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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.

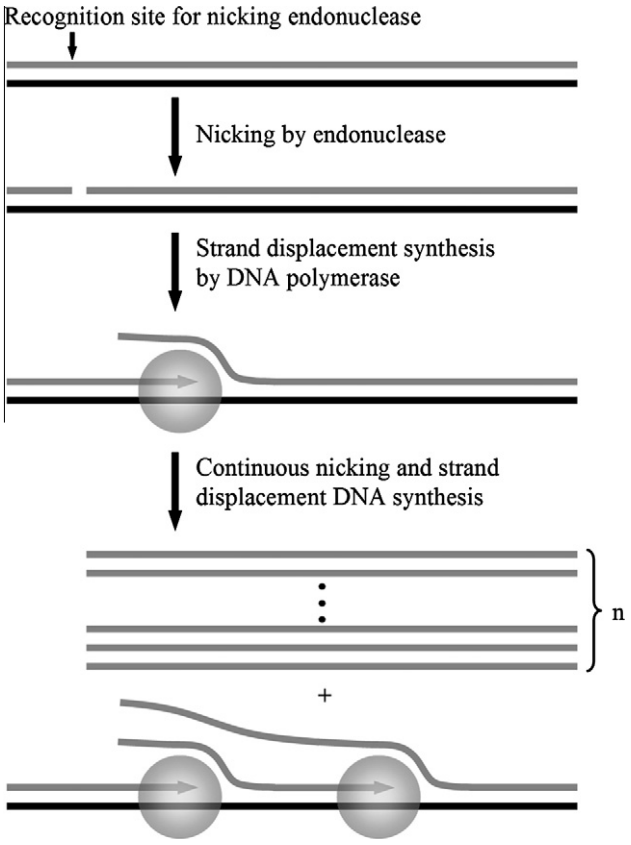

Fig.1. Linear DNA amplification by nicking endonuclease-mediated strand-displacement DNA synthesis. After a nicking endonuclease cleaves a phosphodiester bond in the recognition sequence in one strand of the double-stranded DNA, a DNA polymerase binds to the nicking site and extends from the 3′ OH group, 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

Nt.AlwI, Nt.BbvCI, Nb.BsmI, Nt.BspQI, Nb.BsrDI, and Nt.BstNBI, Exo-Klenow enzyme (*Escherichia coli*. DNA polymerase large fragment), Bst DNA polymerase large fragment, Φ29 DNA polymerase, 9°Nm DNA polymerase, Vent exo- DNA polymerase, and BSA (bovine serum albumin) were acquired from New England Biolabs (NEB). T4 DNA ligase was also obtained from NEB. Sequenase version 2.0, an engineered T7 DNA polymerase [55], and *E. coli* single-stranded DNA binding protein (SSB) were purchased from United States Biochemicals. PCR was performed using a Phusion PCR kit from Finnzymes. Nucleotides were from Sigma Aldrich. Plasmid pET-24(a) was acquired from Novagen, now part of Merck Biosciences. The PCR purification kit (Cat. No. K3100-01) and nucleic acid dyes SYBR Gold, SYBR Green I, and SYBR Green II were obtained from Invitrogen. The PerfeCTa SYBR Green FastMix from Quanta Biosciences was used for the quantitative PCR (qPCR) experiments. Sequencing was provided by Eton Bioscience Inc.

DNA templates

The 0.5, 1, and 5-kilonucleotide (knt) DNA templates were amplified from plasmid pET-24(a) by PCR. The same forward primer was used for the amplification of all the templates. In addition to 21 bp that anneal to the plasmid DNA, this primer contained at its 5′ end the recognition sequences for both Nt.BspQI (GCTCTTCN^) and Nt.BbvCI (CC^TCAGC), and an additional 23 nucleotides of random sequence to increase the melting temperature of the upstream fragment following nicking activity. Each fragment produced from a template created with this forward primer will be 38 nucleotides shorter than the initial template. The forward primer had this sequence: 5′-CTG GAG TCA ACG CAT CGA GCA TAC CTC AGC GCT CTT CCG CTT CCT CGC TCA-3′. The reverse primers were 20- to 21-bp-long oligonucleotides designed to bind at the appropriate locations on the plasmid to provide the desired template lengths. The following primers were used: 5′-CGG GTT GGA CTC AAG ACG ATA-3′ for the 500-nt template, 5′-GAC ATT ATC GCG AGC CCA TT-3′ for the 1-knt template, and 5′-CTG TTC ATC CGC GTC CAG CTC-3′ for the 5-knt template. The GC content of the fragments varied from 52% for the 1- and 5-knt templates to 58% for the 0.5-knt template. All of the templates were amplified by 33 cycles of PCR. Each cycle consisted of 10 s at 98 °C, 15 s at 78 °C, and 15 s/knt at 75 °C. The amplification was finished by a final reaction at 72 °C for 5 min and cooling to 4 °C. The amplified products were purified with a PCR purification kit and quantified by absorption measurement at 260 nm using a spectrophotometer (Nanodrop Model ND-1000 and software version 3.5.2, Thermo Scientific). Templates to test other nicking endonucleases were obtained similarly.

Genomic DNA library construction

A library of randomly fragmented bacteriophage lambda genomic DNA was prepared and used as a model system to demonstrate the ability to amplify a complex library linearly by SDA. The lambda genomic DNA was fragmented by hydrodynamic shearing using a custom-built device [56]. Briefly, linear lambda genomic DNA (48.5 kbp, NEB) at a concentration of 30 ng/μl was passed through a filter screen (1-μm pore size and 1/16 inch in diameter, Product No. 1SR1-10, Valco Instruments Co. Inc.) housed in an internal stainless-steel union with 0.25 mm diameter bore (Product No. ZU1C, Valco Instruments Co. Inc.). The DNA solution was pumped through the screen 20 times at a flow rate of 50 ml/min. The resulting DNA fragments were blunt-ended and 5′ phosphorylated using the NEBNext End Repair Module (Cat. No. E6050S, NEB). Sheared DNA (85 μl) was mixed with 10 μl 10X End-Repair Buffer and 5 μl Enzyme Mix (T4 polymerase and T4 polynucleotide kinase) and incubated at 20 °C for 1 h. The enzymes were removed by membrane purification using the PCR clean-up kit. An 8 μl aliquot of the resulting library was run on a 1% agarose gel to determine the mean size of the fragments, and the molar concentration of the fragments was calculated. Next, both ends of the genomic DNA fragments were ligated to a duplex adapter containing the recognition site for nicking endonucleases Nt.BbvCI and Nt.BspQI. To ensure that the adapter ligation is unidirectional and to avoid self-ligation between the adapters, the duplex adapter is designed to have one blunt end without a 5′ phosphate group and one end with a 1-bp 5′ overhang. The duplex adapter consists of a 51-bp oligonucleotide with sequence 5′-CTG GAG TCA ACG CAT CGA GCA TAC CTC AGC GCT CTT CCG CTT CCT CGC TCA-3′, and a 50-bp complement from the 3′ end. The nicking sites for Nt.BbvCI and Nt.BspQI are located at 25 and 38 nucleotides, respectively, from the 5′ end of the top strand. An 80-fold molar excess of adapters over genomic DNA was used to prevent the ligation between genomic DNA fragments. The ligation reaction contained 2 μM adapters and 25 nM end-repaired genomic DNA in 50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 20 mM KCl, 1 mM ATP, and 10 mM dithiothreitol (DTT). T4 ligase was added to a final concentration of 15 cohesive end units/μl, and ligation was carried out at 26 °C for 12 h. The reaction was stopped by heating at 65 °C for 20 min. Since the adapters do not have a 5′ phosphate, only one strand of the duplex is ligated to the genomic DNA, leaving a nick on the other strand. The nicks were removed by performing a short strand-displacement reaction using the nicks as the priming sites. Nucleotides, BSA, and Bst DNA polymerase large fragment were added to a final concentration of 150 μM, 100 μg/ml, and 35 units/ml, respectively, and the mix was heated to 37 °C for 30 min. The polymerase and excess adapters were removed by two-time purification using a PCR clean-up kit. The final concentration of the DNA library was determined by absorption measurement using the Nanodrop spectrophotometer. The mean fragment size and the molar concentration of the resultant DNA fragments with the adapters were obtained by gel electrophoresis analysis of a small aliquot of the final library.

Strand-displacement amplification reactions

For SDA reactions with only one DNA template, each 25 μl reaction volume was assembled on ice and contained 2 nM DNA template, 40 mM Tris–Cl, pH 8.0, 10 mM $MgCl_2$, 50 mM NaCl, 5 mM DTT, 100 μg/ml BSA, 500 μM each of all four dNTPs, 5 μM SSB, 400 nM (0.45 U/μl) Sequenase 2.0, and 3 nM (0.04 U/μl) Nt.BspQI (the molar concentration of Nt.BspQI was calculated using a specific activity of 370,000 U/mg and a molecular weight of 50 kDa, personal communication from New England Biolabs). The solution was divided into 5-μl aliquots in five microtubes. Immediately, 15 μl of PAGE gel stop buffer (80% formamide, 20 mM EDTA, pH 8.0, 10 mM Tris–borate, 0.0125% bromophenol blue, and 0.0125% xylene cyanol FF) was added to one tube for the zero time point reaction. The remaining tubes were incubated at 37 °C. After 5, 10, 20, and 40 min incubation, one tube was removed and 15 μl of stop buffer was added to terminate the reaction.

For simultaneous amplification of a mixture of three templates at equal molar concentrations, the reaction conditions were the same except that 2 nM of each of the 0.5-, 1-, and 5-knt templates was used. For simultaneous amplification of a mixture of three templates at equal mass or nucleotide concentrations, all conditions were also the same except that the concentrations of the 0.5-, 1-, and 5-knt templates were 3, 1.5, and 0.3 nM, respectively. When expressed in terms of mass or nucleotide, all three templates have the same concentration, 1.5 μM in nucleotide. To investigate the important contribution of SSB to the amplification reaction, two SDA reactions were performed in parallel on 1 nM 0.5-knt

template using the conditions described above without SSB or with 5 μM of SSB. Time points were taken at 0, 5, 10, and 20 min.

For the amplification of the sheared genomic DNA library from bacteriophage lambda, a 15 μl reaction was assembled on ice. The reaction mix contained 2 nM DNA templates, 40 mM Tris–Cl, pH 8.0, 10 mM $MgCl_2$, 50 mM NaCl, 5 mM DTT, 100 μg/ml BSA, 500 μM each of all four dNTPs, 5 μM SSB, 400 nM (0.45 U/μl) Sequenase 2.0, and 3 nM (0.04 U/μl) Nt.BspQI. The solution was divided into 5-μl aliquots in three microtubes. For the zero reaction time point, an agarose gel stop buffer (1.2% SDS, 7% Ficoll-400, 25 mM Tris–Cl, pH 7.5, 25 mM EDTA, 0.0025% bromophenol blue, and 0.0025% xylene cyanol FF at 1×) was added immediately to one tube. The remaining tubes were incubated at 37 °C. After 15 and 30 min, one tube was removed and the stop buffer was added to terminate the reaction.

To observe the effect of nicking enzyme concentration on the SDA reaction, four different molar concentrations of Nt.BspQI were used in parallel SDA reactions on 2 nM 0.5-knt template. The concentrations of the enzyme tested were 0.6, 3, 15, and 75 nM. In addition to a 0 min time point, samples were taken from each reaction at 10 and 20 min and added to PAGE gel stop buffer.

Quantification by gel analysis

The rates and linearity of strand-displacement amplification of the DNA templates prepared by PCR were quantified by electrophoretic analysis with 5% denaturing polyacrylamide gel in 0.5X TBE buffer (45 mM Tris–borate and 1 mM EDTA, pH 8.4). For the 0.5-knt reaction, the entire volume of each time point was loaded onto the gel. For the other templates, half of the sample for each time point was loaded. Additionally, 250 ng of 1 kb DNA ladder (NEB) was run for size comparison and four standard lanes were loaded on each gel for mass quantification. The standards contained known amounts of the template being analyzed and represented 1×, 4×, 16×, and 32× the mass of initial template in the reaction. Each gel was run for 35 min at 400 V constant voltage with a Bio-Rad Mini PROTEAN 3 gel setup. The gels were briefly soaked in deionized water before staining with SYBR Gold in 1X TAE buffer (40 mM Tris, 20 mM acetic acid, 2 mM EDTA, pH 8.0). The gels were imaged with a Gel-Doc XR and 8-bit camera system using Quantity One 1-D analysis software (Bio-Rad Laboratories). After background subtraction, the lanes of known mass were used to create a standard curve so that the amount of DNA in each sample band could be calculated. Following quantification of each sample band, the mass of the product at each time point was plotted vs. time using Microsoft Excel 2007. A linear equation was fitted to the data and the *R*-squared value for each time series was calculated.

Alkaline agarose gel electrophoresis was used to analyze the strand-displacement amplification of the bacteriophage lambda genomic DNA library. Prior to loading onto the gel, the samples were heated to 95 °C for 2 min and then cooled on ice. After NaOH was added to a final concentration of 30 mM, the entire volume of sample for each time point was loaded into a well. Samples from three separate SDA reactions using the same library were loaded onto one gel. For size comparison, one lane was loaded with DNA fragments from the genomic library at 15 times the initial amount used in the amplification. Along with the samples to be analyzed, 400 ng O'Gene Ruler Express DNA Ladder (Fermentas) was loaded onto each gel. The alkaline gel (0.8% agarose, 30 mM NaOH, 2 mM EDTA) was run on ice at 3.5 V/cm for 135 min in 30 mM NaOH and 2 mM EDTA. The gel was briefly soaked in deionized water, neutralized by soaking in 2X SSC (300 mM NaCl, 30 mM sodium citrate, pH 7.0) for 20 min, stained with SYBR Gold in 1X SSC for 15 min, and destained in TAE for 30 min. The gel was imaged as described above. An exponential equation relating fragment length and migration distance on the gel was established from the bands of the DNA Ladder standard. After background subtraction, the raw intensity values of each pixel along the length of each lane were exported to Microsoft Excel, and the average pixel value for each migration distance was calculated from the three samples at each time point. The migration distance was converted to base pair values using the exponential equation, and the average raw intensity vs. fragment length for each of the time points was plotted. By dividing the raw intensity values with their corresponding base pair number, the relative population of each fragment length was calculated. The average fragment length was found by dividing the sum of the raw intensity values by the sum of the relative population values. Using three sets of samples on the gel, we calculated the average mean fragment length for each time point and the standard deviation. The amplification of PCR-generated fragments with hairpin-forming adapters was also visualized by alkaline gel electrophoresis under the same conditions.

Real-time monitoring of strand-displacement amplification

For the real-time detection experiments, the reaction mix contained the DNA template (1 and 3 nM for the 0.5-knt fragment, 0.33 and 1 nM for the 5-knt fragment), 100 nM reverse primer (same primer used to generate the fragment by PCR), 40 mM Tris–Cl, pH 8.0, 10 mM $MgCl_2$, 50 mM NaCl, 5 mM DTT, 100 μg/ml BSA, 500 μM each of all four dNTPs, 5 μM SSB, and 0.5X SYBR Green II. The reaction mix was incubated without enzymes at room temperature for 30 min. After placing the mix on ice, Nt.BspQI and Sequenase 2.0 were added to a final concentration of 3 and 400 nM, respectively. Each template concentration was loaded in triplicate into adjacent wells of a Real-Time PCR Detection System (MiniOpticon, Bio-Rad Laboratories). The samples were preincubated for 2 min at 37 °C, and a reading was then taken every 40 s for 60 min with the temperature maintained at 37 °C. Data were collected using the Bio-Rad CFX Manager Software Version 1.5.

At the conclusion of the run, all data were exported to Microsoft Excel 2007. Baseline subtraction was done by subtracting the relative fluorescent unit (RFU) value of the first read of each well. The resulting data for each template concentration were averaged and the standard deviation was calculated. The mean values were then plotted against time with vertical error bars indicating one standard deviation from the mean. A linear equation was fitted to each set of data and the *R*-squared value for each time series was calculated.

Analysis of amplification bias by qPCR

Three primer pairs were designed to amplify three sequences of lengths 129, 152, and 152 nt from the SDA product. The sequences are located at approximately 5, 30, and 40 knt from one end of the 48.5-knt linear bacteriophage lambda genome. The primer pairs used are 5′-ACA CTG CAG TCC CGG ATG GA-3′ and 5′-ATC AAT GGC CTC CTG ACC GC-3′, 5′-CGC GTC ACC CAC ATG CTG TA-3′ and 5′-TGC TCT CCC GAT GGT TTA TGC A-3′, and 5′-TAC CGC TCA CCG TAT TGC AGG TTG-3′ and 5′-GCC GAC GTA TGG AGT GCC ATA TTT-3′, respectively. A sheared genomic DNA library was amplified for 30 min by SDA as described above and the reaction was stopped by heating at 80 °C for 20 min. qPCR were performed to quantify the three sequences in the SDA product. Each qPCR mix contained 1X qPCR mix, 125 nM primer pair, and a small volume of product from the SDA reaction. The PCR consisted of one initial denaturation/activation cycle of 5 min incubation at 95 °C followed by 40 cycles of 10 s denaturation at 95 °C and 20 s hybridization/synthesis at 68 °C.

Hairpin adapter to produce double-stranded products

The 3′ end of one molecule of the amplified single-stranded products could partially hybridize to certain regions of the same

molecule or other molecules, resulting in chimeric products. To prevent this, we designed an adapter oligonucleotide with self-complementary regions to form a hairpin at the end of the single-stranded SDA product. Once a product is fully synthesized and released from the polymerase, the hairpin forms, allowing the 3′ end to serve as a primer for the synthesis of a double-stranded product. The adapter contains the sequence used in the lambda phage genome library followed by five random nucleotides and 12 additional bp added at the 3′ end that are the reverse complement of the 12 bp immediately following the Nt.BspQI nicking site. One strand of the duplex adapter has a 68-bp sequence of 5′-CTG GAG TCA ACG CAT CGA GCA TAC CTC AGC GCT CTT CCG CTT CCT CGC TCA ATC CAG AGC GAG GAA GC-3′ while the complement strand has a 2-bp overhang (AA) at the 3′ end. The oligonucleotides are not phosphorylated at the 5′ end. We used a 1.5-knt fragment without any Nt.BspQI nicking sites amplified by PCR from the *Saccharomyces cerevisiae* genome as a test template. The forward and reverse PCR primers have the sequences 5′-phosphate-TGC TTT GCC AAG GGT ACC AAT GTT T-3′ and 5′-phosphate-GCA ATT ATG GAC GAC AAC CTG GTT G-3′, respectively. The PCR was performed by denaturation of the genomic DNA at 98 °C for 2 min followed by 30 cycles of PCR, each consisting of 10 s at 98 °C, 20 s at 63 °C, and 25 s at 68 °C. The amplification was completed by a final reaction at 72 °C for 5 min and cooling to 4 °C. The amplified products were purified with a PCR purification kit and quantified by absorption measurement at 260 nm using a Nanodrop spectrophotometer. The duplex adapters were ligated to the PCR product, and SDA reactions were performed by following the same procedures used for genomic DNA library construction and amplification.

Results

We present an approach for isothermal linear amplification of DNA. In order to demonstrate the linear kinetics of this method and to illustrate its ability to amplify long DNA molecules, DNA templates of various lengths were obtained via PCR using primers containing restriction sites and plasmid pET-24(a) as source material. Fragments of length 0.5, 1, and 5 knt were then amplified using Sequenase 2.0 and Nt.BspQI. The results are shown in Fig. 2. First, each fragment was amplified separately and time points were taken at 5, 10, 20, and 40 min. The products were quantified and plotted, and a linear regression line was fitted to each set of data. For each template length, the SDA product is 38 bp shorter than the original molecule since the nicking endonuclease creates a single-stranded break in the DNA 38 bp from the 5′ end of the template. This size difference is seen most clearly in the amplification of the 0.5-knt template, but the polyacrylamide gel is unable to resolve the two bands of higher molecular weight templates. We designed the templates to have a long 38-bp "primer" upstream of the nicking site so that the nicked primer remains

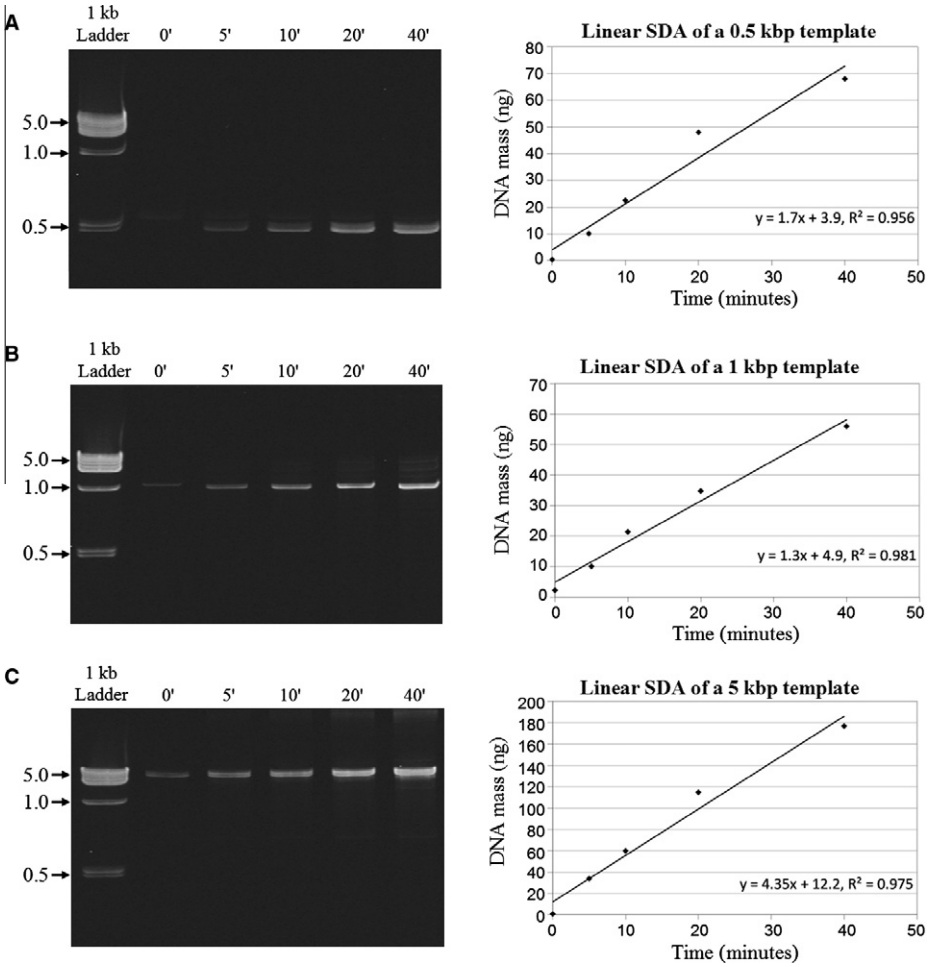

Fig.2. Linear amplification of 0.5- to 5-knt DNA templates by nicking endonuclease-mediated SDA. (A–C) Linear SDA amplification of 0.5-, 1-, and 5-knt templates, respectively. A 1-kb ladder is included for size reference with the 0.5-, 1-, and 5-kb bands indicated by arrows. The SDA product for each template should be 38 nucleotides shorter than the original molecules. Graphs beside each gel are the calculated masses of each sample band plotted vs. time. In each case, a regression line was fit to the data, and the resulting equation and *R*-squared value are shown.

hybridized even at 70 °C for testing amplification at elevated temperatures. At 37 °C, the temperature at which SDA appeared to be most successful, a shorter primer (e.g., 12–14 bp long) would be sufficient. To verify the identity of the amplified fragments, we sequenced the SDA products of the 500-nt and 1-knt fragments by the conventional Sanger dideoxy method. The results confirmed that each product has the same sequence as the initial template.

The amplification reaction could be modeled very well by a linear equation ($R^2 > 0.95$). Additionally, it appeared that each template was amplified about 15–20 times during the 40-min incubation. Unlike in previous iterations of SDA, an increase in target length does not result in an observable decrease in the amplification factor. Furthermore, the doubling time of this reaction is approximately 2–2.5 min. This compares favorably to the original mesophilic SDA using HincII and Klenow exo-, which had a doubling time of 3–5 min [6,28]. Due to the limited dynamic range of the gel imaging instrument and the difficulty in quantifying very low concentrations of DNA, the first data point in each series appears close to zero.

In order to show that our method can be used for linear amplification of a library of targets with fragments of disparate lengths, three target templates with lengths of 0.5, 1, and 5 knt were amplified simultaneously in the same solution. In the first experiment, as illustrated in Fig. 3A, the three templates were initially present at equal molar concentrations. The fluorescent intensity of each stained product band is proportional to the mass or the number of nucleotides in the amplified product. Therefore, if the reaction is linear across the templates of various lengths, the product of

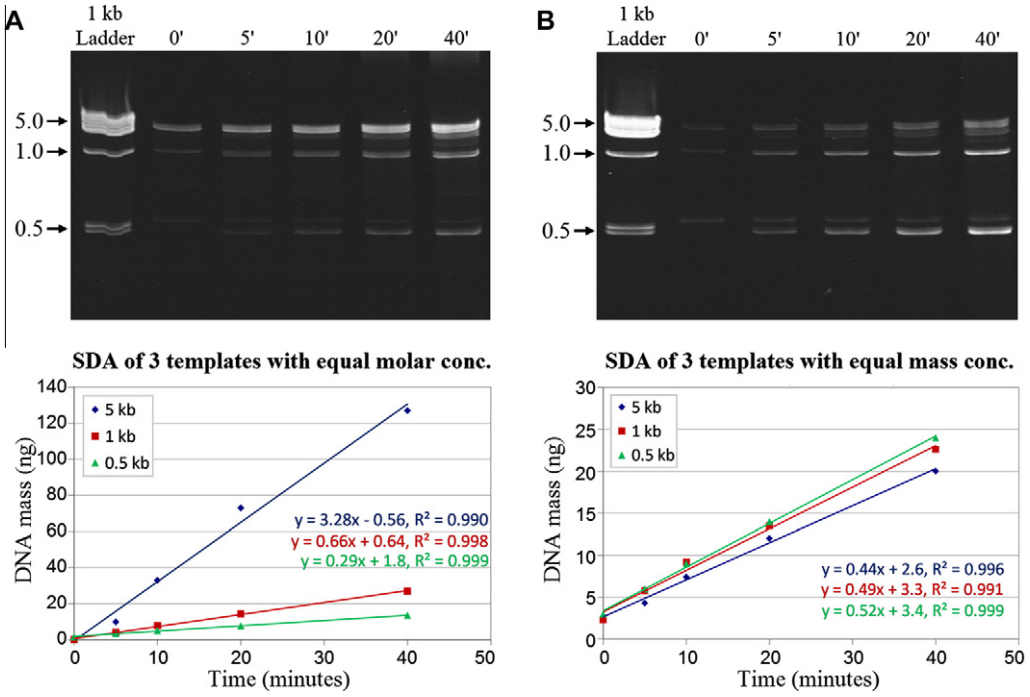

Fig.3. Simultaneous linear amplification of three templates of various lengths. (A) Amplification of three templates of lengths 0.5, 1, and 5 knt with equal initial molar concentration of 2 nM. A 1-kb ladder is included for size reference with the 0.5-, 1-, and 5-kb bands indicated by arrows. A plot of DNA mass vs. time for each template length is shown below each gel image. As anticipated, in mass, the 5-knt product increases 10 times faster than the 0.5-knt product and five times faster than the 1-knt product. (B) Amplification of three templates of lengths 0.5, 1, and 5 knt with equal initial concentration in mass or total nucleotides of 1.5 μM. As expected, the mass or the total number of nucleotides of all three templates increases at the same rate.

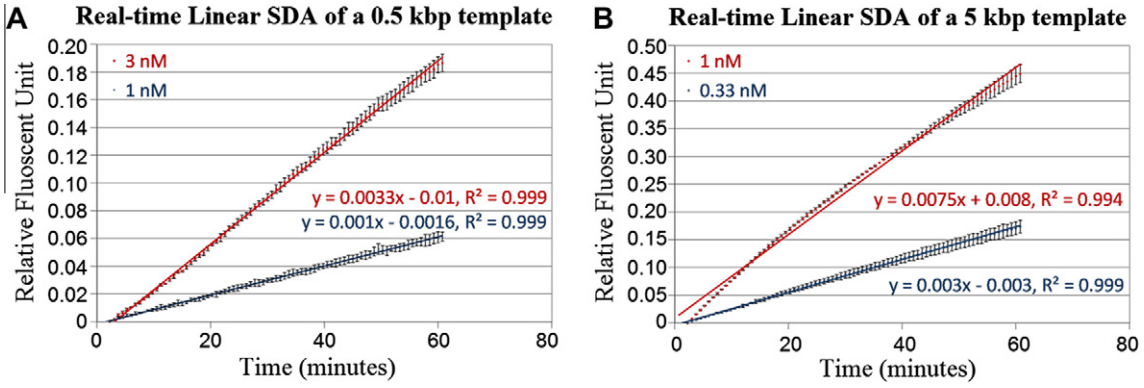

Fig.4. Real-time monitoring of SDA. Linear SDA of 500-nt (A) and 5-knt (B) templates. Relative fluorescent units (RFU) were measured every 40 s for 60 min. After background subtraction, a linear regression line was fitted to each data set. The error bars indicate the standard deviation of measurements from three wells. For both templates, a threefold increase in template concentration, from 1 to 3 nM for the 0.5-knt template and from 0.33 to 1 nM for the 5-knt template, resulted in three times faster increase in the measured RFU.

each template should increase linearly and the slope of the equation fitted to the 5-knt template should be about 10 times that of the 0.5-knt template and five times that of the 1-knt template. As shown, the amplification of each target is linear ($R^2 > 0.99$) and the slopes of the trend lines for both the 5- and 1-knt fragments are within 10–15% of the expected values.

In the second experiment, the three templates are initially present at equal mass concentrations. In this case, the amount (in mass or total number of nucleotides) of DNA in all three bands should increase linearly at the same rate. As shown in Fig. 3B, this is what is observed. The amplification of each template is linear and the slopes of each trend line are within 15–20% of each other, indicating that all three templates are amplified equally in mass.

To prove the linearity of the SDA reaction, the 0.5- and 5-knt templates were amplified using a real-time PCR machine to monitor the increase in DNA mass in real time. Various concentrations of SYBR Gold, SYBR Green I, and SYBR Green II were investigated to find the optimal conditions for the best fluorescent signal. We found that concentrations of SYBR Gold or SYBR Green I above 0.5X significantly inhibited the SDA reaction. Additionally, it had been previously reported that although SYBR Green II is commonly considered a single-strand DNA or RNA binding dye, it exhibits increased fluorescence when bound to double-stranded DNA as opposed to single-stranded DNA [34]. We also found this to be the case and included the reverse primers in the reaction mix to make the SDA product double-stranded.

Plots of relative fluorescent units vs. time for the 500-nt and 5-knt templates are shown in Fig. 4. A straight line is a good fit for each data set ($R^2 > 0.99$). In Fig. 4A, it was anticipated that the slope of the trend line for the 3 nM sample wells would be threefold higher than the slope of the trend line for the 1 nM samples. Likewise, in Fig. 4B, it would be expected that the slope of the 1 nM template would be threefold higher than the slope of the 0.33 nM template samples. In each case, the magnitudes of the slopes are within 10% and 20% of the expected values, respectively. It should be noted that the vertical axes in Figs. 2 and 3 reflect the total amount of DNA in mass or nucleotide while the vertical axes in Fig. 4 represent the increase in relative fluorescent units from the samples.

The data curve for the 5-knt template at 3 nM concentration appears to be slightly less linear than those of the other samples. This moderate decrease in reaction rate is likely due to the depletion of reagents in the reaction, which causes the slope of the regression line to be lower than expected. It should be emphasized, however, that in a mixture of templates with different lengths, each template would experience the same reduction in available reagent concentrations. While the overall reaction rate may diminish somewhat for a long reaction, this would not result in longer templates being underrepresented in the final amplified DNA mix.

After demonstrating that SDA can be used to amplify a mixture of templates of disparate lengths in an unbiased manner, we investigated linear SDA of more complex templates. We used a genomic library constructed from the relatively small genome (48.5 kbp) of the bacteriophage lambda as a model system. A genomic library with a mean fragment length of 1.15 knt was constructed. Fig. 5 shows some typical results from the amplification of the library by SDA using the conditions we established for the PCR-generated templates. After 30 min of incubation with Nt.BspQI and Sequenase 2.0, the template has been replicated about 15 times. When the mean raw intensity from triplicate experiments is plotted as a function of fragment length (Fig. 5B), it is observed that the overall curves of the SDA products shift toward a shorter length relative to the initial template library. The amplified product from each template is expected to be 76 bp shorter than the original template since Nt.BspQI nicks at a site 38 bp from both ends of the adapters. However, the mean fragment length is 810 nt with a standard deviation of 30 nt from triplicate samples, about 200 nt shorter than the expected full-length product. This discrepancy can be explained by the presence of 10 "native" Nt.BspQI nicking sites within the phage genomic library. Amplification from an internal nicking site results in a product shorter than the full-length fragment. The intensity of the bands in the gel image in Fig. 5A and the vertical axis in Fig. 5B depicts the raw intensity which is proportional to the total mass of the DNA, not the relative population of each fragment. Therefore, the centers of the bands on the gel and the peaks of the curves in Fig. 5B do not correspond directly to the true mean fragment sizes reported above. In order to show that the library amplification was unbiased, we selected three short sequences located approximately in the middle and at both ends of the lambda phage genome. We used qPCR to determine the number of molecules in the initial unamplified library and library amplified by SDA. The three fragments were found to be amplified by about 10-, 10-, and 12-fold, respectively.

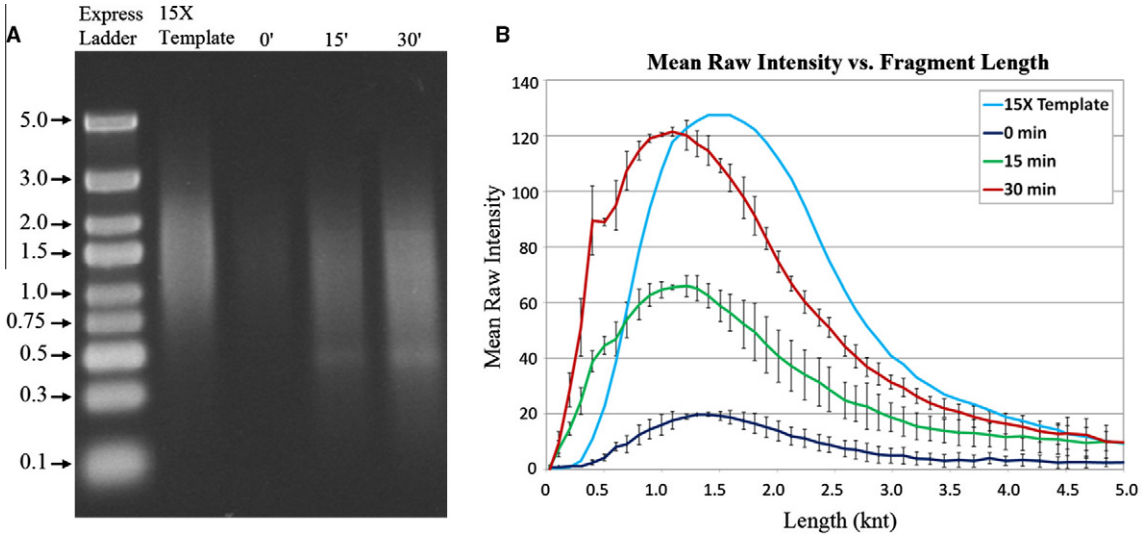

Fig.5. Amplification of a genomic library from bacteriophage lambda. (A) Analysis of the linear amplification of a sheared genome on a denaturing alkaline gel. An Express DNA Ladder is included for size reference. A lane containing 15 times the amount of starting material used for the amplification is included for comparison. (B) Average raw intensity of three reactions at each reaction time plotted as a function of DNA fragment length. Error bars indicate one standard deviation from the mean, calculated from a triplicate data set. The overall shift of the SDA product toward shorter lengths relative to the initial library is likely due to the presence of internal nicking sites within the genomic DNA fragments and the placement of the Nt.BspQI nicking site in the adapter.

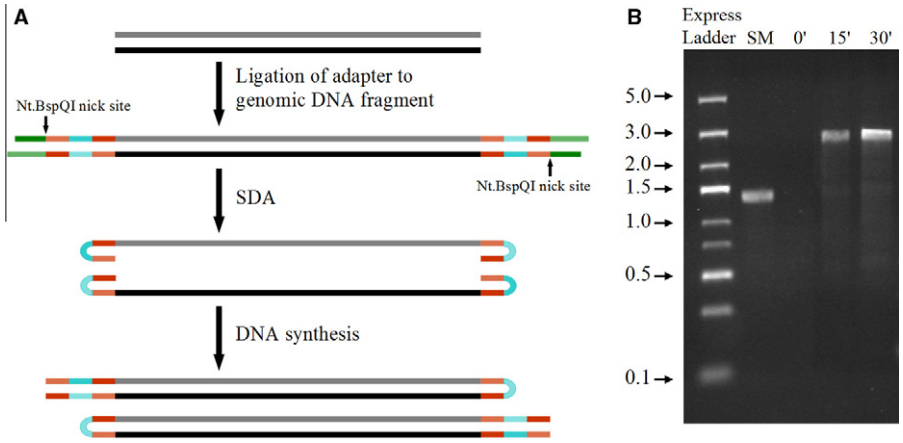

Fig.6. Use of hairpin adapters to convert SDA product to double-stranded DNA. (A) Both ends of the genomic DNA fragment are ligated to an adapter oligonucleotide with two self-complementary regions to form a hairpin at the end of the single-stranded SDA product. Once a product is fully synthesized and released from the polymerase, the 3′ end of each single-stranded SDA product folds into a hairpin and serves as a primer for the synthesis of the double-stranded product with a hairpin at one end. This helps to prevent the nonspecific hybridization of the 3′ end of the single-stranded molecules to regions of itself or other molecules. (B) Strand-displacement amplification of a 1.4-knt template from lambda phage genome. With the 60-bp hairpin adaptors, the product appears as a 3-knt fragment on a denaturing alkaline gel. An Express DNA Ladder is included for size reference. Lane "SM" contains 30 times the amount of starting material used in the amplification. The lane with the small amount of starting materials (0 min lane) is not visible on the gel.

The amplified products of a complex library by SDA are single-stranded. The 3′ end of one product molecule could partially hybridize to certain regions of itself or other molecules, resulting in chimeric products. The use of an adapter sequence that results in a product with a self-priming hairpin structure could alleviate this potential problem. The strategy is illustrated in Fig. 6A. A DNA fragment amplified from the *S. cerevisiae* genome was used as a test template. As shown in Fig. 6B, the amplification of a 1.5-knt template results in a product of twice that length on a denaturing gel. This indicates that the product is converted to a double strand with a hairpin at one end with 100% efficiency.

Discussion

We have established the reaction conditions for strand-displacement amplification of DNA molecules up to 5 knt in length. These conditions differ from previously reported SDA techniques in several important aspects. Past publications have advocated the use of very high concentrations of endonuclease and suggested that a linear relationship exists between endonuclease concentration and reaction rate [22,28]. In our initial experiments, very high concentrations of nicking enzymes were used. Much to our surprise, our numerous attempts at SDA using templates of various lengths and concentrations, and many combinations of DNA polymerases (including Klenow exo-, Φ29, Bst large fragment, 9°Nm, and Vent exo-) and nicking enzymes (including Nt.AlwI, Nt.BstNBI, Nb.BsrDI, Nb.BsmI, Nt.BbvCI, and Nt.BspQI), were not successful. Eventually, we discovered that, in contrast to what was previously reported [28], high concentrations of nicking enzyme in the solution could severely inhibit the SDA reaction. Tan et al. also reported that high concentrations of nicking endonucleases inhibit DNA amplification by EXPAR [34].

We found that the SDA reaction is highly sensitive to nicking enzyme concentration. There exists an optimal concentration unique to each nicking endonuclease. Higher concentrations inhibit the reaction while lower concentrations may not be sufficient to sustain the reaction rate. It has been suggested that some nicking enzymes may bind tightly to the DNA templates at high concentrations [34] and certain nicking endonucleases such as Nt.BbvCI are known to have a tendency to aggregate into higher order species that inhibit the activity of the enzymes [53,54]. However, the mechanism by which high nicking enzyme concentrations inhibit SDA is still not very clear. We found that it is not due to nonspecific nicking by the nuclease or stifling levels of glycerol or BSA contributed by the enzyme storage buffer. The phenomenon was observed for all combinations of nicking enzymes and polymerases we have investigated. The effect is demonstrated in Fig. 7A. A 500-nt template with a starting concentration of 2 nM was amplified by SDA with Sequenase 2.0 and four different concentrations of Nt.BspQI.

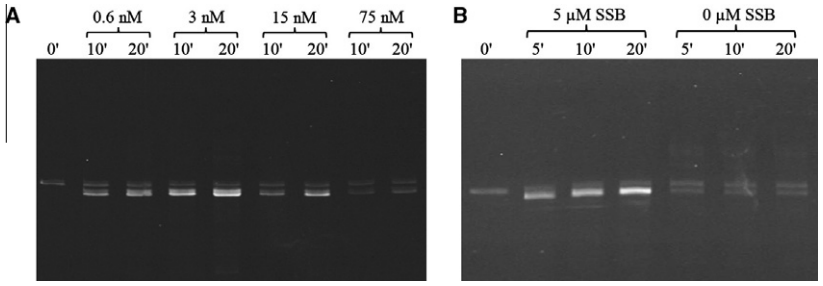

Fig.7. Critical parameters for successful strand-displacement amplification. (A) There exists an optimal concentration for each nicking enzyme for SDA. Shown is a gel image of SDA of a 500-nt template using varying concentrations of Nt.BspQI. Maximal amplification of a 500-nt fragment at a template concentration of 2 nM is achieved with 3 nM Nt.BspQI. This concentration of nuclease is also optimal for other templates of varying lengths and concentration. Amplification yield drops sharply at concentrations above this value. (B) The presence of a high concentration of *E. coli* single-strand DNA binding protein (SSB) is essential for efficient SDA by Sequenase 2.0 DNA polymerase.

An increase in the reaction rate is observed when the Nt.BspQI is increased from 0.6 to 3 nM. However, at 15 nM the reaction rate decreases considerably, and at 75 nM the reaction stops completely even though the DNA templates are still nicked by the endonuclease. Similar experiments were repeated to identify the optimal concentration of each nicking enzyme. The optimal concentration for the nicking enzymes we tested varies by nearly two orders of magnitude. We found that if the optimal concentration of the nicking endonuclease was used, SDA of templates up to 250 nt in length was successful with all the nicking endonucleases, including Nt.AlwI, Nb.BsrDI, Nb.BsmI, Nt.BbvCI, Nt.BstNBI, and Nt.BspQI. It had been previously reported that the ratio of polymerase to nicking enzyme was important [28]. However, this does not seem to be the case for linear SDA under our conditions. We found that the optimal concentration of each nicking endonuclease remains the same regardless of polymerase concentrations. Moreover, the optimal concentration of nicking endonuclease was the same regardless of template concentration. Increasing the template concentration does increase the overall reaction rate. It is quite possible that template concentrations in the 1–15 nM range are below the K_M of the nicking endonuclease; therefore, any change in substrate concentration will have a large effect on the turnover rate of each enzyme.

Once the optimal concentrations for the nicking endonucleases were established, SDA of short templates was successful with all of the nicking enzymes and most of the polymerases. The surprise exception was Φ29 DNA polymerase. With its high processivity, strong strand-displacement activity, and high fidelity, it would seem to be an ideal DNA polymerase for SDA. Unfortunately, Φ29 DNA polymerase is unable to initiate strand-displacement DNA synthesis from an endonuclease-mediated nick in a double-stranded DNA molecule. Similar to what was reported in the original SDA method, SDA with Klenow exo- resulted in specific amplification of short templates, but the amplification factor was highly dependent on target length. SDA with Bst DNA polymerase large fragment gave mixed results. While short templates (<100 nt) were amplified efficiently by Bst DNA polymerase with fast kinetics at high reaction temperatures (50–60 °C), for longer templates we observed very high levels of nonspecific products and the yields of the specific products were much lower. Similar phenomena were observed for other thermophilic polymerases including Vent exo- and 9°Nm. In some cases, a brief period of specific amplification was followed by a rapid increase in nonspecific amplification. Thermophilic polymerases are known to be able to initiate template-independent *de novo* DNA synthesis and amplification [57,58], particularly in the presence of endonucleases [34,59,60]. We chose Sequenase 2.0 as the best polymerase candidate for further investigation because it has a far superior synthesis rate and processivity [55] compared to Klenow exo- and does not exhibit the nonspecific background amplification observed in most thermophilic polymerases. Nt.BspQI was selected as the nicking endonuclease because it has a long 7-bp recognition sequence and has a faster turnover rate than Nt.BbvCI. Table 1 lists the concentrations of the enzymes we have optimized for linear SDA.

Table 1
The concentrations of enzymes for optimal isothermal linear SDA.

Optimal concentrations of enzymes for SDA				
Polymerase	Concentration (nM)	Nicking enzyme	Restriction site	Concentration
Klenow exo-	25	Nt.AlwI	GGATCNNNN^	20 pM
Bst Large Fragment	5	Nt.BstNBI	GAGTCNNNN^	10 nM
Sequenase 2.0	400	Nt.BspQI	GCTCTTCN^	3 nM
Vent exo-	100	Nt.BbvCI	CC^TCAGC	50 nM
9°Nm	5	Nb.BsrDI	^CATTGC	0.08 U/μl
		Nb.BsmI	G^CATTC	0.25 U/μl

For enzymes whose specific activities were not available, the concentrations are given in units per microliter. The usage of higher concentrations of thermophilic polymerases usually leads to nonspecific amplification.

It is known that the addition of single-stranded DNA binding proteins can significantly facilitate DNA synthesis by many DNA polymerases [61–66]. We found that single-stranded DNA binding proteins are essential for SDA with Sequenase 2.0. Even though both T4 gene 32 protein and *E. coli* SSB can be used, amplification with the latter and Sequenase 2.0 gives cleaner bands on polyacrylamide gels. In addition, the use of SSB enhances SDA amplification by Klenow exo- and significantly suppresses background amplification by Bst DNA polymerase. The binding of the displaced DNA strand by SSB facilitates the strand-displacement synthesis by the polymerase and suppresses nonspecific amplification by preventing nonspecific hybridization of the 3′ ends of the single-stranded products. The profound effect of SSB on SDA with Sequenase 2.0 is shown in Fig. 7B. With 5 μM SSB, long DNA molecules can be amplified by Sequenase 2.0. In the absence of SSB, however, no observable amplification occurs. It can be seen that the nicking enzyme did cut one strand of the target, but Sequenase 2.0 was unable to proceed with strand-displacement synthesis in the absence of SSB. As shown in Fig. 7B, the nicked strand appears as a band 38 bp below the original template.

If the nicking site is absent in the internal sequences of the target DNA molecules, all of the target molecules in a complex mixture can be amplified by nicking-endonuclease-mediated strand displacement in a linear fashion regardless of the length or sequence of the molecules. In other words, the molecules are multiplied to the same fold factor with the original ratio among the molecules in the mix maintained. This is because each template molecule contains an identical adaptor sequence that is nicked at an equal rate by the endonuclease with the nicked site in turn serving as the primer for strand-displacement synthesis by the DNA polymerase. The amplification reaction rate could be limited either by the nicking rate of the endonuclease or by the rate of strand-displacement synthesis from the nicks.

The rate-limiting step for SDA under our conditions appears to be the rate of nicking by the endonuclease. This is evidenced by the absence of visible unfinished or truncated products on the gel following the immediate termination of an SDA reaction with stop buffer containing formamide and EDTA. This implies that Sequenase 2.0 can initiate the SDA reaction immediately after each nicking event and can complete the SDA along the entire template in a short amount of time before another nick is made again in the template. A quick calculation supports this theory. The rate of synthesis for Sequenase 2.0 is about 200 nucleotides per second, and the polymerase can incorporate an average of 800 nucleotides without dissociating from the template [55,67]. The presence of at least 100-fold molar excess of polymerase to template and a high concentration of the polymerase (400 nM) ensure that if an enzyme does fall off the template, it is quickly replaced. Therefore, we can expect a 5-knt-long fragment to be completely synthesized in less than 30 s. In contrast, the turnover rate for a mesophilic nicking enzyme appears to range from 30 s for the very active Nt.AlwI [48] to 4.5 min for Nt.BbvCI [53]. Nt.BspQI is considered a thermophilic enzyme with an ideal operating temperature of 50 °C. The K_M and intrinsic turnover rate of the enzyme have not been reported. We determined the intrinsic turnover rate of substrate-saturated Nt.BspQI at 37 °C to be about 45 s under our reaction conditions (data not shown). Interestingly, the enzyme was three times more active in our SDA buffer than in the buffer recommended by New England Biolabs. At a template concentration below 3 nM, the amplification may be operating below the K_M of the nicking enzyme. Therefore, the nicking enzyme is not saturated with substrate and Nt.BspQI would have a lower effective turnover rate. Our observation that 20-fold amplification seems to occur in

40 min indicates that the effective turnover rate under our reaction conditions is about 2 min.

The low turnover rate of the nicking enzyme essentially acts as a method of local isothermal cycling. For each nicking event, each template is replicated once regardless of length or sequence. Complications could arise during SDA of longer DNA molecules if the template was nicked multiple times before the initial polymerization of the strand was complete. This would lead to multiple polymerases performing SDA reactions simultaneously at different sites on the same template. While the amplification would still remain linear, the template would be susceptible to a traffic jam of polymerases if a region of unusual sequence or complexity were encountered by the first polymerase. Additionally, a fraction of truncated products would be present at the conclusion of the reaction. However, in our SDA experiments, very little unfinished product is observed (Figs. 2 and 3). This is due to the rapid kinetics, high processivity, and strong strand-displacement properties of the Sequenase 2.0 DNA polymerase and the slower turnover rate of the nicking enzyme Nt.BspQI used for the SDA reaction.

It is likely that the nicking rate can be increased somewhat without jeopardizing the linearity of the amplification. This would be desirable to obtain greater product yields. Although the optimal temperature for Nt.BspQI is 50 °C, the reaction rate cannot be increased by raising the reaction temperature because Sequenase 2.0 DNA polymerase is not stable above 37 °C. Faster nicking enzymes will be required to increase the speed of linear SDA.

The amplification of a complex library constructed from the lambda phage genome was successful and commensurate to the amplification of single templates. Approximately 10- to 15-fold amplification was achieved in 30 min with the original length distribution of the fragments remaining largely intact. The larger than expected increase in the number of shorter fragments is likely due to the presence of internal Nt.BspQI nicking sites that were not introduced by our adapter fragments but are instead native to the lambda phage genome. The Nt.BspQI recognition site is overrepresented in the lambda phage genome with 10 cutting sites. If the distribution of the native nicking sites follows a Poisson distribution, about 18% of the 1.15-knt fragments contain one or more internal nicking sites. Amplification from the internal native cutting sites results in shorter products, introducing some bias toward certain fragments. This underscores the need for the discovery or engineering of novel nicking endonucleases with much greater specificity. For the amplification of longer DNA molecules and larger genomes, a nicking endonuclease with an even longer recognition sequence (e.g., 12–16 bp) would be ideal. Fortunately, there has been an increased effort in engineering such nicking endonucleases for both DNA amplification and genome engineering [68–70].

There is a relatively bright low molecular weight band at around 0.5 knt in the amplified product which is not present in the original library (Fig. 5A). This band corresponds to the shoulder peak in the curves of Fig. 5B. The source of this is not known. This could be due to the products generated from the internal nicking sites in the genomic DNA fragments, or some nonspecific amplification. Nonspecific amplification is commonly observed in SDA with Bst DNA polymerases even with a single template species. It is, however, rarely observed in SDA by Sequenase 2.0 in the presence of high concentrations of SSB. The use of hairpin adapters may provide a means to improve the specificity of the amplification by converting the free 3′ ends of initial SDA product into a double-stranded form (Fig. 6). An added benefit of using such a design is that the conversion of the single-stranded DNA into a double-strand form also frees up the bound SSB which is essential for strand-displacement replication by Sequenase 2.0.

Given that a 5-knt template can be amplified with ease by SDA using Nt.BspQI and Sequenase 2.0, we expected that longer templates could also be amplified efficiently. Surprisingly, our attempts to amplify a 10-knt fragment from the lambda phage genome were less successful. A large number of shorter DNA fragments are present in the final product. Further optimization of the reaction conditions or the use of a more processive enzyme will be required for the efficient amplification of longer templates.

Conclusions

In summary, we have developed a method for isothermal DNA amplification by nicking endonuclease-mediated DNA polymerase strand displacement. Our technique differs from the SDA method pioneered by Walker et al. in several important aspects. The most significant attribute of our method is the ability to amplify fragments up to 5 knt in length with very little bias. We have shown that 5-knt fragments can be amplified with an efficiency approximately equal to that of 0.5-knt fragments. This is feasible because our method employs a DNA polymerase, Sequenase 2.0, which has very high processivity and strong strand-displacement capability in the presence of single-stranded binding proteins, and a nicking endonuclease, Nt.BspQI, which has a long 7-bp recognition sequence. The other attribute is our use of a nicking endonuclease, which obviates the need for α-phosphorothioate nucleotides in the reaction so the product contains only native nucleotides. We have demonstrated that a mixture of DNA molecules with lengths of 0.5, 1, and 5 knt can be amplified in a linear fashion with the original molar ratio preserved. Using a library of randomly sheared genomic fragments from bacteriophage lambda, we also showed that a complex library was amplified linearly with the original distribution of the fragments largely maintained. Together, these significant improvements open the door to the possibility of using SDA not only for sequence detection but also for linear amplification of long DNA templates and heterogeneous mixtures of templates with little bias and without thermal cycling. Our method is potentially useful for certain applications such as genome sequencing or gene expression profiling from a limited sample source where linear unbiased amplification of a mixture of complex DNA molecules is highly desirable. It is quite possible that our method can be extended to linear amplification of templates longer than 5000 bp, and to exponential SDA amplification by including both the forward and the reverse primers containing a recognition site for the nicking endonuclease in the reaction mix. Further work will be required to demonstrate these capabilities and to enable the unbiased linear amplification of large genomes by SDA.

Acknowledgments

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