

Chapter 2

A Simplified Protocol for Preparing Pyrosequencing Templates Based on 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.

Key words Pyrosequencing, Whole-blood PCR, Linear-after-the-exponential (LATE)-PCR, Genotyping, Fluorouracil

1 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 the synthesis of 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.

2 Materials

1. rTaq DNA Polymerase (TaKaRa, China).
2. AmpliTaq Gold DNA Polymerase (Applied Biosystems, USA).
3. Bovine serum albumin (BSA), D-luciferin, adenosine 5'-phosphosulfate (APS), and apyrase VII (Sigma, USA).

4. ATP sulfurylase and Klenow fragment (obtained by gene engineering in our lab).
5. Polyvinylpyrrolidone (PVP) and QuantiLum Recombinant Luciferase (Promega, USA).
6. 2'-deoxyadenosine-5'-O-(1-thiotriphosphate) sodium salt (dATP α S), dGTP, dTTP, and dCTP (MyChem, USA).
7. Streptavidin Sepharose™ Beads (GE Healthcare, USA).
8. HpH buffer (1×): 100 mM Tris-HCl, 50 mM KCl, pH 9.3–9.5.
9. QIAamp DNA Blood Mini Kit (QIAGEN, Germany).
10. One Drop spectrophotometer (Shanghai, China).
11. Mastercycler PCR system (Eppendorf, Germany).
12. A portable bioluminescence analyzer (HITACHI, Ltd., Central Research Laboratory, Japan).

3 Methods

3.1 DNA Samples Extraction

1. Extract purified genomic DNA from whole blood samples by QIAamp DNA Blood Mini Kit.
2. Determine the DNA concentration by a One Drop spectrophotometer (*see Note 1*).

3.2 Primer Design

1. 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*2A site according to the principles of LATE-PCR [17] (*see Note 2*).

3.3 Whole-Blood LATE-PCR

1. Fifty microliters of PCR contained 1× HpH buffer, 2.0 mM MgCl₂, 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 (*see Notes 3 and 4*).
2. The PCR program was as follows: 94 °C for 3 min, followed by 60 cycles of (90 °C for 10 s; 60 °C for 10 s; 72 °C for 20 s); and finally 72 °C for 7 min.

3.4 Template Preparation for Pyrosequencing

1. Add 40 μL of self-prepared pyrosequencing mixture [18] (*see Note 5*) as well as 3 μL of APS (3 nmol) (*see Note 6*) into the tube having 3 μL of whole-blood LATE-PCR products.
2. Incubate the tube at room temperature for 5 min.
3. Add 10 pmol of sequencing primers anneal at room temperature for 5 min.

3.5 Pyrosequencing

1. We used a portable bioluminescence analyzer for pyrosequencing [14, 15] (*see Note 7*).
2. Pyrosequencing was carried out by the reported method [18]. The reaction volume was 40 μ L, 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 μ M APS, 0.4 mg/mL PVP, 0.4 mM D-Luciferin, 2 μ M ATP sulfurylase, 5.7×10^8 RLU QuantiLum Recombinant Luciferase, 18 U/mL Klenow Fragment, and 1.6 U/mL apyrase.

4 Method Validation

4.1 Genotyping of SNPs Related to Personalized Medicine

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 the 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 [19]. 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 the 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 the starting material directly for LATE-PCR using both buffers. As shown in Fig. 2c, d, it is possible to use blood as the starting material for LATE-PCR, but ssDNA yield from kit-buffer-based PCR is much lower than that from HpH-buffer based PCR [20–21]. Most importantly, the intensities of peaks in the pyrogram from LATE-PCR using rTaq polymerase (Fig. 2d) are very close to those from LATE-PCR using AmpliTaq polymerase (Fig. 1a); thus, HpH-buffer previously developed for conventional blood-PCR is very

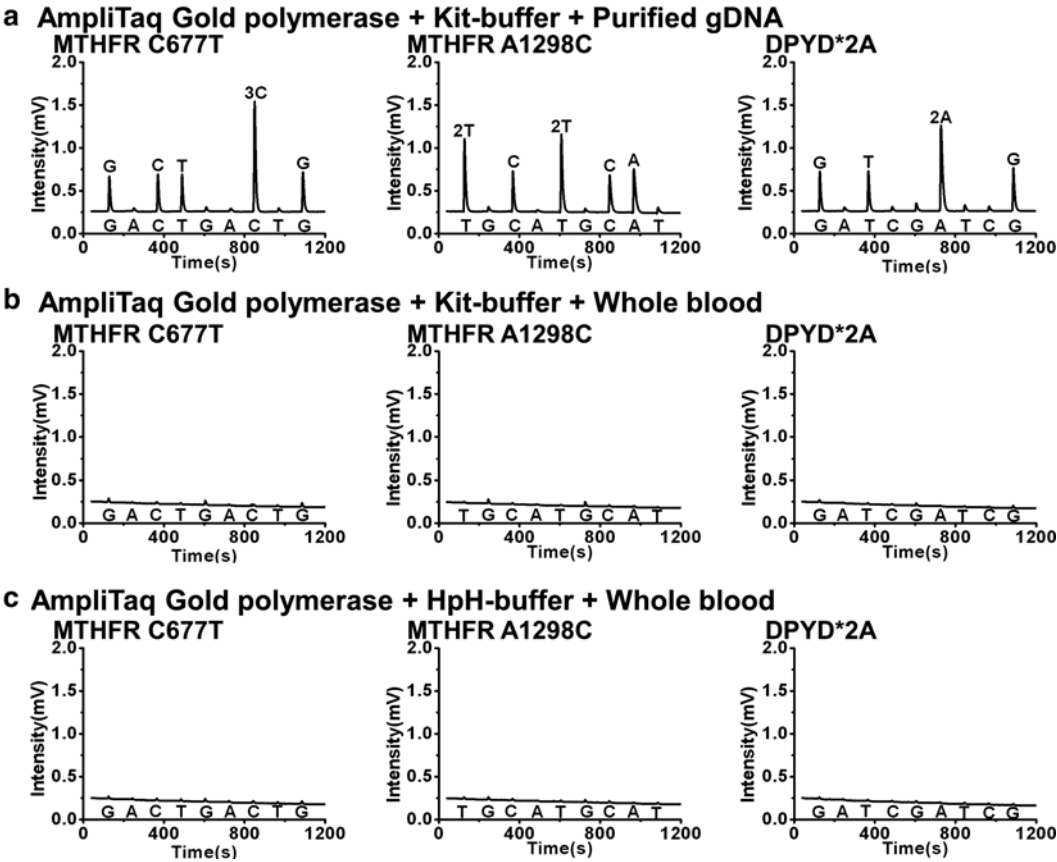
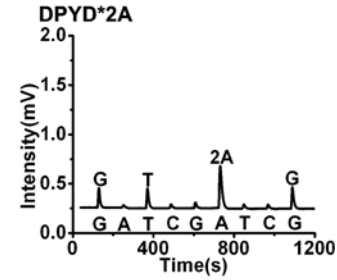
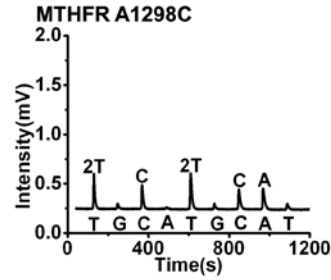
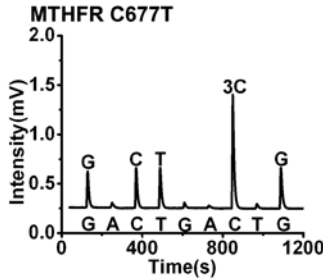


Fig. 1 Pyrograms of amplicons from AmpliTaq Gold polymerase-based PCR using kit-buffer (**a** and **b**) and HpH-buffer (**c**) for amplification as well as using purified gDNA (**a**) and whole blood (**b** and **c**) as starting materials. Three amplified fragments containing three SNPs (MTHFR C677T,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

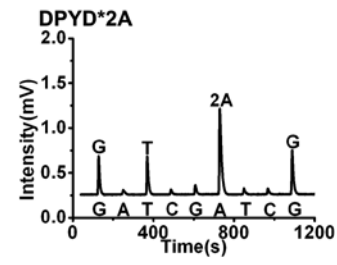
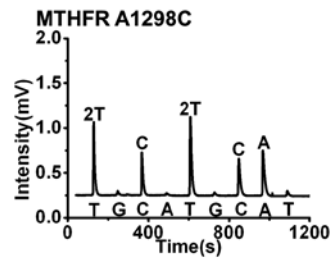
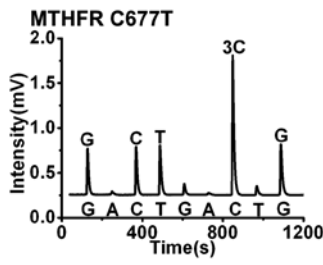
comparable to that used for LATE-PCR. This enables us to avoid the step of extracting gDNA from blood, greatly simplifying the pyrosequencing-based genotyping.

To demonstrate the feasibility of the 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 obtained 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 are shown in Fig. 3. As can be seen in the table, the inactive genotype DPYD*2A with the phenotype of DPYD enzyme deficiency was not found

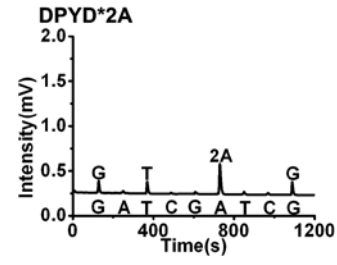
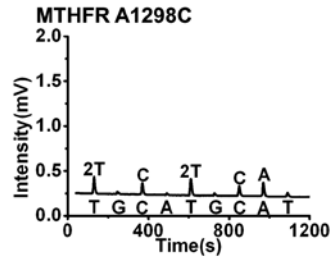
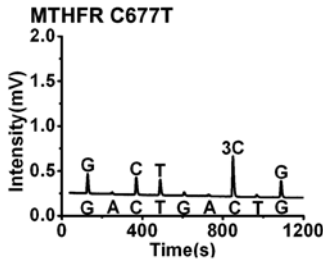
a rTaq polymerase + Kit-buffer + Purified gDNA



b rTaq polymerase + HpH-buffer + Purified gDNA



c rTaq polymerase + Kit-buffer + Whole blood



d rTaq polymerase + HpH-buffer + Whole blood

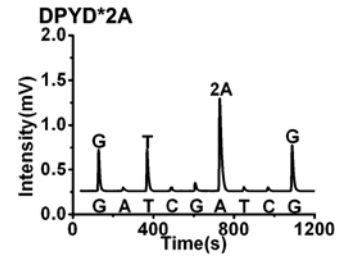
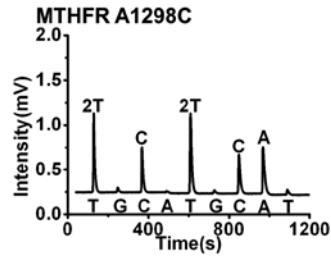
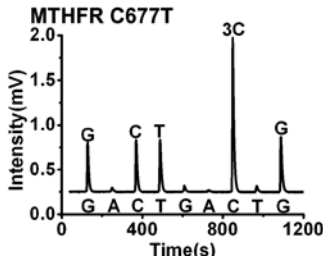


Fig. 2 Pyrograms of amplicons from rTaq polymerase-based LATE-PCR for amplifying purified gDNA (**a, b**) and whole blood (**c, d**) using kit-buffer (**a, c**) and HpH-buffer (**b, d**). Three amplified fragments containing three SNPs (MTHFR C677T, MTHFR A1298C, and DPYD*2A) were pyrosequenced

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

Genes	Alleles/position	Genotype	Enzyme activity
MTHFR	C677T	CC: <i>n</i> = 8	Normal
		CT: <i>n</i> = 8	Decreased
		TT: <i>n</i> = 8	Decreased
	A1298C	AA: <i>n</i> = 18	Normal
		AC: <i>n</i> = 6	Decreased
DPYD	*2A	GG: <i>n</i> = 24	Normal

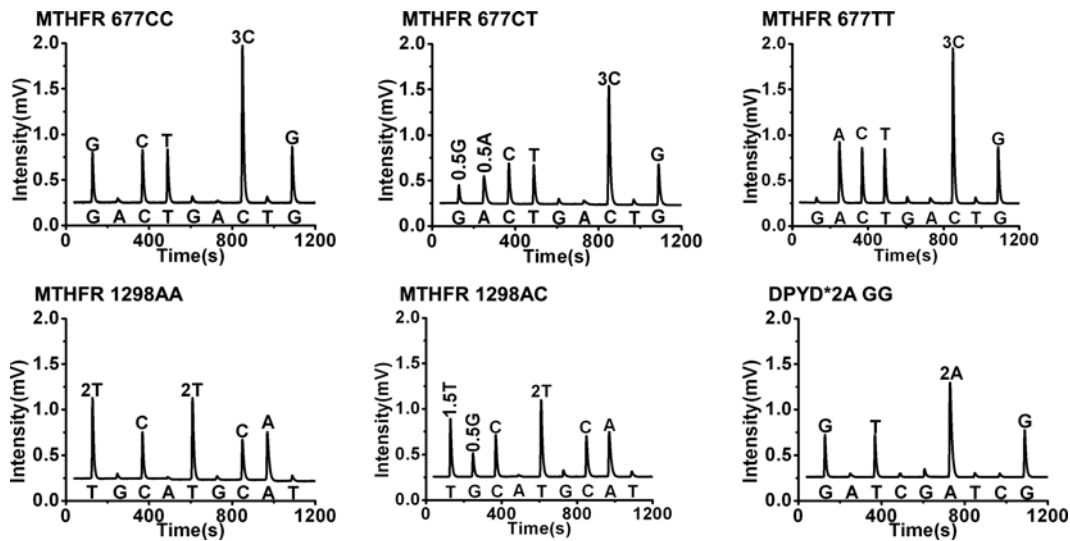


Fig. 3 Pyrograms from typical genotypes of the three polymorphisms in 24 samples

among 24 samples, indicating that the frequency of DPYD*2A is very low in the 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 the Chinese population before the administration of 5-FU. MTHFR C677T in a set of 5 typical samples was typed in triplicate, showing a very good reproducibility of our proposed method.

5 Technical Notes

1. The DNA samples should be stored at -20°C before use.
2. The T_m difference between the limiting primer and the excess primer is greater than 0°C , the T_m difference between amplicon and the excess primer is less than 13°C based on concentration-adjusted values. OligoAnalyzer 3.1 (<http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/>) was used to calculate the primers' T_m values [22].
3. There is no obvious difference in signal intensity among three kinds of anticoagulated blood (EDTA, citrate, and heparin). Therefore, the protocol of the proposed LATE-PCR using HpH buffer is independent of anticoagulant types.
4. Good pyrograms can be obtained when the volume of a blood sample for an assay is larger than $0.1\ \mu\text{L}$.
5. 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 [23]. We can directly employ a conventional pyrosequencing mixture to clean up the amplicons.
6. To compensate for the consumed APS, further addition of APS is necessary. The amount of APS for the compensation is not critical, and we found that it is enough to compensate $1\ \text{nmol}$ of APS for $1\ \mu\text{L}$ of LATE-PCR products.
7. This apparatus has a portable size of $140\ \text{mm (W)} \times 158\ \text{mm (H)} \times 250\ \text{mm (D)}$, with an array of 8 photodiodes (Hamamatsu Photonics K.K, Japan) to detect photo signals, and four separate capillaries to dispense small amounts of dNTPs into the reaction chamber.

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