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Single Nucleotide Polymorphism Genotyping Directly from Whole Blood and Filter Paper by Improved Allele Specific PCR

[1,2]Qingyao Wang, [1,2]Huachun Rao, [1,2]Huiyong Yang and [1,2]Yong Diao
[1]Institute of Molecular Medicine,
[2]Institute of Biomedicine, Huaqiao University, 362021,Quanzhou, China

Abstract: Tri-primer PCR has been developed into a sensitive and specific method for the genotyping of Single Nucleotide Polymorphism (SNP). However, the prior purification of genomic DNA from blood is necessary and time-consuming. In the current study, a special reaction system that leaves out the DNA extraction was investigated. Three different anticoagulant treatment, EDTA, heparin and sodium citrate, all allowed successfully amplication and genotyping directly from blood and filter paper. Spotted on filter paper was less difficult for amplification than crude blood. The methodology described facilitates genotyping with the advantages of immediate sample testing, lower experimental costs and time-saving.

Key words: Single nucleotide polymorphism, whole blood and filter paper, improved allele specific PCR

INTRODUCTION

Several Single Nucleotide Polymorphisms (SNPs) have been identified that associate with disease susceptibility especially complex disease, such as cardiovascular disease, gout and obesity (Merriman and Dalbeth, 2011; Choi *et al*., 2005; Ar'ev *et al*., 2011). The identification and detection of SNPs that are associated with disease that are helpful for understanding the biological mechanisms underlying disease and generating individualized risk profiles that are useful in a public health context (Reimer and Edwards, 2004).

Heme, hemin, lactoferrin and IgG, the known PCR inhibitors of blood, predominantly reduce the activity of DNA Taq polymerase (Al-Soud and Radstrom, 2001; Al-Soud *et al*., 2000). Thus, generally, genomic DNA purified from blood is necessary as template for successful amplification. However, the purification of target DNA requires normally expensive commercial kit. The residual of phenol/chlorine also directly affect PCR efficiency. Therefore, amplification directly from whole blood and filter paper seems to be a time-saving, labor-saving and low-cost method as long as the inhibitions from blood were overcame.

To improve the specificity of genotyping, improved tri-primer PCR was chosen in this study. Conventional tri-primer PCR is based on two inner primers which are designed to amplify the two allelic states and accurately extend only the correctly matched primer. But its poor specificity limits the further application. In order to increase the specificity of the amplification, an additional

MATERIALS AND METHODS

Sample collection: All blood samples were obtained from Quanzhou Osteopathy hospital treated with three different anticoagulant, 4.6 mmol L^{-1} EDTA, 21.3 U mL^{-1} of heparin, or 0.38% sodium citrate. Informed consent was obtained from each volunteer. The samples were anonymously coded and stored at -80°C before further processing. Dried blood spots were made by applying 0.6 μL of blood to 1.5 mm diameter on filter paper. The filter paper cards were air-dried at ambient temperature for minutes and then stored under airtight conditions at 4°C until required for analysis.

DNA extraction: Genomic DNA was extracted and purified from 200 μL peripheral venous blood according to the recommendations of Whole Blood DNA Extraction Kit (Beijing Sunbiotech Co., Ltd). The quantity and quality of the extracted DNA were checked using UV absorption measurement at 260 nm and agarose gel electrophoresis, respectively.

Basis of Tri-primer PCR method: Two SNPs (rs2231142(A/C), rs1165205(A/T)) were conducted to genotyping. Three primers were used in two PCR reactions. Two specific reverse primers, PRN1 and PRN2 (Table 1) (You *et al*., 2013), were designed with complementary 3-terminal nucleotide to the corresponding polymorphism with an additional mismatched base introduced in the third position of the 3th. PRN1 and

Corresponding Author: Huiyong Yang, Institute of Molecular Medicine and Institute of Biomedicine, Huaqiao University, 362021, Quanzhou, China

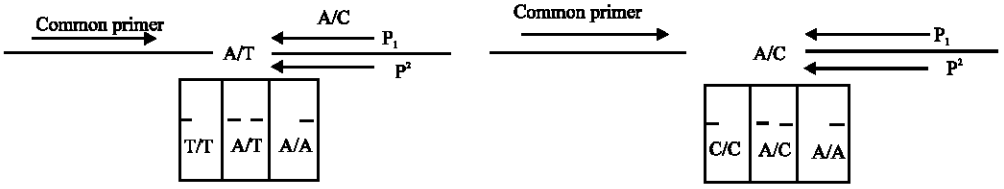
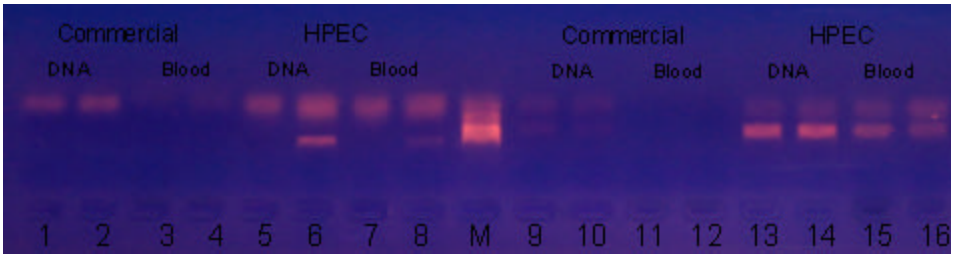


Fig. 1: Basic of Tri-primer PCR method for genotyping



SNP:rs22321142 SNP:rs1165205

Fig. 2(a-b): Tri-primer PCR amplify gnomic DNA and blood with commercial kit and HPEC. Genomic DNA was isolated from the same blood sample treated with EDTA. Two SNPs, 212 bp for the rs1165205, 297 bp for the rs2231142. Lane M, 50 bp DNA marker. PCR products were resolved in 2% agarose gel.

Table 1: Primers used for improved tri-primers PCR

SNP	Primers	Sequences(5'-3')	Tm(°C)	LA(bp)
rs1165205	PFW	CAATCCAAGCAGAGCCCCTAGTAG	59.4	
	PRN1	GCCTGGGAGATATTTTACCAAATGAGAA	57.5	
	PRN2	GCCTGGGAGATATTTTACCAAATGAGAT	57.5	212
rs2231142	PFW	TTGGCAAATCCTTGTATGAAGCAGT	57.6	
	PRN1	AGCCGAAGAGCTGCTGAGAA*T*TT	59.5	
	PRN2	AGCCGAAGAGCTGCTGAGAA*T*TG	60.1	297

PRN2 were added to two different tubes, a common forward primer PFW was used to amplify an allele-specific fragment with P1 or P2, respectively (Fig. 1).

Tri-primer PCR amplification of genomic DNA: The PCR was performed in a 20 μL reaction, containing 20-50 ng of genomic DNA as template, 0.4 μM of each primer, 0.2 mM of each dNTP, 1 U Taq DNA polymerase (Takara, Japan). PCR cycling was carried out in MasterCycler® thermal cycler (Eppendorf, Germany) with an initial denaturation at 96°C for 3 min, followed by 35 cycles of 94°C for 20 sec, 58.5°C for 20 sec and 72°C for 35 sec and a final extension at 72°C for 10 min. The products were analyzed with agarose gel (2%) consisting of 0.5 mg L^{-1} Specific nucleotides were underlined; Specificity-enhancing mismatches were underlined in bold italics.

Gel Red (Biotium, American) with 1×TAE buffer for 7 min at a constant voltage of 110 V. The result were analyzed by LUV-200A UV gel imaging system (Shanghai Luyang Instrument Co., Ltd).

Tri-primer PCR amplification directly from whole blood and filter paper: A special reaction system (HPEC) for whole blood and filter paper as follows: 3% whole blood or 1.5 mm diameter blood spots excised from filter papers as template, 0.4 μ mol L^{-1} of each primer, 0.2 mmol L^{-1} of each dNTP, 3.5 mmol L^{-1} of MgCl2, 20 mmol L^{-1} ammonium sulfate, 0.15% triton X-100, 0.6 mol L^{-1} D-(L)-trehalose and 10% glycerol, 1 U Taq DNA polymerase (Takara, Japan). PCR cycling and products analyses were same to Tri-primer PCR amplification of genomic DNA.

RESULTS

Tri-primer PCR amplification: Tri-primer PCR amplify gnomic DNA with commercial kit (Takara) and special reaction system (HPEC) showed the same specificity, but different in efficiency (Fig. 2). For blood sample, HPEC avoided the false-negative results compare to commercial kit. The fragment lengths for the specific amplicons were as shown: 212 bp for the rs1165205 A/T allele, 297 bp for the rs2231142 A/C allel.

Tri-primer PCR whole blood and filter paper analyses: Blood samples and dried blood spots were successfully

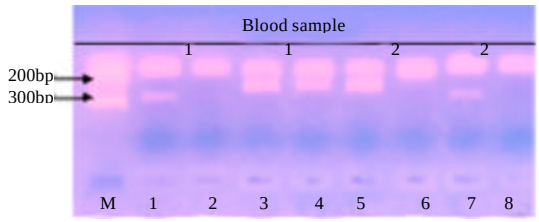


Fig. 3: Genotyping two different blood samples by improved Tri-primer PCR from whole blood treated with EDTA. Lane 1-2: Genotyping blood sample on SNP rs2231142 (297 bp), A/A. Lane 3-4: Genotyping blood sample 1 on SNP rs1165205 (212 bp), A/T. Lane 5-6: Genotyping blood sample 2 on SNP rs1165205 (212 bp), A/A. Lane 7-8: Genotyping blood sample on SNP rs2231142 (297 bp), A/A

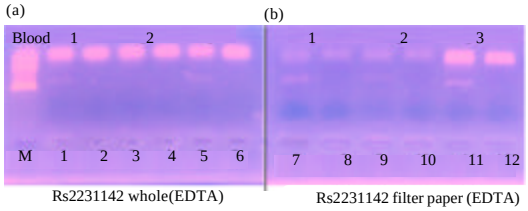


Fig. 4(a-b): Genotyping three different blood samples (EDTA) by improved Tri-primer PCR directly from whole blood and filter paper for SNP rs2231142. Lane 1, 2: Blood sample (a), A/A. Lane 7, 8: Blood sample 1 spotted on filter paper, A/A. Lane 3, 4: Blood sample (b), false-negative. Lane 9, 10: Blood sample 2 spotted on filter paper, A/A. Lane 5, 6: Blood sample 3, A/A. Lane11, 12: Blood sample 3 spotted on filter paper, A/A

genotyped with improved Tri-primer PCR under the facilitator of special reaction system (HEPC). This reaction system was also successfully challenged different anticoagulant treatments (sodium citrate, heparin and EDTA) (Fig. 3, 4, 5 and 6). Citrate, EDTA and heparin are the most common anticoagulants used for blood treatment in diagnostics. To our known, anticoagulants themselves were also inhibitors that affecting amplification efficiency. Among these anticoagulants, heparin was the most powerful inhibitor because of its binding and inactivation to Taq DNA polymerase with hight negative charge (Al-Soud and Radstrom, 2001). That determines blood sample treated with heparin was more difficult to amplify than citrate and EDTA (Fig. 3 and 6). Compared to whole blood, dried spot was much easier to observe successful amplification (Fig. 4 and 5).

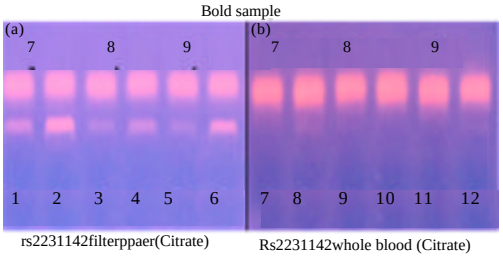


Fig. 5(a-b): Genotyping three different blood samples (Citrate) by improved Tri-primer PCR directly from whole blood and filter paper for SNP rs2231142. Lane 1, 2: Blood sample 7 spotted on filter paper, C/C. Lane 1 was false-positive. Lane 7, 8: Blood sample 7, C/C. Lane 3-4: Blood sample 8 spotted on filter paper, A/C. Lane 9, 10: Blood sample 8, false-negative. Lane 5, 6: Blood sample 9 spotted on filter paper, C/C, lane 5 was false-positive. Lane 11, 12: Blood sample 9, C/C.

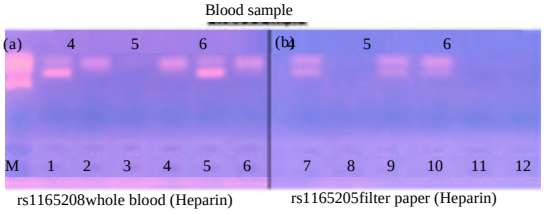


Fig. 6(a-b): Genotyping three different blood samples (Heparin) by improved Tri-primer PCR directly from whole blood and filter paper for SNP rs1165205. Lane 1, 2: Blood sample 4, A/A. Lane 7, 8: Blood sample 4 spotted on filter paper, A/A, lane 8 was false-negative. Lane 3, 4: Blood sample 5, false-negative. Lane 9, 10: Blood sample 5 spotted on filter paper, A/T. Lane 5-6: Blood sample 6, A/A. Lane 11, 12: Blood sample 6 spotted on filter paper, false-negative

DISCUSSION

For some crud sample, presence of inhibitors potently reduce the amplification efficiency. One strategy is using an alternative thermostable DNA polymerase or mutants of Taq DNA polymerase which is more resistant to inhibitors (Zhang *et al*., 2010). The other strategy is using amplification facilitators to enhance the amplification capacities of polymerases or neutralize inhibitory effect (Sharma *et al*., 2012). PCR additives reported previously usually only cover one aspect, helping either the

amplification efficiency or specificity. So different concentrations and combination of facilitators are tested in different labs to find novel efficient PCR enhancer cocktail systems for different difficult sample (Horakova *et al*., 2011).

For genotyping of SNP, allele specific PCR is a simple and reliable method includes tri-primer PCR and tetra-primer PCR. Compare to tri-primer method, tetra-primer method is more likely to produce false positive results because of a third common non-indicative PCR amplicon which can compete with the amplification of the indicative specific amplicons in a single tube (Yang *et al*., 2011). That means the method is more difficult to optimize. In order to improve the specificity of tri-prime allele PCR, an additional mismatched base was introduced in the third position of the 3'nd. However, the efficiency reduces 100 times or more accordingly (Koizumi *et al*., 2005).

Genotyping directly from whole blood eliminates the need of time-consuming and hight-cost DNA extraction and purification. But needs compatible PCR enhancer cocktail systems and suitable method. Amplification from dried blood spotted on filter papers is similar to amplification from whole blood. However, filter paper is less difficult for amplification than crude blood because heme and protein are fixed on paper rath than suspended in the PCR mixture. Suspended inhibitors may more likely to capture DNA polymerase giving a lower yield (Bu *et al*., 2008). Furthermore, filter paper is easy for sample collection, storage and shippment. Therefore, dried blood spot is a simple and reliable procedure for blood sampling and analysis especially in field studies (Aubry *et al*., 2012).

CONCLUSION

In summary, direct PCR from whole blood and filter paper provide a convenience for genotyping and other detection. Filter paper will be the most ideal choose for genotyping and detection because of more easier sampling, storage and transportation.

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