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Amplification of closed circular DNA *in vitro*

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ABSTRACT

The polymerase chain reaction is a powerful technique used to amplify nucleic acids *in vitro*. The reaction produces linear products, and as of yet, closed circular products have not been possible. Since the replicatively competent form of many DNA molecules is the closed circular form, it would be adventitious to amplify closed circular DNA as closed circular molecules. Until now, these molecules could only be amplified *in vivo* in appropriate host cells. Here, we describe an *in vitro* procedure, ligation-during-amplification (LDA), for selective amplification of closed circular DNA using sequence-specific primers. LDA is useful for site-directed mutagenesis, mutation detection, DNA modification, DNA library screening and circular DNA production.

The polymerase chain reaction (PCR) (1–3) is a powerful technique for *in vitro* amplification of nucleic acids. Although circular and linear nucleic acids can serve as templates for PCR, the resulting products have always been linear molecules. Until now, closed circular DNA, the replicatively competent form of many DNA molecules, could only be amplified in appropriate host cells. Here, we describe and demonstrate an *in vitro* procedure, ligation-during-amplification (LDA), for selective amplification of closed circular DNA using sequence-specific primers. The essence of LDA is the inclusion of a thermostable DNA ligase in a PCR reaction that uses a closed circular DNA as template. After a primer is fully extended on the circular template, the ligase closes the gap to form a double-stranded DNA. Following thermal denaturation, the two circular DNA strands serve as templates for the next round of extension and ligation. Through thermal cycling, closed circular DNA is amplified exponentially.

To demonstrate the feasibility of using LDA to generate and amplify closed circular DNA, two 5′ phosphorylated primers (16 and 17 nt long) were used to mutate and amplify a circular plasmid of 1990 bp. The primers are complementary to different strands of the plasmid in an inward orientation (Fig. 1A). One possesses a single G to A mismatch on an *Hph*I site in the plasmid. The reaction mixture (50 µl) contained 10 ng of native plasmid, 10 pmol of each primer, 10 nmol of dNTPs, 5 nmol of ATP, 2.5 U *Pfu* DNA polymerase (Stratagene, La Jolla, CA), 4 U *Pfu* DNA ligase (Stratagene), in 1× cloned *pfu* DNA polymerase reaction buffer consisting of 20 mM Tris–HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 1% Triton X-100 and 100 µg/ml BSA. The mixture was pre-incubated at 70°C for 10 min allowing the ligase to repair any nicks in the template. It was then subjected to thermal cycling at 95°C for 1 s (denaturation), 50°C for 1 s (annealing), 72°C for 4 min (extension), 95°C for 1 s (denaturation) and 72°C for 4 min (annealing, extension and ligation) for 20 cycles. As a control, a PCR was performed under similar conditions, except that the DNA ligase was not included. The reaction mixtures were analyzed directly by electrophoresis into 1% agarose gels followed by staining with ethidium bromide. As shown in Figure 1B, inclusion of *Pfu* DNA ligase in the reaction produced a product having mobility slightly faster than that of the relaxed form (RF II) of the plasmid isolated directly from a bacterial culture. In the absence of *Pfu* DNA ligase, this same closed circular product was not produced. The yield of amplified plasmid increased as the number of LDA cycles increased (Fig. 1C).

To examine the functional competence of the LDA product, the amplified plasmid was used to transform bacterial cells. The LDA amplified plasmid was first digested with *Dpn*I restriction enzyme to remove the starting template DNA and subsequently introduced into *Escherichia coli* strain DH5α by electroporation. The yield of transformed cells was very high. All 10 clones tested possessed the desired mutation.

LDA can be used to introduce multiple mutations simultaneously. On a 5192 bp plasmid, three mutagenic primers were used to eliminate a *Bsg*I, *Bpm*I and a *Bsm*I site, simultaneously. The *Bsg*I primer was complementary to one strand, and the *Bpm*I and *Bsm*I primers to the other strand. The 3′-end of the *Bsg*I primer is 3890 and 4742 bp from the 3′-ends of the *Bsm*I and *Bpm*I primers, respectively. The plasmid was subjected to LDA in the presence of the three mutagenic primers, and after transformation, 10 clones were analyzed. Of the 10 clones, all had the *Bsg*I mutation, eight had both *Bsg*I and *Bpm*I mutations, and six had all three mutations.

We have successfully amplified larger plasmids. Using two primers separated by 95 bp between their 3′-ends, a 9516 bp plasmid was amplified (Fig. 1D). The amount of input DNA was similar to that used in Figure 1C. The cycling conditions used were: 94°C for 10 s, 50°C for 2 min, 72°C for 10 min, 94°C for 10 s and 72°C for 10 min, for 20 cycles.

Adding thermostable DNA ligase to a PCR reaction and using standard PCR cycling conditions, Michael (4,5) observed the formation of linear PCR products only. We found that adding a

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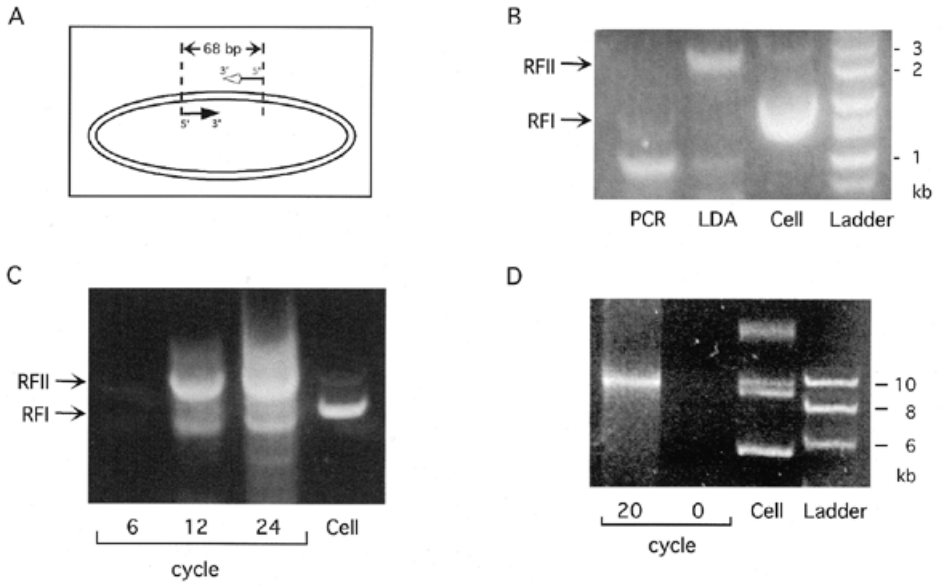

Figure 1. Amplification of closed circular plasmids by LDA. (**A**) The primer configuration used in the amplifications shown in (**B**) and (**C**). The open arrow represents the mutagenic primer. (B)–(D) Ethidium bromide stained 1% agarose gels containing the products of LDA and PCR reactions. (**B**) Comparison of the amplification products from a 1990 bp plasmid obtained in the presence (LDA) and absence (PCR) of a thermostable DNA ligase. (**C**) Amplification of the plasmid as the cycle number increases. (**D**) The amplification of a 9516 bp plasmid. The migration positions of the supercoiled (RF I) and relaxed (RF II) forms of the native plasmid isolated directly from bacterial culture (Cell) and the DNA size makers (Ladder) are as indicated.

72°C annealing step, immediately following a 95°C denaturation step, is critical for the formation of closed circular plasmid. This inhibits the annealing of the input primers, and instead, allows the two strands of the standard PCR product (a 68 bp fragment in the example in Fig. 1A–C) to anneal to the template and serve as 'megaprimers' (6) for the LDA reaction. This prevents accumulation of the standard PCR product (i.e. that defined by the 5′-ends of the two primers), and favors amplification of the entire circular molecule. In fact, if this thermal cycling protocol is used, circular DNA can be amplified using either inward or outward primer orientations that are tens to thousands of base pairs apart. However, we have also found that using two 20 nt primers having 10 nt of complementarity at their 5′-ends, a two-step LDA cycle (95°C, denaturation; 68°C, extension/ligation) will produce the same result.

Allowing the use of sequence-specific primers to amplify replicatively competent closed circular DNA directly, without the need of subcloning, makes LDA a powerful tool. Besides site-directed mutagenesis, many other applications are also possible. LDA can be used to detect point mutations. The use of a primer with a single mismatched nucleotide at its 5′-end will allow only the correspondingly mutated DNA to be amplified. LDA can be used to prepare plasmid containing modified nucleotides. The modifications can be randomly introduced in both DNA strands by inclusion of suitably modified nucleotide triphosphates in the LDA reaction. Alternatively, modifications can be introduced at specific site(s) by using primer(s) containing the modified nucleotides. Most significantly, LDA can be used to isolate vectors carrying a gene or DNA fragment of interest directly from a genomic or cDNA library. This eliminates the need for time consuming screening by the commonly used colony and plaque hybridization methods. Finally, LDA can be used to produce replicatively competent DNA, including viral DNA, safely in a cell-free system.

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