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Deoxyribonuclease Treatment Improves the Homogeneity of Single-Stranded DNA Preparations

BioTechniques 22:105-110 (January 1997)

ABSTRACT

The isolation of single-stranded (ss) phagemid DNA using standard protocols often results in impure preparations, which contain undesirable quantities of chromosomal and/or double-stranded (ds) phagemid DNA. Here we report a simple and efficient method for elimination of virtually all dsDNA by incubation of phagemid viral particles with deoxyribonuclease I. In addition to analyzing the ratio of linear-to-circular topological forms of ssDNA after deoxyribonuclease I treatment, we verified that no decrease in transformation efficiency occurred and demonstrated that ssDNA molecules covered by capsid proteins remained intact following such treatment.

INTRODUCTION

Single-stranded DNA (ssDNA) molecules of filamentous phages or phagemid vectors are widely used in molecular biology experiments. Since it was first described, helper phage mediated single-stranded phagemid DNA isolation (6) has become a routine tool used in the preparation of template DNA for site-specific mutagenesis and sequencing.

The ssDNAs isolated by standard techniques always contain some amount of chromosomal and/or double-stranded phagemid DNA (dsDNA). The presence of dsDNA may cause mispriming events that result in ambi-

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guity in the sequencing ladder or, in the case of mutagenesis, a lower yield of mutant progeny. Most of these protocols call for the cells to be incubated for 6–12 h following helper phage infection. However, since contaminating dsDNA is accumulated as a result of cell lysis, the longer the cells are cultivated, the greater the amount of contaminating dsDNA. Shorter incubation periods, which usually result in the isolation of ssDNA containing less dsDNA, may in certain cases be insufficient to obtain good yields of ssDNA. This may be caused by differences in the rates of synthesis and accumulation of ss phagemid DNA of different structures. Thus, the isolation of larger amounts of ssDNA molecules may necessitate longer periods of cell cultivation, which in turn may be accompanied by an unfavorable accumulation of cellular and phagemid dsDNA.

We have developed a method for the elimination of dsDNA from ssDNA preparations by including a deoxyribonuclease I (DNase I) treatment step in the ssDNA isolation protocol. This has enabled us to obtain ssDNA of higher purity without degradation of the ssDNA molecules themselves.

MATERIALS AND METHODS

Phagemid pOPf1 was chosen for the isolation of ssDNA. This phagemid (5.8 kb in length) was constructed in our laboratory by insertion of the f1 intergenic region into the *Eco*RI site of the plasmid pOP540 (2).

Single-Stranded DNA Isolation

XL-1 Blue cells (1) (Stratagene, La Jolla, CA, USA) were transformed by pOPf1 double-stranded phagemid and plated on 2× YT agar plate (16 g tryptone, 10 g yeast extract, 5 g NaCl, 17 g bacto-agar per liter) supplemented with 70 µg/mL ampicillin (Amp) and 15 µg/mL tetracycline (Tet). Several colonies were selected for inoculation into 10 mL of 2× YT broth (Tet, Amp), and the culture was incubated overnight at 37°C. Using 0.5 mL of the overnight culture, 50 mL of 2× YT medium (without antibiotics) were inoculated. The cells were grown until they reached optical density (OD)₅₅₀ = 0.3,

Table 1. Results of Transformation of Competent XL-1 Cells by pOPf1 ssDNA Purified from Agarose Gel

	Control ssDNA	DNase I-treated ssDNA			
		DNase I concentration (mg/mL)			
		0.006	0.03	0.06	0.6
Number of colonies	64 ± 11	73 ± 8	70 ± 15	56 ± 7	61 ± 12

Control ssDNA sample is pOPf1 ssDNA from viral particles subjected to phenol/chloroform extraction immediately after PEG precipitation, omitting treatment with DNase I. Results represent the mean ± standard deviation of three separate experiments.

which corresponded to about 2.5×10^8 cells/mL. At this point, fresh M13KO7 helper phage (Promega, Madison, WI, USA) was added to a multiplicity of infection of 10 plaque-forming units per cell and incubated for 1 h at 37°C with moderate agitation (145 rpm). Kanamycin was added to a final concentration of 70 µg/mL, and cells were grown for 10 h at 37°C and 180 rpm.

The cells were pelleted (17 000× *g*, 15 min, 4°C), and the supernatant was retained and subjected to an additional centrifugation (17 000× *g*, 15 min, 4°C) to pellet remaining cells. The volume of the supernatant was measured, and a

solution (one-fourth of the measured volume) containing 20% polyethylene glycol (PEG 6000) and 3.5 M ammonium acetate was added. The phage suspension was left to precipitate for 1 h at 4°C. The precipitated phagemid viral particles were pelleted by centrifugation as above. The supernatant was poured out, and the remaining PEG was taken off with a drawn out pipet connected to a vacuum. The phage pellet was resuspended in 2 mL of sterile Milli-Q™ water (Millipore, Bedford, MA, USA).

Aliquots of the phage solution (130 µL) were subjected to DNase I treatment (see below) followed by extrac-

Figure 1. Agarose gel (1%) with ssDNA samples following DNase I treatment. Lane 1: the phagemid pOPf1 dsDNA control sample; lane 2: ssDNA from viral particles that were subjected to phenol/chloroform extraction immediately after PEG precipitation (without incubation in Tris-magnesium buffer and DNase I treatment); lanes 3–7: ssDNA from viral particles that were incubated after PEG precipitation in Tris-magnesium buffer for 1 h at 37°C in the presence of DNase I at the following concentrations (mg/mL): lane 3, 0; lane 4, 0.006; lane 5, 0.03; lane 6, 0.06; and lane 7, 0.6; lane 8: M13KO7 helper phage ssDNA.

Figure 2. Electrophoretic (1.5% alkaline agarose gel) separation of circular and linear topological forms of ssDNA samples isolated from viral particles following DNase I treatment. Lane 1: ssDNA from viral particles that were subjected to phenol/chloroform extraction immediately after PEG precipitation; lanes 2–5: ssDNA from viral particles that were incubated after PEG precipitation in Tris-magnesium buffer for 1 h at 37°C in the presence of DNase I at the following concentrations (mg/mL): lane 2, 0.006; lane 3, 0.03; lane 4, 0.06; and lane 5, 0.6.

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tion with Tris-HCl buffer, pH 8.0, saturated phenol/chloroform (3:1). The extraction was repeated four times until no interface material was observed and then followed by two chloroform extractions. The DNA was then precipitated by addition of one-half volume of 7.5 M ammonium acetate (pH 7.5) and 3 volumes of ethanol. After precipitation, the DNA pellet was dissolved in sterile water and reprecipitated. Finally, it was dissolved in 20 μ L of sterile Milli-Q water.

The control 130- μ L aliquot was treated in parallel with those subjected to the experimental protocol with the exception that it was not incubated with DNase I.

DNase I Treatment

Tris-HCl, pH 7.5 and MgCl_2 were added to 130 μ L of phage solution to the final concentrations of 50 and 10 mM, respectively. DNase I from bovine

pancreas (stock solution 10 mg/mL; Serva Feinbiochemica GmbH, Heidelberg, Germany) was added to the suspension of phagemid viral particles to achieve final concentrations of 0, 0.006, 0.03, 0.06 and 0.6 mg/mL. The reaction mixture (150 μ L) was incubated at 37°C for 1 h.

Gel Electrophoresis

Agarose gel electrophoresis (1% gel, 1 $\times$ TAE buffer) was used to analyze the ssDNA samples from DNase I-treated and untreated viral particles. Two microliters of ssDNA (1/10 of the volume) were used per lane.

Alkaline agarose gel electrophoresis was performed in 1.5% gel at 3 V/cm for 4 h according to the procedure described by Shin and Day (5). Three microliters of ssDNA were loaded into each lane.

Both gels were stained with ethidium bromide to visualize DNA bands.

Agarose Gel Purification of ssDNA

After electrophoretic separation, the TAE buffer was removed from the electrophoresis chamber until the level of buffer was just below the top surface of the gel. A piece of agarose gel was excised just ahead of the ssDNA bands to form a window that was filled with 3 $\times$ TAE buffer. Slightly behind the bands, a strip of NA45 membrane (Schleicher & Schuell, Dassel, Germany) was inserted to catch larger molecules. Electrophoresis was initiated and run for 3 min at 6 V/cm to elute the ssDNA out of the gel and into the window. When all the DNA had entered the window, the DNA-containing 3 $\times$ TAE buffer was recovered from the window, and the DNA was precipitated with ethanol.

Cell Transformation by ssDNA

ssDNA recovered from the agarose gel was dissolved in 10 μ L of sterile water. The concentration of ssDNA was

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measured using a Model TKO 100 DNA Fluorometer (Hoefer Pharmacia Biotech, San Francisco, CA, USA) according to the manufacturer's instructions.

One nanogram of ssDNA was added to 200 μ L of competent XL-1 cells, which were prepared using the calcium chloride method (4). The cells with ssDNA were incubated on ice for 1 h then heat-shocked for 90 s at 42°C. After that, 800 μ L of 2 $\times$ YT medium were added to the cells, and the cells were incubated at 37°C for 2 h before spreading on 2 $\times$ YT/agar/ampicillin plates. Two hundred microliters of cells were spread. The plates were incubated overnight at 37°C.

RESULTS AND DISCUSSION

ssDNA samples following DNase I treatment of viral particles, phenol/chloroform extraction and electrophoretic separation are presented in Figure 1. Comparison of lanes 2 and 3 with lanes 4–7 demonstrates that DNase I treatment is sufficient to eliminate phagemid dsDNA, which comprises the upper bands of the gel (lanes 2 and 3). It can also be seen that the intensity of ssDNA bands in the case of DNase I treatment equals that of the untreated sample even when the highest enzyme concentration was used for DNase I treatment. The appearance of fragments of smaller size, which may result from ssDNA degradation by DNase I, is not observed in lanes 4–7.

Both the ssDNA samples from viral particles treated with DNase I as well as the control ssDNA sample from untreated particles were subjected to alkaline agarose gel electrophoresis (Figure 2). This was done to exclude the possibility that DNase I had introduced single-stranded breaks into the circular DNA molecules, thus converting them to their linear form. According to the observations of Shin and Day (5), this procedure can be used to determine the topology of ssDNA molecules, as the circular and linear forms of ssDNA are easily resolved under alkaline conditions. Following the authors' recommendations, we performed the separation in a 1.5% agarose gel in order to achieve higher resolution of the two

forms (the linear ssDNA to circular mobility ratio increases with increasing gel concentration). The upper bands (Figure 2) correspond to circular ssDNA molecules, while the lower ones correspond to linear. It can be seen that the share of circular ssDNA molecules in lanes 2–5 is not less than for the control ssDNA sample and even exceeds it. This experiment also showed that the ratio of the two forms does not depend on the concentration of DNase I used during treatment.

The analysis of ssDNA topology, as well as the observation that no DNA smear is visualized in Figures 1 and 2, allows us to come to the conclusion that the ssDNA inside the viral capsid is not susceptible to DNase I degradation.

To further confirm the supposition that ssDNA molecules remain intact following DNase I digestion, we studied the ability of ssDNA molecules obtained using this method to transform XL-1 competent cells. ssDNA from the untreated sample was gel-purified and used as a control. This was done to prevent the appearance of colonies caused by the transformation of recipient cells with double-stranded phagemid DNA in the control sample. Taking into account that double-stranded molecules transform cells up to 10 times better than single-stranded molecules (3), it was impossible to disregard this fact. To standardize the experimental conditions, the DNase I-treated ssDNA was also gel-purified.

The results of these transformations are presented in Table 1. We observed no significant differences in the transformation efficiencies of ssDNA molecules from treated and untreated samples. This confirmed that DNase I does not damage ssDNA molecules, which are covered by capsid proteins.

In conclusion, the method presented here allows a rapid and efficient purification of ssDNA isolates from concomitant phagemid dsDNA without reducing the yield and quality of the ssDNA molecules. This method eliminates any necessity to tightly control the conditions of cell cultivation for ssDNA accumulation. Any preparation, even those having a remarkable content of dsDNA forms, can be used for mutagenesis and sequencing following DNase I treatment.

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The authors thank Prof. James R. Wild for generously providing the plasmid pOP 540 as well as Dr. Melinda Wales and Barbara D. Kuhlmann for their help in the preparation of the manuscript (Prof. James R. Wild, Dr. Melinda Wales and Barbara D. Kuhlmann are from the Department of Biochemistry and Biophysics, Texas A&M University, College Station, TX USA). Address correspondence to Teimur K. Aliev, Department of Chemical Enzymology, Chemical Faculty, Moscow State University, 119899 Moscow, Russia. Internet: aliev@ibch.siobc.ras.ru

Received 15 December 1995; accepted 10 June 1996.

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