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Method to eliminate linear DNA from mixture containing nicked-circular, supercoiled, and linear plasmid DNA¹

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Abstract

Preparations of circular plasmid DNA in either supercoiled or nicked-circular form often are contaminated with undesired linear DNA fragments arising from shearing/degradation of chromosomal DNA or linearization of plasmid DNA itself. We report a simple enzymatic method, using a combination of λ exonuclease and RecJ_F, for the selective removal of linear DNA from such mixtures. λ exonuclease digests one strand of linear duplex DNA in the 5' to 3' direction, whereas RecJ_F, a single-strand-specific exonuclease, digests the remaining complementary single strand into mononucleotides. This combination of exonucleases can remove linear DNA from a mixture of linear and supercoiled DNA, leaving the supercoiled form intact. Furthermore, the inability of λ exonuclease to initiate digestion at nicks or gaps enables the removal of undesired linear DNA when nicked-circular DNA has been enzymatically prepared from supercoiled DNA. This method can be useful in the preparation of homogeneous circular plasmid DNA required for therapeutic applications and biophysical studies.

Keywords

λ exonuclease; RecJ_F; Nicked-circular DNA; Linear DNA; Supercoiled DNA

Introduction

Plasmid DNA exists in three topological forms: supercoiled, nicked-circular (open-circular), and linear. The replicatively competent form of plasmid DNA is either supercoiled or open-circular, and active circular plasmid DNA plays an important role in many DNA-based biologic and therapeutic applications [1]. Plasmid vectors and artificial bacterial chromosomes, used to clone DNA inserts ranging in size from a few hundred basepairs to thousands of basepairs, are circular, as is suitably engineered plasmid DNA employed in DNA vaccination [2] and gene therapy [3] strategies. Direct injection into target tissue of circular plasmid DNA containing a therapeutic gene of interest has also been attempted [4]. In these applications, it is essential

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that all the plasmid DNA be circular in order to be transformed effectively into bacteria or transfected into mammalian cells [5].

Although the bacterial host strains used to amplify plasmid DNA maintain the DNA in a negatively supercoiled state, subsequent isolation procedures and downstream processing result in a portion of the supercoiled plasmid DNA molecules becoming nicked in either one or both strands to form open-circular and linear DNA, respectively. Agarose gel electrophoresis and chromatographic methods [6,7] can separate circular forms of plasmid DNA from the linear variant and subsequently the circular form can be extracted from the gel. However, these methods are limited to processing DNA in small quantities and are not suitable for removing linear DNA from large-scale (mg) plasmid preparations.

We describe herein a simple method that is based on using a combination of λ exonuclease and RecJ_F to remove selectively linear DNA contaminants from circular plasmid DNA. This method was developed in connection with our ongoing exploration of the role of DNA topology in radiation-induced DNA strand breaks [8,9]. As an example of the usefulness of this method, we demonstrate that the minor portion of undesired linear DNA formed in the large-scale preparation of nicked-circular pUC19 from supercoiled DNA can be simply and efficiently removed. We anticipate that this simple procedure for digesting linear DNA completely from mixtures containing DNA in various topologies will be applicable in a variety of situations in which linear DNA is viewed as a contaminant.

Materials and methods

Preparation of ³H-dThd-pUC19 plasmid DNA

Stocks of *E. coli* DH5 α bacterial cultures with pUC19 were grown in Luria broth for 16 h at 37 °C in the presence of ampicillin (50 μ g/mL) and ³H-thymidine (³H-dThd, 37 MBq). The plasmids (³H-dThd-pUC19) were isolated using the Qiagen Maxi preparation kit and dissolved in PBS (pH 7.4). The concentration, determined at A₂₆₀, was 0.748 μ g/ μ L. The plasmid DNA was stored at -20 °C.

Nicking of ³H-dThd-pUC19 plasmid DNA

Restriction endonuclease *Eco*R1 (New England Biolabs) cuts only one of the two DNA strands in its recognition sequence in the presence of ethidium bromide and leads to a nicked-circular molecule [10]. Since our model pUC19 plasmid DNA contains one *Eco*R1 site, we digested supercoiled ³H-dThd plasmid DNA (100 μ g in 134 μ L 1X PBS) with *Eco*R1 (800 units, 40 μ L) in the presence of ethidium bromide (0.4 mg/mL) and *Eco*R1 buffer (1X, 400 μ L, New England Biolabs) for 24 h at 37 °C. The samples were assessed on agarose gel for nicked-circular DNA formation. To ensure the complete digestion of supercoiled DNA, another aliquot of nicking reaction cocktail was added and the incubation continued for an additional 24 h. Subsequently, the reaction was arrested by addition of EDTA to a final concentration of 10 mM. Finally, the reaction mixture was extracted with phenol (pH >7.5) and chloroform:isoamyl alcohol (24:1, v/v), followed by five *n*-butanol extractions to remove ethidium bromide completely. DNA was precipitated with ethanol and redissolved in 1X PBS.

λ exonuclease and RecJ_F digestion

The enzymes λ exonuclease and RecJ_F were purchased from New England Biolabs. A mixture of supercoiled (3.7 μ g) and linear (3.3 μ g) DNA was incubated with λ exonuclease (1 μ L, 5 units/ μ L) and RecJ_F (3 μ L, 30 units/ μ L) in a total volume of 100 μ L containing 1X λ exonuclease buffer (New England Biolabs) at 37 °C for 16 h. The nicked-circular DNA preparation (14 μ g) containing traces of linear DNA was then incubated again with λ exonuclease (0.5 μ L) and RecJ_F (3 μ L) in a total volume of 100 μ L containing 1X λ exonuclease

buffer at 37 °C for 16 h. Next, the λ exonuclease was heat-inactivated (65 °C, 10 min), and the reaction mixture was extracted with phenol and chloroform:amyl alcohol (24:1, v/v). DNA was evaluated on 1% agarose gel. Homogeneous nicked-circular ^3H -dThd-pUC19 plasmid DNA was precipitated with ethanol and dissolved in 1X PBS.

Results and discussion

The ability of λ exonuclease and RecJ_F to selectively digest linear plasmid DNA in a mixture containing both linear and supercoiled forms is shown in Fig. 1. The supercoiled pUC19 DNA (7.4 μg) was mixed with linearized pUC19 DNA (6.6 μg) and divided into two equal portions. One aliquot was kept aside as a control and the second aliquot was digested overnight at 37 °C with λ exonuclease (5 units) and RecJ_F (90 units). Subsequently, the digested DNA sample was analyzed on 1% agarose gel (Fig. 1, lane 2) along with the undigested DNA sample (supercoiled and linear forms) as a control (Fig. 1, lane 1). Prior to its digestion with λ exonuclease and RecJ_F (lane 1), the DNA mixture has two discrete bands corresponding to supercoiled and linear forms of pUC19. After digestion (lane 2) the linear form disappears completely, indicating that the combination of λ exonuclease and RecJ_F is able to digest completely linear plasmid DNA into mononucleotides while leaving the supercoiled DNA initially present in the mixture intact. These results demonstrate that the combination of λ exonuclease and RecJ_F can digest the linear form selectively and efficiently in a mixture containing supercoiled (closed-circular) and linear forms.

Under normal conditions of digestion, restriction endonuclease *Eco*R1 (New England Biolabs), a homodimer, cuts both strands in DNA between the bases G and A in the recognition sequence 5' GAATTC 3', leading to a double-strand break. However, when the DNA intercalator ethidium bromide is added to the supercoiled DNA sample, *Eco*R1 severs only one of the two DNA strands in its recognition sequence, resulting in the relaxation of supercoiling and the production of a nicked-circular molecule [10]. To examine whether the exonuclease treatment can successfully remove linear plasmid DNA produced during the preparation of nicked-circular plasmid DNA, the supercoiled form of pUC19 (100 μg) was digested with *Eco*R1 (800 units) in the presence of ethidium bromide (10 mg/mL). After an overnight incubation at 37 °C, the mixture was analyzed on 1% agarose gel. Only ~20% of the supercoiled form is converted into nicked-circular DNA with traces of linear DNA resulting from scission in both strands at the *Eco*R1 site (Fig. 2A). Another aliquot of the cocktail containing *Eco*R1 and ethidium bromide was added to the reaction mixture, the incubation continued another 24 h, and the nicking reaction was assessed again on agarose gel. As seen in Fig. 2B, lane 2, a significant amount of nicked-circular DNA (~85%) is formed with no supercoiled DNA left undigested. However, a small but significant amount of linear DNA is also produced due to scission in both strands of DNA, leaving this preparation unsuitable for our studies.

To remove the linear DNA contaminant from the nicked-circular DNA preparation, we used the λ exonuclease treatment. The inability of this exonuclease to initiate digestion at nicks or gaps enables the removal of undesired linear DNA whereas nicked-circular DNA is left intact. In Fig. 2C, lanes 1 and 2 contain control supercoiled and linear pUC19 DNA, respectively, and lane 3 has nicked-circular DNA containing traces of linear DNA. Lane 4 shows the same nicked-circular DNA preparation after treatment with λ exonuclease and RecJ_F in which case the undesired linear DNA is completely removed leaving pure nicked-circular DNA suitable for biophysical studies. Using the method described here, we are able to obtain pure nicked-circular plasmid DNA without resorting to cumbersome cesium chloride or sucrose density gradient centrifugation methods.

Conclusions

We have developed an efficient enzyme-mediated method to remove linear plasmid DNA from a mixture containing various topological forms of plasmid DNA. Further, we have shown that the enzymes λ exonuclease and RecJ_f are highly selective in digesting linear DNA leaving behind pure, biologically active and replicatively competent, intact circular (supercoiled or nicked-circular) plasmid DNA [5]. This method would be useful for removing contaminant linear DNA (for example, bacterial chromosomal DNA) from plasmid DNA preparations intended for therapeutic applications and biophysical studies.

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Fig. 1. Agarose gel electrophoresis demonstrating potential of λ exonuclease and RecJ_f to selectively digest linear (L) form of plasmid DNA. Mixture of supercoiled (SC) and linear forms of pUC19 before (lane 1) and after (lane 2) digestion with λ exonuclease and RecJ_f. DNA size marker (lane 3). Note that after digestion linear form disappears completely leaving only supercoiled DNA.

Fig. 2.

(A) Nicking of supercoiled (SC) pUC19 by *EcoR*I in presence of ethidium bromide assessed by agarose gel electrophoresis after overnight incubation at 37°C. (B) After incubation continued an additional 24 h. Control supercoiled DNA (lane 1); aliquot drawn from nicking reaction (lane 2). Note that at end of second incubation (B, lane 2) supercoiled DNA is completely converted into nicked-circular (N) and linear (L) forms. (C) Agarose gel showing digestion of nicked-circular (N) DNA preparation with λ exonuclease and *RecJ*_f to remove undesired linear DNA. Control supercoiled (SC) pUC19 DNA (lane 1); control linear (N) pUC19 DNA (lane 2); nicked-circular DNA preparation before exonuclease digestion (note presence of contaminating linear DNA) (lane 3); nicked-circular DNA preparation after exonuclease digestion (note disappearance of contaminating linear DNA) (lane 4); DNA size marker (lane M).