 1 μl of LATE-PCR products.

Effect of anticoagulants on LATE-PCR using blood as starting material

As there are various types of anticoagulants added in blood, it is necessary to look for whether or not the efficiency or specificity of LATE-PCR was affected by anticoagulant types. Typically, three types of anticoagulants, EDTA, citrate and heparin are most used clinically. For the investigation, three aliquots of a fresh blood sample were added with anticoagulants of EDTA, citrate and heparin respectively. Then, the anticoagulated blood samples were individually added into three different tubes for LATE-PCR. Pyrograms of ssDNAs from three LATE-PCR tubes showed that no obvious difference in signal intensity was observed among three kinds of anticoagulated blood (Fig. 4). Therefore, the protocol of the proposed LATE-PCR using HpH buffer is independent of anticoagulant types.

The minimum volume of a blood sample required for a successful genotyping by pyrosequencing

In clinical assay, one blood sample may be used for various diagnoses, so a method with a small amount of blood sample is preferable. Therefore, it is necessary to investigate the minimum amount of blood required for a successful genotyping by pyrosequencing. In a 25-μl reaction, 1 μl,

0.5 μl, 0.1 μl and 0.05 μl of a blood sample were individually added for LATE-PCR. As shown in Fig. 5 (A, B, C), good pyrograms were obtained when the volume of a blood sample for an assay is larger than 0.1 μl. Although pyrosequencing signals are obvious in the pyrogram with 0.05 μl of blood (Fig. 5D), the decreased signal-to-noise may affect the correct genotyping. The signal intensities of one base extension are nearly same if more than 0.1 μl of blood is employed for genotyping, implying that the template amount in a 0.1 μl of blood is enough for a successful PCR. Usually, around 100 copies of DNA template are enough for a successful PCR. After optimization, the sensitivity of LATE-PCR is down to single cells[19]. Averagely, 0.1 μl of human blood contains 700 white cells; thus a good quality of a pyrogram can be obtained by using 0.1 μl of blood as starting material of LATE-PCR. In a convention PCR based on purified genomic DNA, several hundred microliters of blood are required for a successful genomic DNA purification. Therefore our proposed method is much template-saving and time-saving. In addition, the proof-of-concept experiment using finger-blood or dried paper-blood as the starting amplification material is successful (data not shown). For a simple operation, 1 μl of blood was employed in a conventional protocol.

Genotyping of SNPs related to personalized medicine of 5-FU

5-FU is a widely used chemotherapeutic agent for the treatment of colon and breast cancers, and the modes of action of 5-FU are critically dependent on cellular composition of folates. As the C677T and the A1298C polymorphisms in the MTHFR gene affect the concentration and intracellular distribution of folates, genotyping of these two pharmacogenomics biomarkers may be useful in personalized chemotherapy of 5-FU.[20, 21] In addition, more than 80% of the administered 5-FU is primarily catabolized by the DPYD in the liver.[22] If DPYD is of deficiency, levels of 5-FU and its active metabolites would significantly increase in blood; thus, patients with DPYD deficiency will suffer from serious toxicity after the 5-FU administration.[23] As the most common DPYD mutation (termed as DPYD*2A) in the splice site of intron 14 (IVS14+1G>A) creates a non-functional DPYD enzyme, it is necessary to detect this mutation before 5-FU administration.

To demonstrate the feasibility of proposed method, the three polymorphisms of MTHFR C677T, MTHFR A1298C and DPYD IVS14+1G>A, which relate to the efficacy and toxicity of 5-FU, were used as the detection targets for the method evaluation. A total of 24 blood samples

were from people who voluntarily joined this study with an informed consent form, and the typing results are listed in Table 1. Pyrograms from typical genotypes of the 3 polymorphisms in 24 samples were showed in Fig. 6. As can be seen in the table, the inactive genotype DPYD*2A with the phenotype of DPYD enzyme deficiency was not found among 24 samples, indicating that the frequency of DPYD*2A is much low in Chinese population. However, the frequency of MTHFR 677TT with the phenotype of decreased MTHFR activity is as high as 33.3% in our study. Although the sample size is very small, the preliminary results did imply that it is necessary to detect the genotypes of the 3 polymorphisms in Chinese population before the administration of 5-FU.

MTHFR C677T in a set of 5 typical samples was typed intriplicate, showing a very good reproducibility of our proposed method (see Supplementary Fig. S2 for details). To validate the accuracy of our proposed method, the 24 samples were also typed by conventional Sanger sequencing, giving the same results as our proposed pyrosequencing. Supplementary Fig. S3 showed the comparison of the two methods for typing samples with different genotypes. So the simplified pyrosequencing protocol is feasible for typing real biological samples.

Conclusions

In this study, we have developed a simplified method for genotyping by pyrosequencing. The method is based on LATE-PCR using whole blood as starting material. As a large amount of ssDNA is produced from LATE-PCR, the amplicons could be directly pyrosequenced after a simple treatment. For a clinical assay, an important concern is the cost and labor used for genotyping. The comprehensive comparison of the labor and cost between whole-blood LATE-PCR based pyrosequencing and conventional PCR based pyrosequencing is demonstrated by typing 3 polymorphisms in a sample. As shown in Table 2, the cost of the proposed method is about 40% lower than that of conventional pyrosequencing in China, and the labor of the proposed method is more saving than that of conventional pyrosequencing, indicating that pyrosequencing coupled with whole-blood LATE-PCR is cost-effective and labor-saving. Most importantly, less steps of post-PCR will greatly avoid the contamination risk from amplicons, especially for space-limited labs. The potential issue of the present method in the future clinical application is the difficulty in the design of LATE-PCR primers,[17] and this can be solved by improving the LATE-PCR system with a larger amount of longer primer, but small amount of shorter primer.

This modification along with different cycling conditions allows the primer design more flexible.[19] Therefore our proposed method is more valuable for clinical use.

Acknowledgments

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Tables

Table 1. Genotyping results of 3 SNPs in 24 clinical samples by the proposed method.

Genes	SNPs	Genotypes	Enzyme activity
MTHFR	C677T	CC : n=8	Normal
		CT : n=8	Decreased
		TT : n=8	Decreased
	A1298C	AA : n=18	Normal
		AC : n=6	Decreased
DPYD	IVS14+1G>A	GG : n=24	Normal

Table 2. Comparison of labor and cost in the typing of 3 SNPs between whole-blood LATE-PCR-based pyrosequencing and conventional PCR-based pyrosequencing.

Steps	Conventional PCR-based pyrosequencing		LATE-PCR-based pyrosequencing[15]		Whole-blood LATE-PCR -based pyrosequencing	
	Time (h)	Cost (RMB)	Time (h)	Cost (RMB)	Time (h)	Cost (RMB)
gDNA extraction [a]	~0.5	24	~0.5	24	-	-
PCR [b]	~2	5	~2	48	~2	5
ssDNA preparation [c]	~0.5	15	0.2	18	0.2	0.5
Pyrosequencing [d]	~0.5	54	~0.5	54	~0.5	54
Total	~3.5	98	~3.2	144	~2.7	59.5

[a] QIAamp DNA Blood Mini Kit (QIAGEN) was used for gDNA extraction. [b] rTaq DNA polymerase (TaKaRa) was used for conventional PCR and whole-blood LATE-PCR; AmpliTaq Gold polymerase (Applied Biosystems) was used for LATE-PCR. [c] Streptavidin Sepharose beads (GE Healthcare) was used for ssDNA preparation in conventional PCR-based pyrosequencing; PyroMark Gold Q96 Reagents (QIAGEN) was used for the cleanup of LATE-PCR products before pyrosequencing; 3 μl of APS was added into the pyrosequencing mixture before the cleanup of the whole-blood LATE-PCR products. [d] PyroMark Gold Q96 Reagents(QIAGEN) was used for pyrosquencing.

Figure Captions

Fig. 1. Pyrograms of amplicons from AmpliTaq Gold polymerase-based PCR using kit-buffer (A, B) and HpH-buffer (C) for amplification as well as using purified gDNA (A) and whole blood (B, C) as starting material. Three amplified fragments containing 3 SNPs (MTHFR C677T, MTHFR A1298C and DPYD IVS14+1G>A) were pyrosequenced.

Fig. 2. Pyrograms of amplicons from rTaq polymerase-based LATE-PCR for amplifying purified gDNA (A, B) and whole blood (C, D) using kit-buffer (A, C) and HpH-buffer (B, D). Three amplified fragments containing 3 SNPs (MTHFR C677T, MTHFR A1298C and DPYD IVS14+1G>A) were pyrosequenced.

Fig. 3. Real-time baseline monitoring and pyrograms when adding LATE-PCR products (3 μl) only (A) and together with 3 nmol of APS (B) into conventional pyrosequencing mixture.

Fig. 4. Pyrograms of amplicons from PCR directly using EDTA (A), citrate (B) and heparin (C)-anticoagulated blood as starting material, respectively. The template for pyrosequencing is the amplified fragment containing the SNP of DPYD IVS14+1G>A.

Fig. 5. Pyrograms of amplicons from PCR using 1.0 μl (A), 0.5 μl (B), 0.1 μl (C) , and 0.05 μl (A) EDTA-anticoagulated blood as starting material, respectively. The template for pyrosequencing is the amplified fragment containing the MTHFR C677T.

Fig. 6. Pyrograms from typical genotypes of the 3 polymorphisms in 24 samples.

Figures

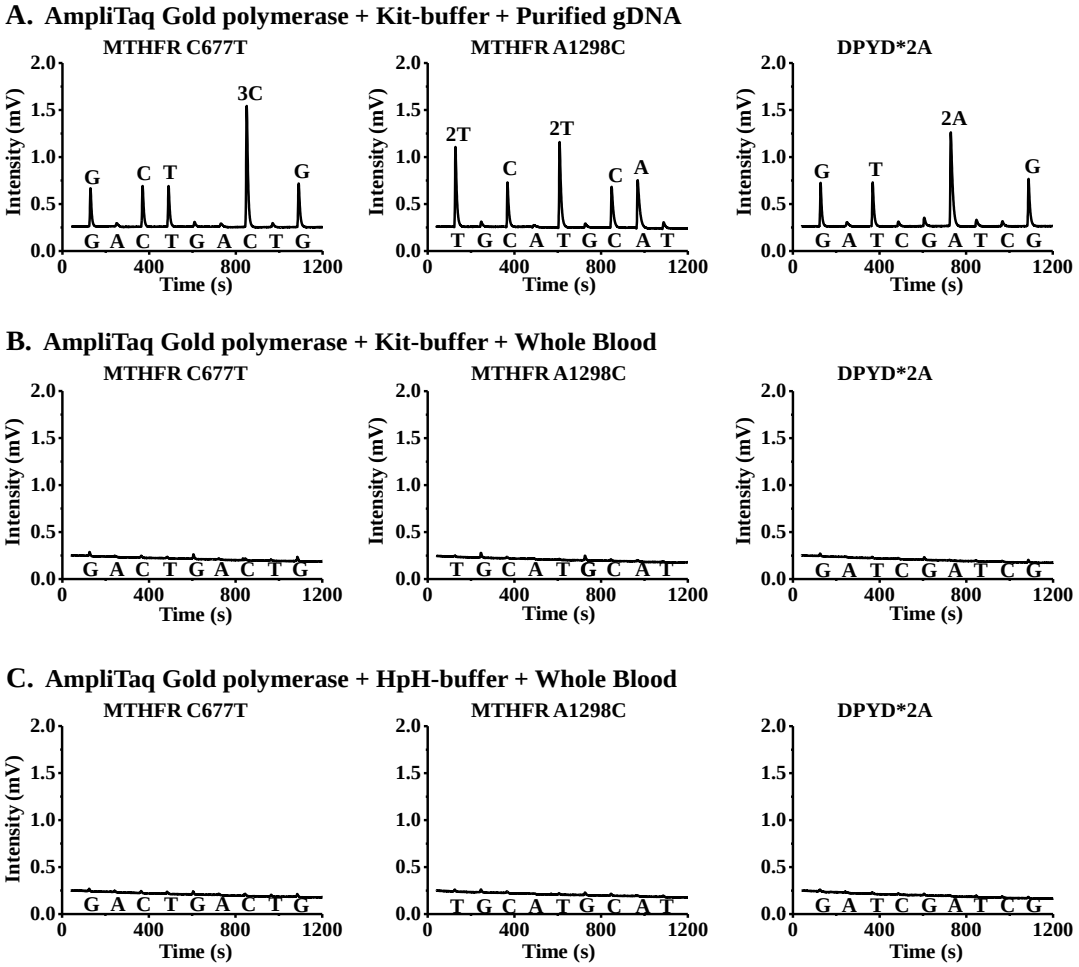

Fig. 1. Pyrograms of amplicons from AmpliTaq Gold polymerase-based PCR using kit-buffer (A, B) and HpH-buffer (C) for amplification as well as using purified gDNA (A) and whole blood (B, C) as starting material. Three amplified fragments containing 3 SNPs (MTHFR C677T, MTHFR A1298C and DPYD IVS14+1G>A) were pyrosequenced.

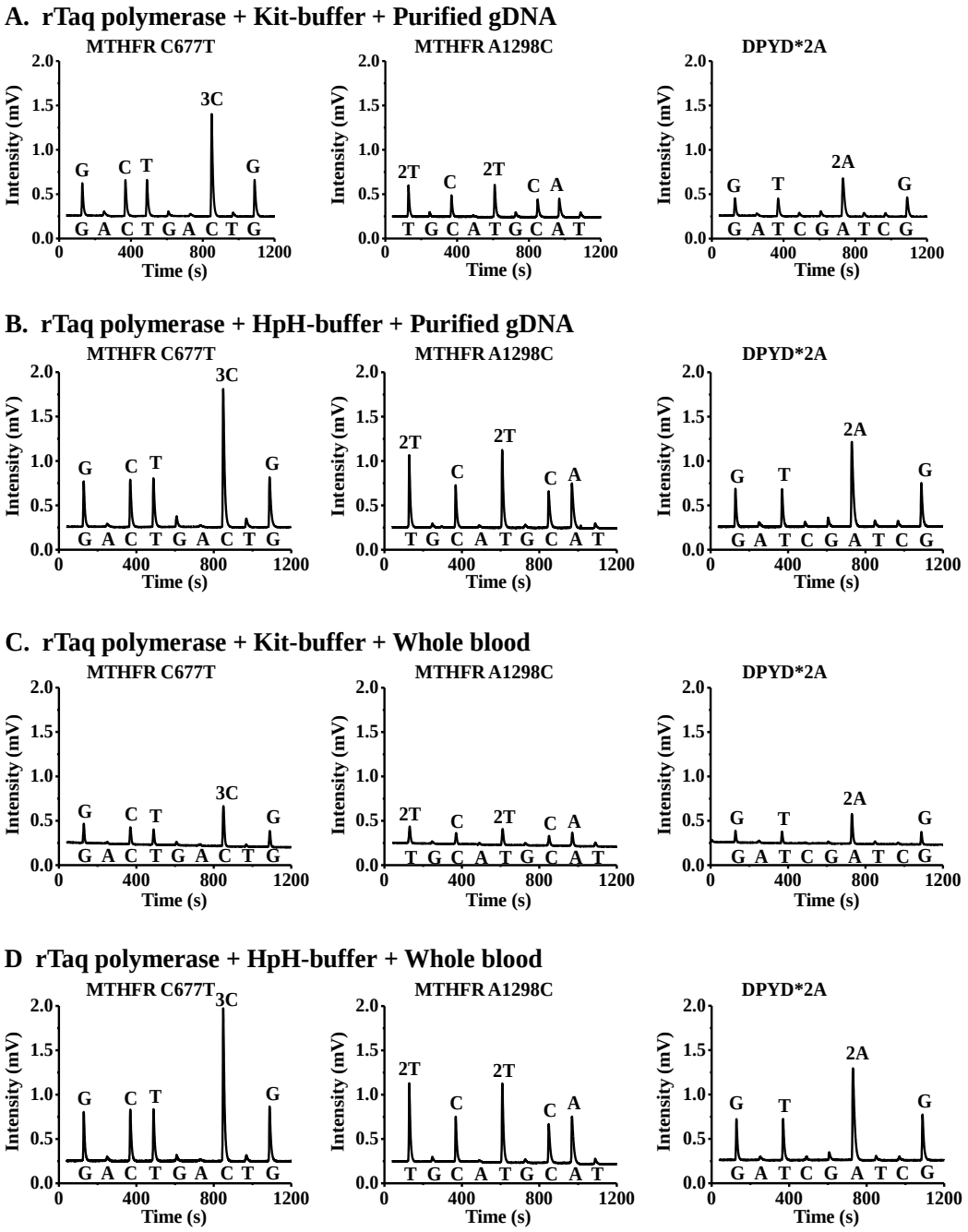

Fig. 2. Pyrograms of amplicons from rTaq polymerase-based LATE-PCR for amplifying purified gDNA (A, B) and whole blood (C, D) using kit-buffer (A, C) and HpH-buffer (B, D). Three amplified fragments containing 3 SNPs (MTHFR C677T, MTHFR A1298C and DPYD IVS14+1G>A) were pyrosequenced.

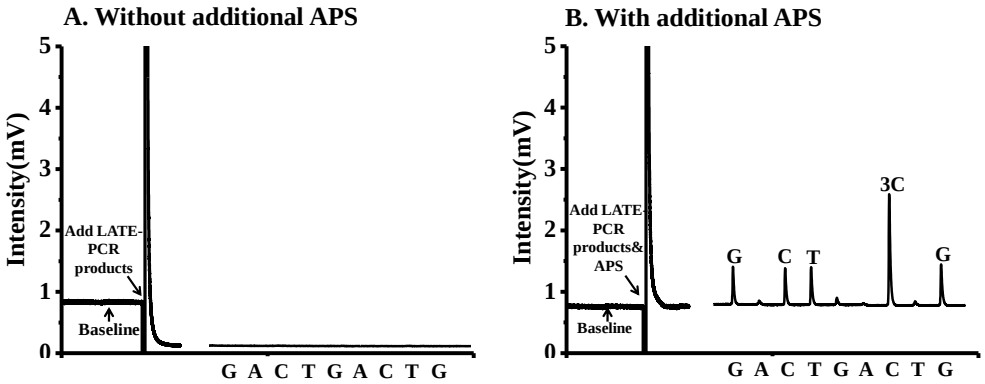

Fig. 3. Real-time baseline monitoring and pyrograms when adding LATE-PCR products (3 μl) only (A) and together with 3 nmol of APS (B) into conventional pyrosequencing mixture.

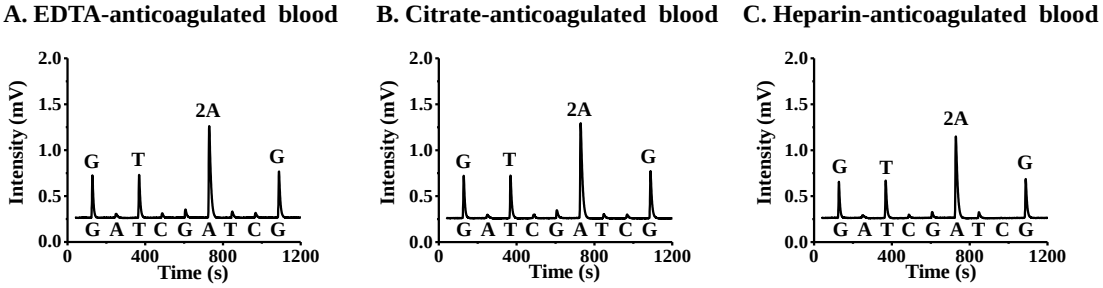

Fig. 4. Pyrograms of amplicons from PCR directly using EDTA (A), citrate (B) and heparin (C)-anticoagulated blood as starting material, respectively. The template for pyrosequencing is the amplified fragment containing the SNP of DPYD IVS14+1G>A.

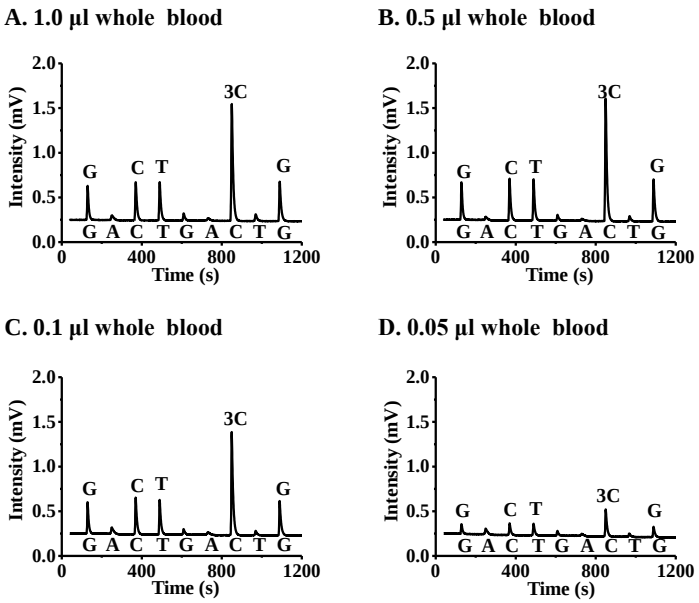

Fig. 5. Pyrograms of amplicons from PCR using 1.0 µl (A), 0.5 µl (B), 0.1 µl (C) , and 0.05 µl (A) EDTA-anticoagulated blood as starting material, respectively. The template for pyrosequencing is the amplified fragment containing the MTHFR C677T.

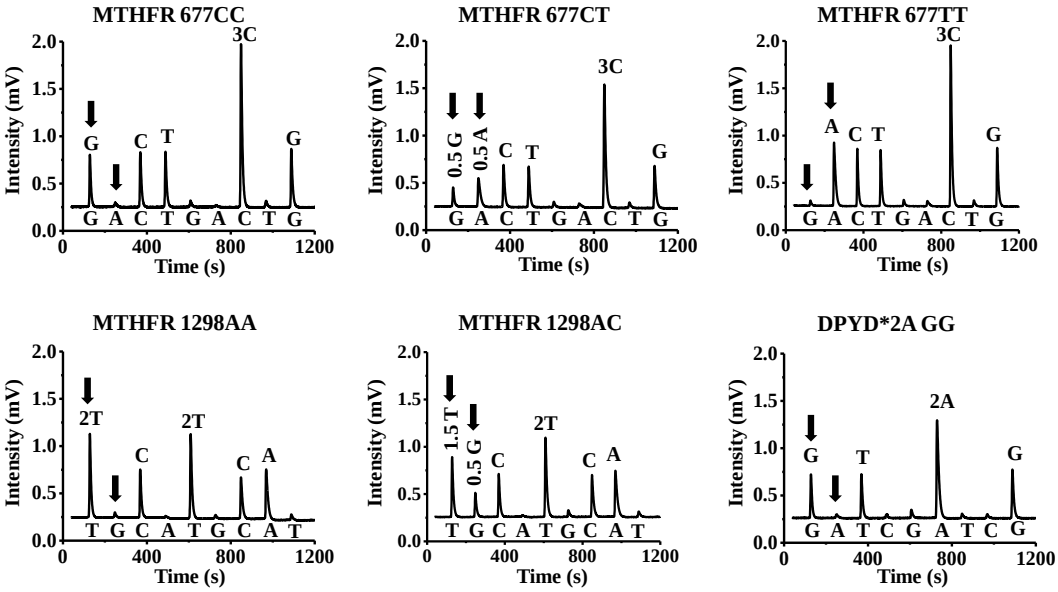

Fig. 6. Pyrograms from typical genotypes of the 3 polymorphisms in 24 samples.