Robust and Efficient Direct Multiplex Amplification Method for Large-Scale DNA Detection of Blood Samples on FTA Cards (Postprint)

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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.

Full Text

Preamble

Robust and Efficient Direct Multiplex Amplification Method for Large-Scale DNA Detection of Blood Samples on FTA Cards

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Abstract
DNA damage from radiation exposure has become increasingly common with the development of nuclear weapons and the widespread clinical use of CT scans and nuclear medicine. All ionizing radiations (X-rays, γ-rays, alpha particles, etc.) and ultraviolet radiation cause DNA damage. Polymerase chain reaction (PCR) is one of the most widely used techniques for detecting DNA damage, as amplification terminates at the site of damage. Enhancing PCR efficiency remains a critical challenge. Here we establish a multiplex PCR assay system (MPAS) that serves as a robust and efficient method for direct detection of target DNA sequences in genomic DNA. This system is developed by adding a combination of PCR enhancers to standard PCR buffer. MPAS performance was demonstrated through direct PCR amplification on 1.2 mm human blood punches using commercially available primer sets containing multiple primer pairs. The optimized PCR system produced high-quality genotyping results without any inhibitory effects, achieving a full-profile success rate of 98.13%. Our studies demonstrate that 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

Introduction

DNA is one of the key targets for radiation-induced damage across various organisms, from bacteria to humans [1]. Radiation-induced DNA damage can lead to serious hereditary diseases such as non-polyposis colon cancer, as well as non-hereditary diseases like breast cancer [2,3]. Over the past few decades, numerous DNA damage detection methods have been investigated based on various mechanisms [4-7]. PCR is among the most reliable methods for detecting DNA damage because amplification stops at the damage site [8,9]. While PCR-based methods are generally very sensitive and facilitate easy measurement of gene-specific DNA damage, they suffer from limitations in reproducibility [10]. For instance, the analysis depends entirely on the template activity of damaged DNA during amplification and relies on the intensity of amplified bands.

Other factors, such as pipetting of different PCR mixture components and the amount of starting template material, may also affect band intensity. Therefore, an efficient and robust PCR method is required to overcome these difficulties.

Fluorescent multiplex PCR is a widespread molecular biology technique for amplifying multiple loci in a single reaction [8,11]. Over the last decades, this technique has been actively explored for applications in numerous areas including forensic DNA analysis [12,13], SNP analysis [14], and clinical molecular

diagnostics [15,16], providing a highly effective way to generate PCR products from target sequences. Despite their widespread use, standard procedures often fail to produce meaningful amplification in DNA detection due to the presence of inhibitors such as immunoglobulin G [17], heme [18], hemoglobin, and $FeCl_3$, which inhibit multiplex PCR amplification. These inhibitors can often be removed, at least partially, through sample preparation and extraction steps that are either time-consuming or laborious [20]. Therefore, further application of fluorescent multiplex PCR for DNA detection necessitates novel methods to efficiently meet requirements for effectiveness, sensitivity, and reproducibility.

[Figure 1: see original paper] 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 g/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).

To overcome these shortcomings and improve sample processing efficiency for DNA detection, a variant termed direct multiplex PCR was introduced to the field [21-23]. With direct PCR techniques, human biological samples can be amplified directly without prior DNA extraction. Direct multiplex PCR offers considerable savings in time, expense, and effort for biological sample detection by eliminating DNA extraction and quantification procedures. This approach simplifies detection workflows, enables effective processing of large numbers of samples at minimal cost, and improves sample processing efficiency for PCR-based DNA detection methods. Unfortunately, complete failure of DNA detection often occurred with certain samples such as blood stored on FTA cards. FTA cards are poor substrates for direct amplification due to the presence of inhibitors (EDTA, sodium dodecyl sulfate (SDS), uric acid, etc.). These inhibitors can significantly affect PCR result quality and genotyping success rates for large-scale human blood samples [24,25]. Several factors often contribute to these failures: 1) characteristics of substrates used for sample storage, 2) sample size added to the PCR reaction, 3) inappropriate sampling position, and 4) PCR inhibition caused by various inhibitors. Among these factors, PCR inhibition appears to be the major obstacle to successful direct PCR on "difficult" FTA samples [21,26]. Accordingly, a straightforward solution involves improving PCR amplification efficiency.

Improvement of amplification has been the focus of many research efforts. It has been found that various additives can often yield significant improvements in PCR amplification. The most commonly reported additives tested include dimethyl sulfoxide (DMSO), polyethylene glycol (PEG), betaine, and formamide [19,20,27,28], among others.

Here we developed an effective direct multiplex PCR assay that broadly improves the performance of PCR-based DNA detection. Complete DNA profiles could be obtained from 1.2-mm diameter punches in 10 µL reaction volume using the established direct PCR reaction system. This system contains all components necessary for direct multiplex PCR. Furthermore, the performance

of this reaction system was compared with commercialized kits.

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2.1 Materials

Blood samples were collected from anonymous volunteers. FTA® cards and Harris Micro-Punch® items were purchased from Whatman®, Inc. Fifty µL of whole blood from anonymous donors was applied onto FTA® cards and dried according to manufacturer instructions. 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.

[Figure 2: see original paper] 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 Setup

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 selected PCR enhancers were added to the standard reactions. All reagents used were of PCR grade. Direct multiplex PCR reactions consist of the standard reaction and various PCR enhancers.

Direct multiplex PCR amplifications were performed in 10 µL or 25 µL final reaction volumes. For 25 µL reactions, the mixture consisted of 12.5 µL enzyme-containing direct multiplex PCR buffer and 12.5 µL of primer set. For 10 µL reaction volumes, it consisted of 5 µL enzyme-containing direct multiplex PCR buffer and 5 µL of primer sets. Commercially available direct amplification reactions were carried out in 10 µL reaction volume as recommended by the manufacturer.

[Figure 3: see original paper] Effect of various polysorbitan 20 concentrations on direct multiplex amplification. Nine concentrations were tested: 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, and 0.9%, respectively.

[Figure 4: see original paper] (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 polysorbitan 20-containing buffer and (c) PEG 6000-containing buffer.

2.3 PCR Amplification

All amplifications were performed on the GeneAmp PCR System 9700 (Applied Biosystems). The amplification parameters were as follows: 95°C for 11 min, 28 cycles of 94°C for 20 s, 59°C for 2 min, and 72°C for 1 min, followed by a final extension at 60°C for 30 min, and permanent hold at 4°C. The DNATyper15 kit was obtained from the 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 blood-spotted FTA paper card was placed directly into 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 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°C for 3 min and cooled at 0°C for 3 min. Electrophoresis was conducted at 3 kV for 10 s and data was collected by ABI Collection Software v1.1. Allele sizes were determined using GeneMapper® ID Software v3.2.1 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 standard reactions to characterize their effects on direct PCR performance. Various PCR additives were included in standard buffer at the following concentrations: 2% DMSO, 4% polyethylene glycol, 800 µg/mL bovine serum albumin (BSA), 0.5% 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 evaluated through STR profile quality.

2.6 Comparative Studies

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

2.7 Concordance of Genotyping Profiles

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

3.1 Optimization of PCR Reaction Buffer

PCR additives can help decrease inhibitor effects by complexing them, nullifying their effects, and positively influencing amplification through multiple mechanisms. Some commonly used PCR additives such as DMSO, glycerol, BSA, and nonionic detergents can have varying effects on PCR reactions. Several reactions incorporating these additives were formulated, along with one reaction containing no additives. A 1.2 mm FTA blood punch was directly amplified using these formulated reactions with various standard additives. Each PCR was carried out in a 10 µL reaction volume. Neither of these "difficult" samples amplified in the presence of standard 10× PCR buffer alone, and addition of DMSO, BSA, betaine, gelatin, formamide, trehalose, D-sorbitol, or DTT did not greatly improve results (Fig.1). Only partial DNA profiles were obtained in the presence of nonionic detergents and polyethylene glycol (Fig.2). The effectiveness of tested buffers was evaluated by the number of allele peaks (Fig.2b). However, results presented in Fig.3 and Fig.4a indicated that profile quality could be improved by increasing concentrations of polysorbitan 20 and PEG 6000 in standard PCR reactions, respectively. The effects of these two additives on multiplex PCR reaction could vary from non-specific amplification to unusual peak morphology and suboptimal peak heights (Figs.4b and 4c).

Notably, successful direct amplification could only be obtained from reactions containing a combination of multiple PCR enhancers (0.9% polysorbitan 20 and 5% PEG 6000) (Fig.5). The experiments were repeated three times. The inclusion of these two PCR enhancers resulted in high-quality profiles without any inhibitory effects.

[Figure 5: see original paper] 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-containing 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 punches without DNA purification or pre-washing. As anticipated, in 25 µL or 15 µL amplification volumes, full STR profiles could be obtained from 1.2 mm FTA discs using both PPD and IDD kits (Fig.6), although some

inhibitory effects were indicated for IDD in Fig.6. For 1.2 mm diameter FTA card punches in 10 µL amplification volume, IDD completely failed to produce DNA profiles (Fig.6). However, the P-P enhancer-containing reagent systems generally improved the ability to type "difficult" samples and yielded reliable, high-quality results (Fig.6). Each experiment was run in triplicate. Accordingly, relatively larger FTA disc sizes may lead to complete profile failure due to overwhelming quantities of PCR inhibitors present in lower amplification volumes. These results indicated that PPD buffer performance was comparable to other commercial direct PCR amplification kits.

[Figure 6: see original paper] 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 volumes.

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 for evaluating PPD system performance. A total of 107 blood samples were collected to examine direct PCR amplification success rate. Of these 107 FTA blood samples, 105 yielded full genotyping profiles without any inhibitory effects, 2 generated partial profiles, and no samples resulted in complete PCR amplification failure. Overall, FTA blood punches achieved a full-profile success rate of 98.13%. The majority of heterozygous peak heights ranged from 1000–6800 RFU (data not shown). Genotyping profiles obtained from these samples showed 100% concordance with previously obtained results, suggesting that the PPD buffer system provides a consistent and reliable method for detecting DNA from human samples.

References

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 approach that significantly simplifies sample handling procedures. It is clear that combining different PCR enhancers is necessary 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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Note: Figure translations are in progress. See original paper for figures.