Forensic Science International: Genetics 6 (2012) 185–190

Contents lists available at ScienceDirect

Forensic Science International: Genetics

journal homepage: www.elsevier.com/locate/fsig

Evaluation of circular DNA substrates for whole genome amplification prior to forensic analysis

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ARTICLE INFO

Article history:
Received 27 November 2010
Received in revised form 3 March 2011
Accepted 4 April 2011

Keywords:
Whole genome amplification (WGA)
Rolling circle amplification (RCA)
Multiple displacement amplification (MDA)
Forensic DNA analysis

ABSTRACT

Forensic biological evidence often contains low quantities of DNA or substantially degraded DNA which makes samples refractory to genotype analysis. One approach that shows promise to overcome the limited quantity of DNA is whole genome amplification (WGA). One WGA technique, termed rolling circle amplification (RCA), involves the amplification of circular DNA fragments and this study evaluates a single-stranded (ss) DNA ligase enzyme for generating circular DNA templates for RCA WGA. Fast, efficient ligation of several sizes of ssDNA templates was achieved. The enzyme also ligated double-stranded (ds) DNA templates, a novel activity not previously reported. Adapter sequences containing optimal terminal nucleotide ends for increased ligation efficiency were designed and ligation of adapters to template DNA was optimized. Increased amplification of DNA templates was observed following WGA; however, no amplification advantage for ssDNA ligase treatment of templates was evident compared to linear templates. A multi-step process to utilize ssDNA ligase prior to WGA was developed and short tandem repeat (STR) analysis of simulated low template (LT) and fragmented DNA was evaluated. The process resulted in the loss of template DNA and failed STR analysis whereas input of linear genomic DNA template directly into WGA prior to STR analysis improved STR genotyping results compared to non-WGA treated samples. Inclusion of an extreme thermostable single-stranded DNA binding protein (SSB) during WGA also increased DNA yields. While STR artifacts such as peak imbalance, drop-in, and dropout persisted, WGA shows potential for successful genetic profiling of LT and fragmented DNA samples. Further research and development is warranted prior to use of WGA in forensic casework.

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1. Introduction

DNA has many characteristics that make it a robust material for individual identification. However, DNA samples obtained for forensic casework can contain very limited amounts of DNA referred to as low template (LT) DNA or can be highly degraded. Obtaining complete genetic profiles may be challenging or even impossible using standard short tandem repeat (STR) amplification or mitochondrial DNA (mtDNA) sequencing. Forensic DNA analysis has advanced considerably over the past 20 years through the development of new techniques, such as the use of miniSTR analysis, which involves amplification of smaller DNA targets [1]. This technique has enabled genotype analysis of some samples which contain degraded nuclear DNA [2,3]. Several proposed assays which target single nucleotide polymorphisms (SNPs) also evaluate small DNA regions and represent alternative approaches to forensic analysis of challenging samples [4].

Whole genome amplification (WGA), a method intended to increase the quantity of starting DNA template, has the potential to improve genetic profiling of refractory forensic DNA samples. In principle, WGA can amplify DNA template in an unbiased manner with complete genome coverage and high accuracy in an isothermal reaction [5,6]. WGA applications have promise for clinical samples [7,8], single cells [9–13], formalin-fixed, paraffin-embedded samples [14–18], post-mortem brain samples [19], and archived samples [20,21]. WGA has also successfully amplified DNA for STR genetic profiling from LT or degraded DNA samples, including touch DNA obtained from single dermal ridge fingerprints [22–26], for mtDNA sequencing [27], and for SNP genotyping [4,16,28–30]. However, the application of WGA to forensic casework is complicated by the occurrence of STR technical

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1872-4973/$ – see front matter. Published by Elsevier Ireland Ltd.
doi:10.1016/j.fsigen.2011.04.011

artifacts, such as preferential allele amplification (leading to heterozygous peak imbalance), allele dropout, increased stutter, and non-specific generation of extra alleles (allele drop-in) [22,31–33]. In addition, some reports have suggested poor performance of WGA on forensic casework samples [22,31].

WGA is most successful when using high quality template but can fail to yield sufficient representation of amplified DNA from highly degraded template [5,34]. However, two variations of WGA, rolling circle amplification (RCA) and multiple displacement amplification (MDA), offer the potential to improve STR genotyping results even from highly degraded samples [31]. RCA and MDA utilize random primers, which can recognize any genomic sequence without bias, and Φ29 DNA polymerase [35,36], a DNA polymerase with high processivity, strong strand displacement activity, and 3′–5′ exonuclease proofreading activity that can provide high-fidelity amplification [33,37]. The RCA method is highly robust and is best applied to the replication of circular DNA, such as plasmids or viral genomes [35,36]. The RCA technique exploits circular DNA templates that are essentially infinite-length templates where the DNA polymerase will recurrently synthesize linear replicas of the circular DNA [38,39]. Alternatively, a primase-based WGA method (pWGA) performs fast isothermal DNA amplification but employs a helicase and primase [40].

Since circular templates, other than intact mtDNA, are not typically found in the human genome, other techniques have been applied to achieve template circularization for effective RCA. One technique uses T4 DNA ligase; however, this enzyme exhibits inefficient self-ligation of double-stranded (ds) and single-stranded (ss) DNA and has been shown to generate concatemers of dsDNA [41]. As an alternative, this study examined the use of ssDNA ligase for DNA circularization. The ssDNA ligase enzyme catalyzes the intramolecular ligation of the 5′-monophosphate group and the 3′-hydroxyl group of ssDNA molecules greater than 15 bases and up to 1.9 kb (kilobases) without inter-strand ligation [42]. Circular DNA probes created by ssDNA ligase have been used as signal amplification tools to detect specific nucleic acid sequences [43], to enhance signal for immunoassays [39], and for an inverse-rapid amplification of cDNA ends (RACE) method used to produce template to isolate the 3′ and 5′ ends of transcripts [44]. Therefore, ssDNA ligase could potentially produce circular DNA products suitable for RCA of LT or highly fragmented DNA [45].

The purpose of this study was to evaluate and optimize the application of ssDNA ligase for the circularization of individual ss- and dsDNA to provide a more suitable DNA template for RCA. Adapters that contain optimal terminal nucleotide ends (5′ G and 3′ T) for ssDNA ligase activity were evaluated and optimized to produce efficient ligation of DNA templates. DNA amplification yield following WGA of mock-treated linear and ssDNA ligase-treated ss- and dsDNA templates were also evaluated. In addition, an experimental methodology for the treatment of simulated LT or degraded genomic DNA, which mimic forensic samples refractory to genotype analysis, was used to investigate whether this technique improved STR profile analysis following WGA.

2. Materials and methods

All DNA templates evaluated in this study contained 5′-phosphate groups to facilitate ligation. Single-stranded DNA templates included a 55-mer oligonucleotide (5′ G and 3′A) provided as a control for the ssDNA enzyme (Epicentre Biotechnologies, Madison, WI) and random 100-mer and 200-mer oligonucleotides each possessing a 5′ G and 3′ T (Integrated DNA Technologies, Coralville, IA). Double-stranded templates included the 100-mer and 200-mer templates annealed to their respective reverse complementary oligonucleotides (Integrated DNA Technologies) and a ~1.2 kb template amplified from the human mitochondrial DNA control region of HL-60 genomic DNA (Promega Corp., Madison, WI). Simulated LT DNA and simulated fragmented DNA were prepared from 9947A genomic DNA (Promega Corp.). DNA ligation was carried out with CircLigase™II ssDNA ligase (Epicentre Biotechnologies, Madison, WI).

All WGA methods were carried out according to the manufacturer's recommendations except 1 μl of template DNA (instead of the recommended DNA input concentration) was used for amplification. WGA was performed using the Illustra TempliPhi™ Amplification Kit (GE Healthcare, Piscataway, NJ), Repli-g Mini Kit (Qiagen, Valencia, CA), Illustra GenomiPhi™V2 DNA Amplification Kit (GE Healthcare), GenomePlex® Complete Whole Genome Amplification Kit, Omniplex® Library (Sigma–Aldrich, St. Louis, MO), or Rapisome™ pWGA Kit (Biohelix, Beverly, MA). Heat denaturation was utilized for the Illustra GenomiPhi™V2 DNA Amplification Kit (GE Healthcare).

Detailed descriptions of these and all other materials and methods are provided in the Supplementary Material.

3. Results

Detailed results are provided in the Supplementary Material.

3.1. Circularization of single-stranded DNA templates utilizing ssDNA ligase

T4 DNA ligase, which has been utilized to circularize DNA templates prior to RCA [46,47], was assessed for circularization of small ssDNA templates. 100-mer template was treated with T4 DNA ligase followed by exonuclease I to digest linear ssDNA template, and the reaction products were separated using denaturing polyacrylamide gel electrophoresis (PAGE). However, no exonuclease I-resistant circular products were observed following this treatment (Supplemental Fig. 1a (Fig. S1A)). In addition, manipulation of template concentration resulted in the formation of concatemers or inefficient ligation (data not shown), a result consistent with previous findings [41].

The observed inefficiency of T4 DNA ligase prompted an evaluation of ssDNA ligase for generating circular products utilizing 100-mer template, as well as 55-mer and 200-mer templates to mimic various degrees of fragmented DNA. Following ssDNA ligase and exonuclease I treatment, denaturing PAGE analysis revealed efficient circularization of each ssDNA template (SF1B). Furthermore, a mixture of all three templates produced the respective circular forms upon ssDNA ligase treatment (SF1C), demonstrating the robustness and fidelity of this reaction. Importantly, no unligated template remained and no unintended ligation products, such as concatemers or heterogeneous products, were observed.

3.2. Efficient ligation of double-stranded DNA templates utilizing ssDNA ligase

T4 DNA ligase and ssDNA ligase were evaluated for efficient ligation of a ds 100 bp template. Following treatment, samples were subjected to exonuclease III digestion to eliminate linear dsDNA template. T4 DNA ligase treatment produced linear concatemers but no exonuclease III-resistant product (SF2A). In contrast, ssDNA ligase treatment produced an exonuclease III-resistant product for the ds 100 bp, ds 200 bp, and ds 1.2 kb templates (SF2B). Interestingly, the exonuclease III-resistant ligation products migrated unlike that of the corresponding circular ssDNA products but similar to their respective ds linear templates. The exact molecular conformations of the ds reaction products are presently under investigation.