# Robust and efficient direct multiplex amplification method for large-scale DNA detection of blood samples on FTA cards

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**Abstract** Deoxyribonucleic acid (DNA) damage arising from radiations widely occurred along with the development of nuclear weapons and clinically wide application of computed tomography (CT) scan and nuclear medicine. All ionizing radiations (X-rays, γ-rays, alpha particles, etc.) and ultraviolet (UV) radiation lead to the DNA damage. Polymerase chain reaction (PCR) is one of the most wildly used techniques for detecting DNA damage as the amplification stops at the site of the damage. Improvements to enhance the efficiency of PCR are always required and remain a great challenge. Here we establish a multiplex PCR assay system (MPAS) that is served as a robust and efficient method for direct detection of target DNA sequences in genomic DNA. The establishment of the system is performed by adding a combination of PCR enhancers to standard PCR buffer. The performance of MPAS was demonstrated by carrying out the direct PCR amplification on 1.2 mm human blood punch using commercially available primer sets which include multiple primer pairs. The optimized PCR system resulted in high quality genotyping results without any inhibitory effect indicated and led to a full-profile success rate of 98.13%. Our studies demonstrate that the MPAS provides an efficient and robust method for obtaining sensitive, reliable and reproducible PCR results from human blood samples.

**Key words** PCR amplification, Multiplex PCR assay system, DNA detection

## 1 Introduction

DNA is one of the key targets for radiation-induced damage in a variety of organisms ranging from bacteria to human[1]. In humans, these radiation-induced DNA damage may lead to seriously hereditary diseases such as non-polyposis colon cancer as well as non-hereditary diseases, such as breast cancer[2,3]. Over the past few decades, a number of methods had been investigated for DNA damage detection based on various mechanisms[4-7]. PCR is one of the most reliable methods for detecting DNA damage as the amplification stops at the site of the damage[8,9]. PCR-based methods are generally very sensitive and easy to measure the gene-specific DNA damage, however they suffer from some limitations of sensitivity, stability and reproducibility[10]. For instance, it is completely based on template activity of the damaged DNA during amplification and the analysis depends on the intensity of an amplified band. Other factors like pippetting of different component of PCR mixture and amount of starting material (template) for amplification may also affect the band intensity and therefore an efficient and robust PCR method is required to overcome the above difficulties.

Fluorescent multiplex PCR is a widespread molecular biology technique for amplification of multiple loci in a single PCR reaction[8,11]. During the last decades, this technique has been actively explored for applications in numerous areas including forensic DNA analysis[12,13], SNP analysis[14], clinical molec ular diagnostic[15,16] and provides a highly effective way of generating PCR products from target sequences. Despite their widespread use, however, in the field of

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DNA detection, the standard procedure often fails to produce meaningful amplification due to the presence of inhibitors, such as immunoglobulin G[17], heme[18], hemoglobin and $FeCl_3$[19] that have inhibitory effect on the multiplex PCR amplification. These inhibitors can often be removed, at least partly, in the sample preparation and extraction steps, which are either time-consuming or laborious[20]. Therefore, further application of fluorescent multiplex PCR for DNA detection makes it necessary to take novel methods to efficiently meet the requirements of effectiveness, sensitivity and reproducibility.

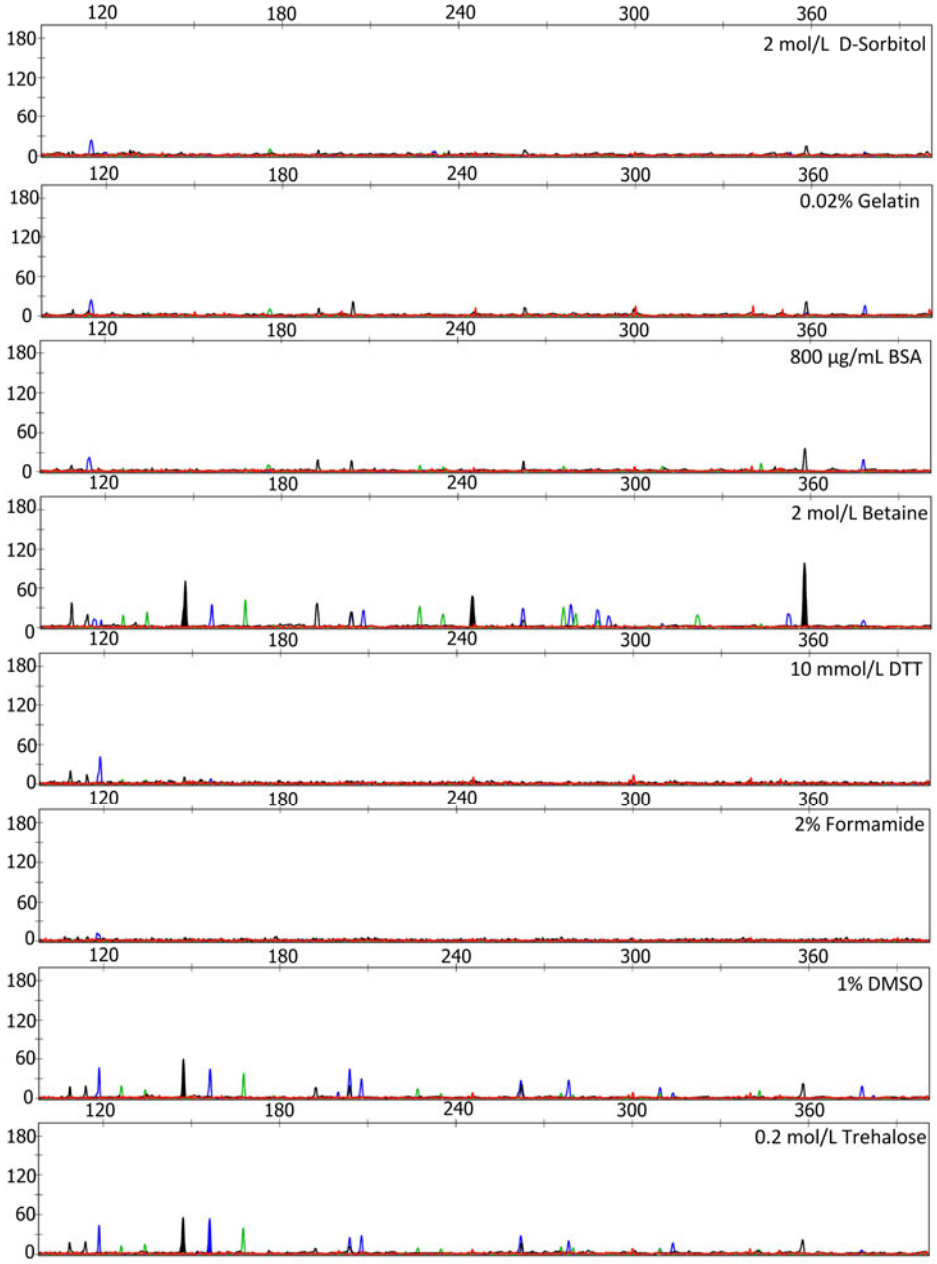


**Fig.1** Genotyping of 1.2-mm FTA blood punches using newly formulated reaction buffer systems based on different PCR enhancers. Eight different routine PCR enhancers were tested：2 mol/L D-Sorbitol, 0.02% Gelatin, 800 μg/mL BSA, 2 mol/L Betaine, 10 mmol/L DTT, 2% Formamide, 1% DMSO and 0.2 mol/L Trehalose (from top to bottom, respectively).

In an attempt to overcome these shortcomings and also to increase the efficiency of sample processing for DNA detection, a variant termed direct multiplex PCR was introduced to the field of DNA detection[21-23]. With a direct PCR technique, human biological samples can be amplified directly without the need for DNA extraction prior to PCR. The direct multiplex PCR has the potential to produce considerable savings of time, expense and effort on detection of biological samples by eliminating the

procedures involved in DNA extraction and quantification. The use of the direct multiplex PCR simplifies detection procedures, enables the effective processing of a large number of samples at minimal costs, and makes the possible improvement of sample processing efficiency for PCR-based DNA detection methods. Unfortunately, complete failure of DNA detection was often happened for certain samples such as blood samples stored on FTA cards. FTA cards are poor substrates for direct amplification due to the presence of inhibitors such as Ethylenediaminetetraacetic acid (EDTA), Sodium dodecyl sulfate (SDS), uric acid, etc. These inhibitors can significantly influence the quality of PCR results and the genotyping success rates of large-scale human blood samples[24,25]. The following factors often result in the failure of PCR amplification: 1) the characteristic of substrates used for storage of samples, 2) the size of samples added to the PCR reaction, 3) inappropriate sampling position, 4) inhibition of PCR caused by the presence of various inhibitors. Of many factors above, PCR inhibition appears to be the major obstacle to success of direct PCR on "difficulty" FTA samples[21,26]. Accordingly, a straightforward solution to problems described above has been the improvements of the efficiency of PCR amplification.

Improvement of amplification has been the focus of many research efforts. It has been found that various additives can often yield significant improvements in this regard, the most commonly reported additives tested being Dimethyl sulfoxide (DMSO), polyethylene glycol (PEG), betaine and formamide[19,20,27,28], etc.

Here we developed an effective direct multiplex PCR assay that can broadly improve the performance of PCR-based DNA detection. Complete DNA profiles could be obtained from the 1.2-mm diameter punch in 10 μL reaction volume using the established direct PCR reaction system. This system contains all the components necessary for the direct multiplex PCR. Furthermore, the performances of this reaction system was compared with those of commercialized kits.

## 2 Materials and methods

### 2.1 Materials

Blood samples were collected from anonymous volunteers. FTA® cards and Harris Micro-Punch® items were purchased from Whatman®, Inc. 50 μL of whole blood from anonymous donor was applied onto the FTA® cards and dried according to the manufacturer's instructions. The control DNA 9947A was purchased from Applied Biosystems. Paper card discs were prepared using the Harris Manual Punch. All other reagents were purchased from Sigma and were of PCR grade.

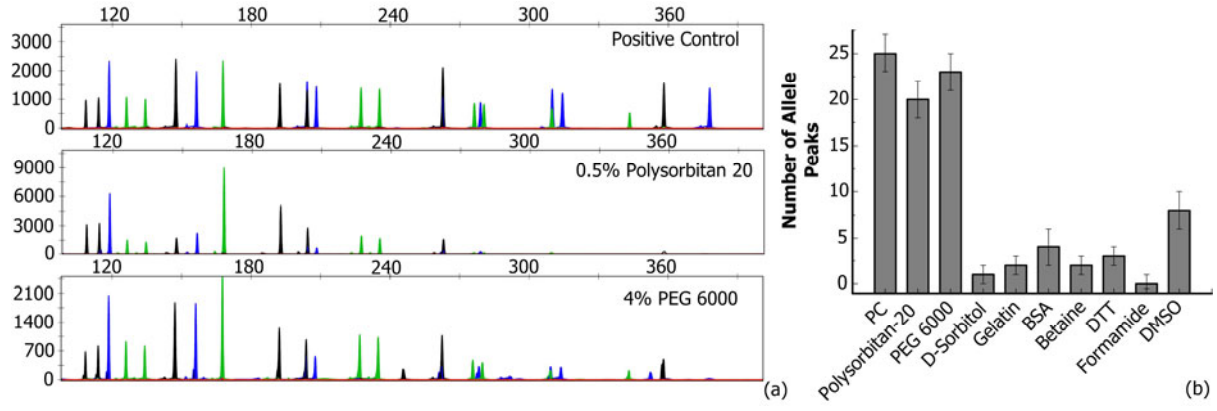


**Fig.2** Effect of 4% PEG 6000 and 0.5% Polysorbitan 20 on direct multiplex amplification. (a) Genotyping of 1.2 mm FTA blood punches using newly formulated reaction buffer systems based on 4% PEG 6000 and 0.5% Polysorbitan 20, and (b) the effect of different PCR enhancers on the number of allele peaks.

### 2.2 Direct multiplex PCR reaction set up

The standard 10×multiplex PCR reaction is composed of 500 mmol/L potassium chloride, 100 mmol/L Tris-HCl (pH 8.3 at room temperature), 15 mmol/L magnesium chloride, 2 mmol/L of each dNTP and 1000 U/mL of AmpliTaq Gold (Applied Biosystems).

Several different selected PCR enhancers were added to the standard reactions. All other reagents used were of PCR grade. Direct multiplex PCR reactions are composed of the standard reaction and a variety of PCR enhancers.

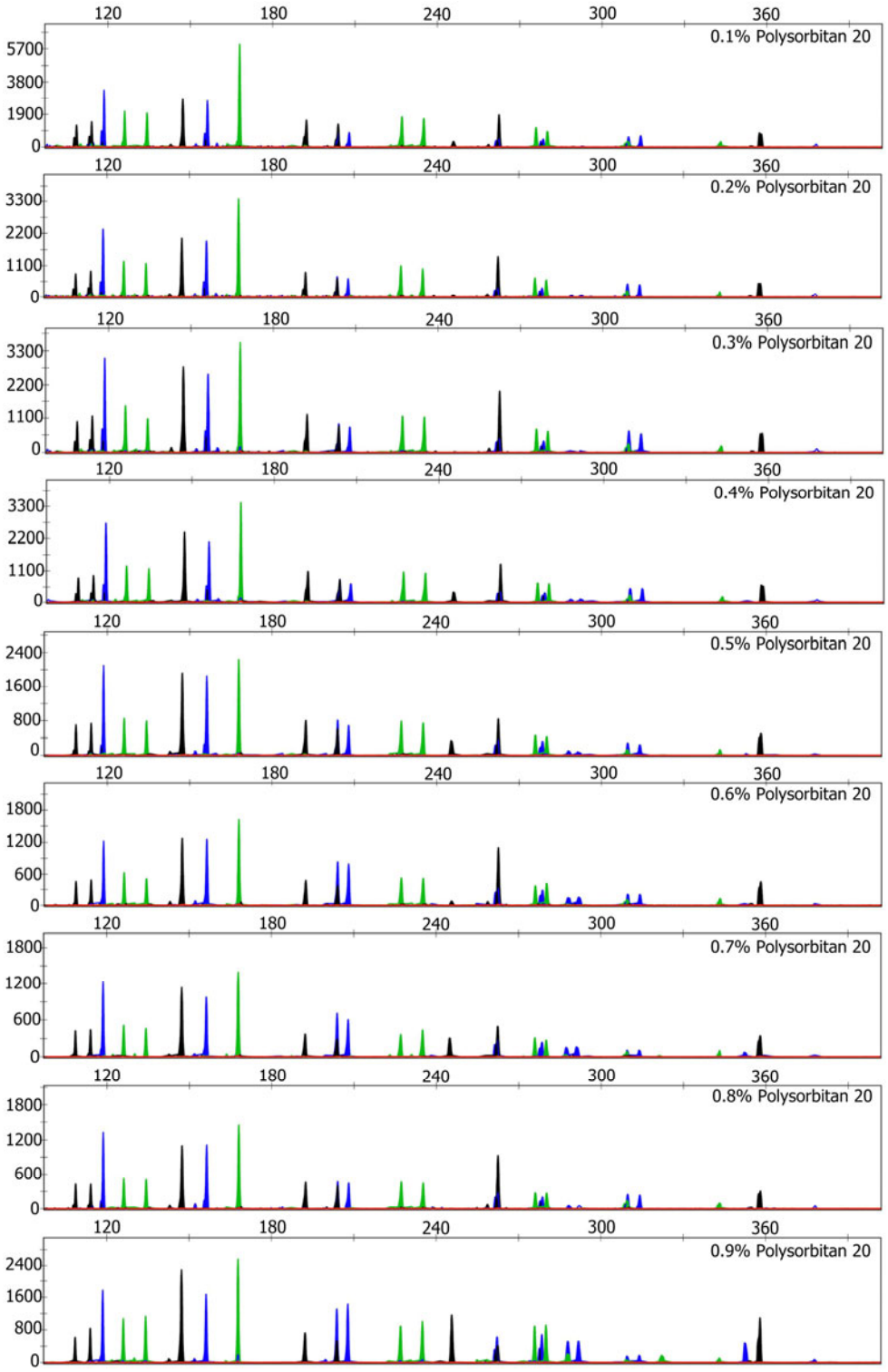


**Fig.3** Effect of various polysorbitan 20 concentrations on direct multiplex amplification. Nine concentrations were tested as 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8% and 0.9%, respectively.

Direct multiplex PCR amplifications were performed in a 10 μL or 25 μL final reaction volumes. For 25 μL reaction, it was consisted of 12.5 μL enzyme-containing direct multiplex PCR buffer, 12.5 μL of the primer set. And it was consisted of 5 μL enzyme-containing direct multiplex PCR buffer, 5 μL

of the primer sets for a 10-μL reaction volume. In addition to other special descriptions, the commercially available direct amplification reactions were carried out in a 10 μL reaction volume. Set-up occurred as recommended by the manufacturer.

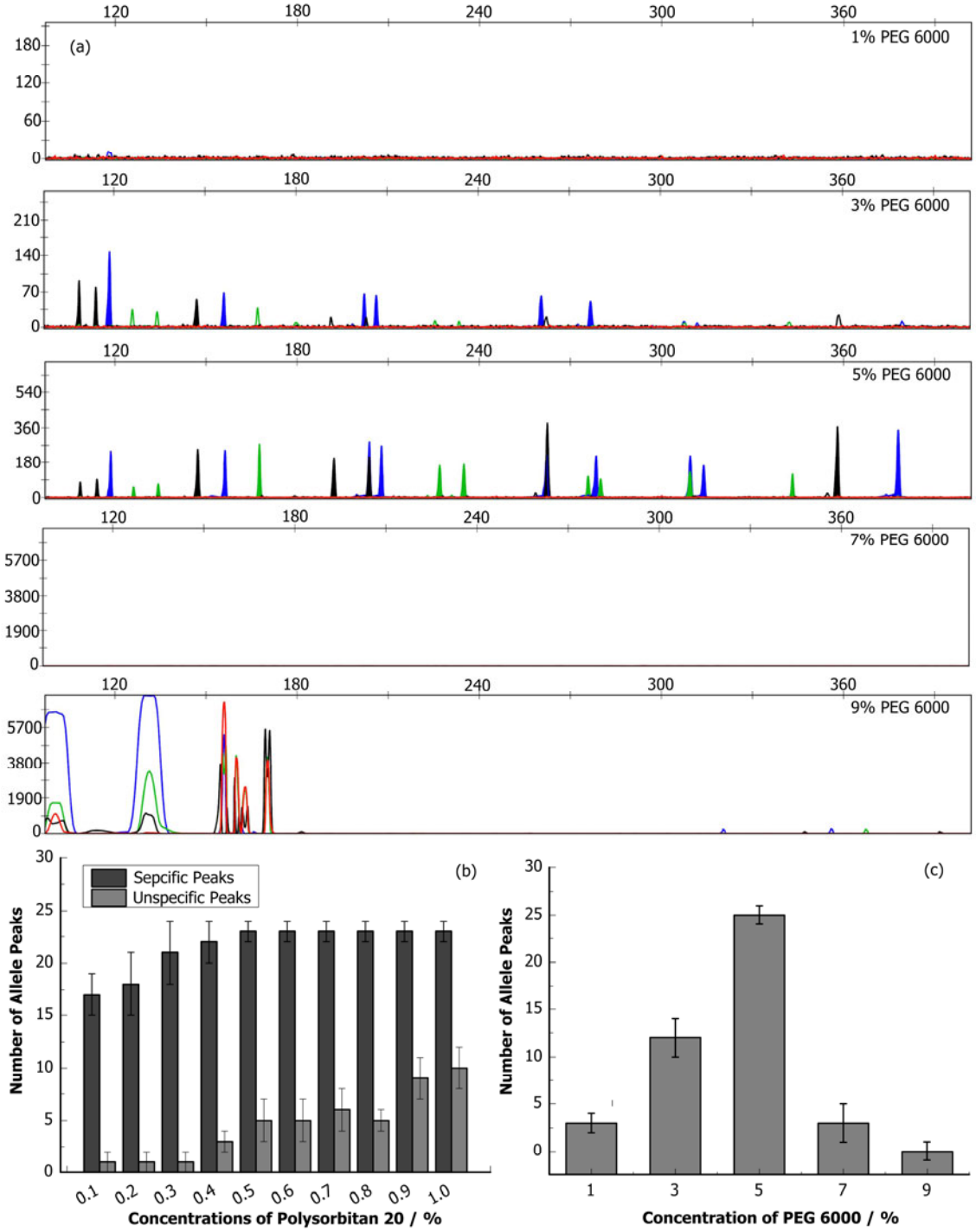


**Fig.4** (a) Effect of various PEG 6000 concentrations on direct multiplex amplification. Five concentrations were tested: 1%, 3%, 5%, 7% and 9%, (b) the average numbers of alleles including specific and unspecific peaks generated by polysortitan 20 contained buffer and (c) PEG 6000 contained buffer.

## 2.3 PCR amplification

All amplifications were performed on the GeneAmp PCR system 9700 (Applied Biosystems). The amplification parameters were as follows: 95℃ for 11 min, 28 cycles of 94℃ for 20 s, 59℃ for 2 min, and 72℃ for 1 min, a final extension at 60℃ for 30 min,

and 4℃ hold permanently. The DNATyper15 kit was obtained from Institute of Forensic Science Ministry of Public Security China. The Identifiler® PCR amplification kit was obtained from Applied Biosystems. A 1.2 mm diameter punch of a blood spotted FTA paper card was directly placed into direct PCR reactions.

### 2.4 Capillary electrophoresis

Amplification products were electrophoresed on an ABI 3130xl Genetic Analyzer (Applied Biosystems) and analyzed using GeneMapper® ID Software v3.2.1 (Applied Biosystems). Capillary electrophoresis was performed on ABI 3130xl Genetic Analyzer (Applied Biosystems) using POP-4™ polymer (Applied Biosystems). An aliquot of 1-3 μL of each PCR product was mixed with 10 μL of Hi-Di™ Formamide and 0.3 μL of ABI GeneScan-500 LIZ Size Standard, denatured at 95℃ for 3 min and cooled at 0℃ for 3 min. Electrophoresis was conducted at 3 kV 10 s and the data was collected by ABI Collection Software v1.1. Allele sizes were determined using the GeneMapper® ID Software v3.2.1 software and the Local Southern size calling method. The detection threshold was set at 50 relative fluorescence units (RFU).

### 2.5 Optimization of PCR reaction buffer

Several PCR enhancers were added to the standard reactions to characterize the effect of PCR enhancers on the performance of direct PCR reaction. Various PCR additives were contained in standard buffer at following concentrations, 2% DMSO, 5% polyethylene glycol, 800 μg/mL of bovine serum albium (BSA) and 0.5% of non-ionic detergents (polysorbitan 20), 2 mol/L Betaine, 0.02% gelatin, 10 mmol/L DTT, 0.2 mol/L trehalose, 2% formamide etc. The effectiveness of various additives was described through the quality of STR profiles.

### 2.6 Comparative studies

A comparative study was made between the newly established direct amplification PCR system and the commercially available direct amplification kit on human blood samples. All samples were amplified according to the protocols recommended. Human blood on FTA cards were directly placed to the amplification reactions. The present system was evaluated and compared to the commercially available kit by the qualities of profiles obtained from above samples. The reaction volume for all methods was 10 μL. Concordance of genotyping profiles was examined by comparison with those previously obtained results.

### 2.7 Concordance of genotyping profiles

A total of 107 DNA database samples on FTA cards were collected for performing concordance study of the current method. Direct amplifications were performed in a 10 μL of PCR reaction volumes using 1.2 mm discs punched out of the sample in the FTA card. Amplification products were electrophoresed on an ABI 3130xl Genetic Analyzer (Applied Biosystems) and analyzed using GeneMapper® ID Software v3.2.1 (Applied Biosystems).

## 3 Results and discussion

### 3.1 Optimization of PCR reaction buffer

The use of PCR additives can help to decrease the effect of inhibitors in the reaction by complexing them, annulling their effects and positively influencing amplification efficiency (based on multiple mechanisms). Some of the most commonly used PCR additives, such as DMSO, glycerol, BSA, nonionic detergents can have varying effects on PCR reactions. Several reactions incorporating these additives were formulated. One reaction containing no additives was also assembled. A 1.2 mm FTA blood punch was directly amplified using the newly formulated reactions above, with the inclusion of various standard additives. Each PCR was carried out in a reaction volume of 10 μL. In addition to polysorbitan 20 and PEG 6000, neither of these “difficulty” samples amplified in the presence of the standard 10×PCR buffer alone, and the addition of either DMSO, BSA, Betaine, Gelatin, Formamide, Trehalose, D-Sorbitol and DTT did not greatly improve the results (Fig.1). Only partial DNA profiles were obtained in the presence of nonionic detergents and polyethylene glycol (Fig.2). The effectiveness of tested buffer was evaluated by the number of allele peaks (Fig.2b).

However, results presented in Fig.3 and Fig.4a indicated that the quality of profiles can be improved by increasing concentrations of polysobitan 20 and PEG 6000 to standard PCR reactions respectively. The effects of these two additives on the multiplex PCR reaction can vary from non-specific amplification to unusual peak morphology and suboptimal peak heights (Figs.4b and 4c).

It is worth noting of that successful direct amplification could only be obtained from the reactions containing a combination of multiple PCR enhancers (0.9% polysorbitan 20 and 5% PEG 6000) (P-P enhancers) (Fig.5). The experiments were repeated for 3 times. The inclusion of the two PCR enhancers resulted in high quality profiles without any inhibitory effects indicated.

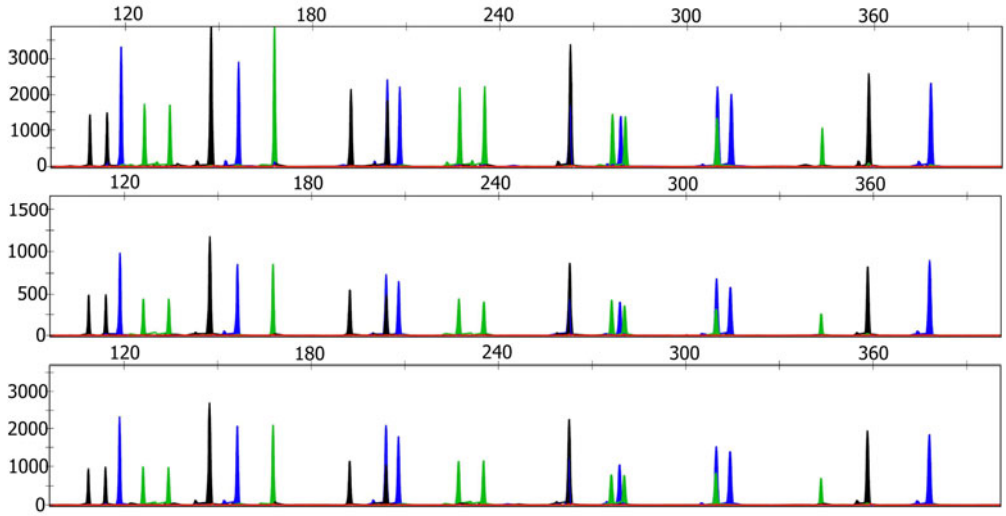


**Fig.5** Direct PCR amplification of database samples preserved on FTA cards using PPD. Each experiment was repeated in triplicate.

### 3.2 Comparative studies

To further examine the performance of the P-P enhancer-contained direct buffer (PPD), a commercially available kit, AmpFlSTR Identifiler direct amplification kit (IDD), was used to amplify and type STR loci directly from 1.2 mm FTA blood punch without the procedure for DNA purification and pre-washing. As anticipated, in a 25 μL or 15 μL of amplification volume, a full STR profile can be obtained from 1.2 mm FTA discs using both PPD and IDD kit (Fig.6), even though some inhibitory effects for IDD indicated in Fig.6. For the inputs of 1.2 mm diameter punch FTA cards in a 10 μL of amplification volume, IDD failed completely to produce a DNA profile (Fig.6). However, the P-P enhancer contained in reagent systems generally improved the ability to type "difficulty" sample and gave reliable and high quality results (Fig.6). Each set of experiment was run in triplicate. Accordingly, the relatively enlargement of the size of FTA discs may lead to a complete failure of profiles due to the overwhelming quantity of PCR inhibitors present in the lower amplification volumes. These results indicated that the PPD buffer is comparable to other commercial direct PCR amplification kits.

### 3.3 Success rate of direct amplification and concordance of genotyping profiles

The success rate of direct multiplex PCR was one of the most critical factors need to be considered for the evaluation of the performance of PPD system. A total of 107 blood samples, were collected to examine the success rate of direct PCR amplification. Of the 107 FTA blood samples, 105 resulted in full genotyping profiles without any inhibitory effects indicated. 2 generate partial profiles and without any sample resulted in complete failure of PCR amplification. Overall, FTA blood punches resulted in a full-profile success rate of 98.13%. The majority of heterozygous peaks heights were in the range of 1000–6800RFU (data not shown).

The genotyping profiles obtained from the above samples showed 100% concordance with previously obtained results, suggesting that PPD buffer system is able to provide a consistent and reliable method for detecting DNA from human samples.

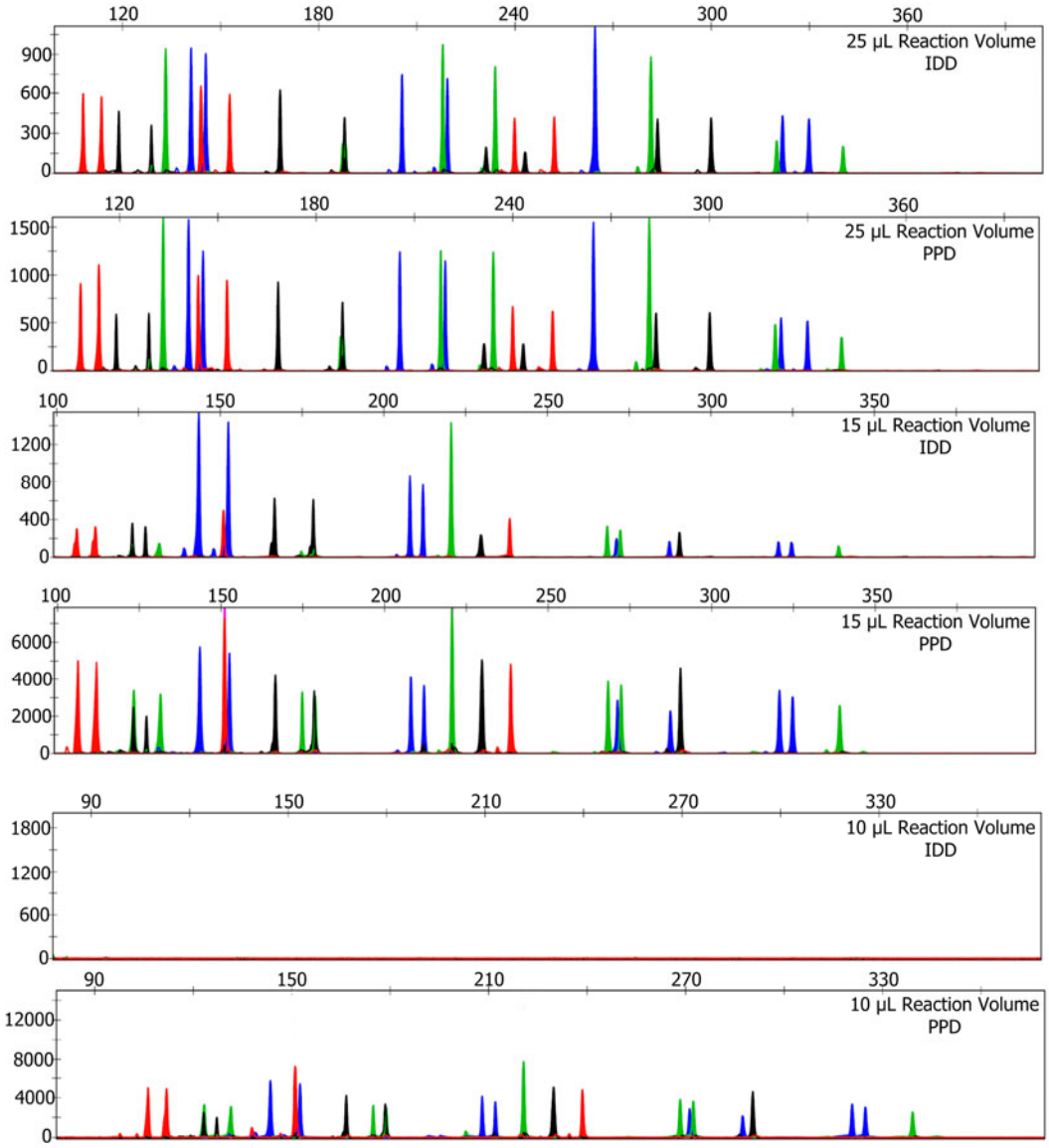


**Fig.6** Comparison of the performance of P-P Direct buffer (PPD) with commercially available identifiler direct kit (IDD) by amplifying 1.2 mm FTA blood punch in different reaction volume.

## 4 Conclusion

In this work, we established an optimum method for direct detection of human DNA from blood stored on FTA cards. The method provides an efficient way which can significantly simplify the sample handling procedure. It is clear that there is a need to combine the application of different PCR enhancers to develop a robust assay that can overcome PCR inhibition. This may be helpful in developing excellent strategies and providing better solutions for large-scale DNA detection from human samples.

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