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Linear nicking endonuclease-mediated strand-displacement DNA amplification

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ABSTRACT

We describe a method for linear isothermal DNA amplification using nicking endonuclease-mediated strand displacement by a DNA polymerase. The nicking of one strand of a DNA target by the endonuclease produces a primer for the polymerase to initiate synthesis. As the polymerization proceeds, the downstream strand is displaced into a single-stranded form while the nicking site is also regenerated. The combined continuous repetitive action of nicking by the endonuclease and strand-displacement synthesis by the polymerase results in linear amplification of one strand of the DNA molecule. We demonstrate that DNA templates up to 5000 nucleotides can be linearly amplified using a nicking endonuclease with 7-bp recognition sequence and Sequenase version 2.0 in the presence of single-stranded DNA binding proteins. We also show that a mixture of three templates of 500, 1000, and 5000 nucleotides in length is linearly amplified with the original molar ratios of the templates preserved. Moreover, we demonstrate that a complex library of hydrodynamically sheared genomic DNA from bacteriophage lambda can be amplified linearly.

Many recent advances in biomedical research and applications such as genome sequencing and genetic diagnosis can be attributed, to a large extent, to the invention of many ingenious methods for DNA amplification, such as the cloning of plasmid DNA in bacteria [1] and the polymerase chain reaction (PCR)[1] [2,3]. In addition to the revolutionary PCR and the ligase chain reaction methods [4] which are based on thermal cycling, a variety of other methods have been developed for isothermal amplification [5]. Notable ones include strand-displacement amplification (SDA) with linear templates [6] or rolling circle amplification (RCA) with circular templates [7,8], transcription-mediated amplification (TMA) [5,9,10], multiple-displacement amplification (MDA) [11,12], helicase-dependent amplification (HDA) [13,14], and primase-based amplification (pWGA) [15]. SDA, RCA, TMA, and HDA can be used for both linear and exponential amplification with sequence-specific primers. MDA and pWGA can only be used for exponential amplification. Short degenerate oligonucleotide (6–8 nt) primers are required for MDA while no primers are needed for pWGA. The powerful MDA has been the method of choice for whole genome amplification from a limited amount of genomic DNA [16] and has been applied to the amplification of genomic DNA from single cells for genome sequencing [17–20]. Unfortunately, a high degree of amplification by MDA could produce significant amplification bias and artifactual chimeras, which are very likely the results of the stochastic random priming events and the formation of primer–dimers due to the use of the short degenerate primers [17,20,21].

The nicking-mediated SDA method initially described by Walker et al. utilizes a polymerase working in concert with a restriction enzyme and a set of specific primers to amplify target DNA molecules isothermally [6,22,23]. However, there are substantial drawbacks. First, the method relies on the incorporation of an α-phosphorothioate into one strand of the partially palindromic recognition site of a double-stranded cutting restriction enzyme to prevent the hydrolysis action of the enzyme on the strand containing the α-phosphorothioate. The incorporation of the α-phosphorothioate essentially transforms the cutting site introduced by the primers into a nicking site for the restriction enzyme. Only a very limited number of restriction enzymes (usually HincII or BsoBI) can be used for SDA. Second, a DNA product containing a significant fraction of bases with α-phosphorothioate may not be desirable for certain downstream applications since the nonnative nucleotide may interfere with further manipulations of the DNA such as digestion by nucleases [24–27]. Third, it has not been demonstrated that DNA molecules with length greater than 100–200 nucleotides (nt) can be amplified with the method [22,28]. This may have been due to the use of DNA polymerases that do not possess both high processivity and strand-displacement capability in the earlier SDA experiments. In addition, the restriction enzymes used have recognition sequences equivalent to only 5 bp. Therefore, it is expected that in general the method cannot be employed

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[1] *Abbreviations used:* BSA, bovine serum albumin; DTT, dithiothreitol; HDA, helicase-dependent amplification; MDA, multiple-displacement amplification; PCR, polymerase chain reaction; pWGA, primase-based amplification; RCA, rolling circle amplification; SDA, strand-displacement amplification; TMA, transcription-mediated amplification.

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to amplify targets over a 1000 nucleotides since there exists an average of one cutting site per 1024 nt, assuming that the template sequence is random. While SDA with endonucleases and phosphorothioate nucleotides has proven to be very useful for signal amplification and sequence detection [29,30], so far its utility for amplifying longer DNA sequences has yet to be demonstrated.

To circumvent the use of α-phosphorothioates and double-stranded cutting restriction enzymes in SDA, two groups have reported attempts at utilizing nicking enzymes for SDA [31,32]. In a method called nicking-endonuclease-mediated DNA amplification (NEMDA) reported by Chan et al., an engineered nicking nuclease with only a 3-bp recognition sequence was used in combination with a DNA polymerase for the amplification of genomic DNA [31]. Through extensive optimization, Ehses et al. also demonstrated some success in the amplification of a 93-nt fragment by SDA with engineered nicking endonuclease Nt.BstNBI, which has a 5-bp recognition sequence [32]. However, so far there has not been any report of success in the amplification of DNA molecules greater than 200 bp by SDA [28,32]. Other methods such as NESA [33] and EXPAR [34,35] that employ polymerases and nicking enzymes are limited to even shorter targets and rely on the spontaneous dissociation of DNA strands following nicking rather than the strand-displacement activity of the polymerase. Techniques that use cycles of nicking and polymerization to stimulate the aggregation of nanoparticles or light emission have also been developed [36,37]. Much like SDA with phosphorothioate nucleotides, these methods are excellent for signal amplification but cannot be used for amplifying long DNA strands.

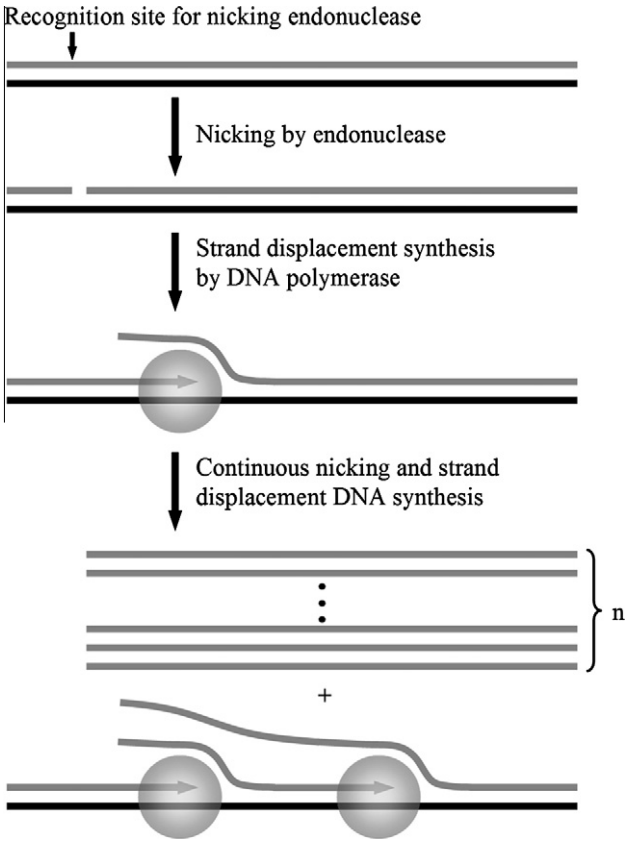

Fig.1. Linear DNA amplification by nicking endonuclease-mediated strand-displacement DNA synthesis. After a nicking endonuclease cleaves a phosphodiester bond in the recognition sequence in one strand of the double-stranded DNA, a DNA polymerase binds to the nicking site and extends from the 3′ OH group, displacing the downstream strand. The extension by the DNA polymerase from the nick regenerates the double-stranded recognition site for the nicking enzyme. The continuous combined actions of the nicking endonuclease and DNA polymerase result in the linear amplification of one strand of the DNA. The recognition site for a nicking enzyme is either an endogenous site on the target DNA, or a site added to the end of the target DNA by the ligation of an oligonucleotide duplex containing the recognition sequence or by PCR using a primer containing the recognition sequence.

Other similar approaches have been developed for the isothermal linear amplification of RNA and DNA as well. Small quantities of RNA can be amplified with little bias by T7 transcriptional amplification [38–40], and adaptations of this method for DNA amplification [41] have demonstrated replication of genomic DNA. Recently, a method called circular nicking endonuclease-dependent amplification (cNDA) demonstrated the ability to combine a nicking enzyme with the T4 replisome to amplify plasmid DNA [42]. However, these methods require the concerted action of several enzymes. There is still a potential for bias introduced by the T7 primer sequence [43] and RNA intermediates or final products are susceptible to degradation [44], while cNDA requires a circular template. Another group has exploited the nicking activity of the DNA mismatch-repair enzyme endonuclease V to enable linear SDA of target molecules [45], but did not demonstrate the ability to amplify long DNA molecules.

Very long DNA molecules in a complex mixture such as a whole genome can be amplified by RCA [8,46] and MDA presumably due to the use of Φ29 DNA polymerase which has extremely high processivity and strong strand-displacement capability [16,47]. However, the construction of circular DNA templates for RCA could be cumbersome or not practical and large DNA molecules may not be amplified efficiently by RCA, while MDA may still produce significant amplification bias and chimeras due to the use of short degenerate primers [17,20,21]. Methods for unbiased linear or exponential amplification of long DNA molecules in a complex mixture are useful for many applications. SDA appears to be an ideal method because of its unique mechanism of amplification.

In this study, we investigated the use of DNA polymerases with high processivity and strong strand-displacement capability in combination with nicking endonucleases with long recognition sequences for linear amplification of long DNA molecules by SDA. A number of DNA polymerases and nicking endonucleases were examined. The DNA polymerases include Bst (large fragment), Φ29, and Sequenase 2.0, all of which seem to possess the desired characteristics for SDA. To enable the specific amplification of long DNA targets, nicking endonucleases with long recognition sequences are essential. Fortunately, several nicking endonucleases have been recently engineered and are commercially available [31,48–54]. The engineered nicking enzymes Nt.BspQI and Nt.BbvCI both have 7-bp recognition sequences [52–54], which are 16× more specific than an enzyme with a 5-bp recognition sequence. On average, they would nick only once every 16,000 bp (4^7 = 16,384) in a DNA molecule with random sequence, ensuring that for most templates amplification only occurs at nicking sites introduced by the primers. We report the use of these commercially available nicking endonucleases for linear amplification of DNA molecules by SDA. The basic principle of linear strand-displacement amplification (LSDA) is illustrated in Fig. 1. We have demonstrated for the first time that a mixture of DNA molecules from 500 to 5000 nucleotides can be amplified in a linear fashion independent of the lengths and sequences of the DNA molecules. We have also demonstrated that a complex library of bacteriophage lambda genomic DNA can be amplified linearly with the original distribution of the fragments largely preserved.

Materials and methods

Oligonucleotides, enzymes, and other reagents

All oligonucleotides were purchased from Integrated DNA Technologies. All nicking enzymes and several polymerases, including