

Full Length Research Paper

A simple, fast and efficient method for cloning blunt DNA fragments

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To increase a recombinant ratio of cloning blunt DNA fragments, for example, polymerase chain reaction (PCR) products, a vector DNA is usually digested by a blunt-end producing restriction endonuclease and then dephosphorylated in order to avoid self-ligation of the linearized vector. In this paper, we described a simple method to directly ligate a blunt DNA fragment to a non-dephosphorylated vector with a high recombinant ratio. Firstly, a vector DNA was digested by a blunt-end producing restriction enzyme, such as SmaI in our experiment. The linearized vector was then directly ligated to a blunt DNA fragment in a standard ligation buffer in the presence of SmaI. The results showed that the restriction enzyme SmaI in the ligation reaction can efficiently minimize the self-ligated vector and keep a very high recombinant ratio.

Key words: Blunt-end ligation, non-dephosphorylation, high efficiency.

INTRODUCTION

Ligation of blunt-end DNA fragments is a daily work in molecular biology laboratory. For a higher recombination, an endonuclease-cut vector is usually dephosphorylated in order to avoid self-ligation of the linearized vector. For that, a vector DNA is usually dephosphorylated by a phosphatase after restriction endonuclease digestion. After dephosphorylation of a vector, the phosphatase is removed from the reaction by phenol-extraction before ligation. If the dephosphorylated vector is used for cloning blunt-end polymerase chain reaction (PCR) products, the products are phosphorylated by a kinase before ligation. These procedures make a cloning experiment both time-consuming and expensive. Liu and Schwartz (1992) reported that end-filled PCR products could be ligated to a SmaI-cut vector at 22°C for 4 h in the presence of SmaI and the recombinant colonies were dramatically increased from 1.2 to 30%. Here, a similar ligation was set up but at 16°C overnight, and a much higher recombinant ratio, 86.8%, was acquired. The previous report (Liu and Schwartz, 1992) and our results proved that the method was really simple, fast, and efficient by adding a

restriction endonuclease in a ligation buffer, of which the endonuclease cut-site will not be regenerated after ligation.

MATERIALS AND METHODS

Preparation of cloning vector DNA and linearization

The vector, pUC19 was used for the experiment. It was prepared by the Alkaline Lysis method (Sambrook and Russell, 2001). Its quality and concentration were checked by agarose gel electrophoresis and an UV spectrometer, respectively. The vector DNA was then digested with SmaI (TaKaRa Biotechnology (Dalian) Co., Ltd.) into the blunt-end DNA fragment.

The digestion reaction was: 3 µg of pUC19 DNA, 3 µl of 10 x restriction buffer (330 mM Tris-Ac, pH7.9, 5 mM Dithiothreitol, 100 mM Mg-Ac, 660 mM K-Ac), 3 µl of SmaI (10 u/µl), deionized water up to 30 µl. The reaction mixture was incubated at 30°C for 1 h. After digestion, the linearized vector was extracted with the Tris-saturated phenol (pH 7.6), and then precipitated with ethanol (Sambrook and Russell, 2001). The air-dried vector DNA was re-dissolved in 30 µl of deionized water and determined by an UV spectrometer for concentration.

Preparation of the cloned foreign gene fragment

The open reading frame for AtDPBF4, one of the Dc3 promoter

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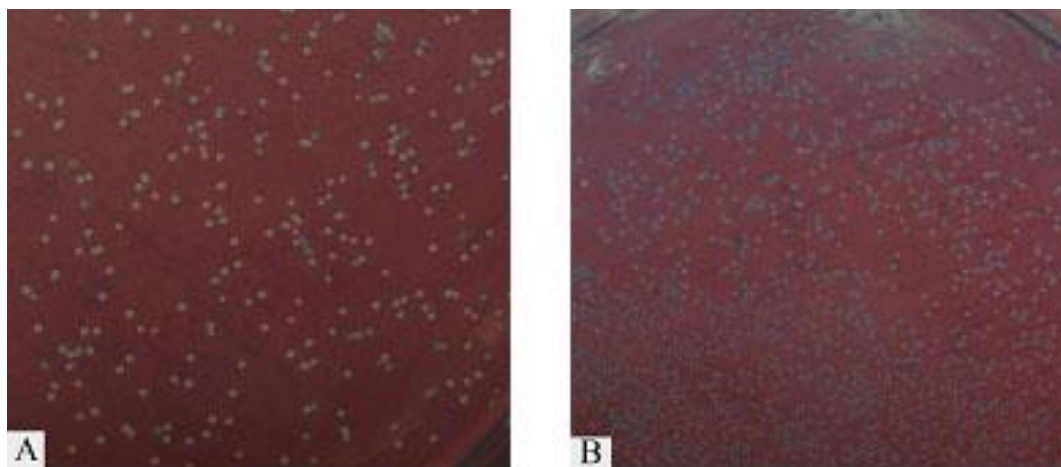


Figure 1. The transformation results of *E. coli* DH5 α with 15 μ l of the ligation reaction A (A) or of the ligation reaction B (B).

Table 1. The transformation results of the two ligation reactions.

Reaction	Total colony	White colony	Blue colony	Ratio of white colony to total colony (%)
Reaction A	577	500	77	86.6
Reaction B	1325	34	1291	1.8

binding factors, was amplified with the PrimeSTAR HS DNA polymerase (TaKaRa Biotechnology (Dalian) Co., Ltd.) from *Arabidopsis thaliana* cDNAs. The PCR primers used in the amplification were: AtDPBF4F-NotI (5'-atttgcggccgcatgggttctattagaggaaac), and AtDPBF4R-NotI (5'-aaatgcggccgctcagagagaagcagagtttggtc). After PCR, the blunt-end products were purified by agarose gel electrophoresis and then recovered from the gel with the Gel Purification Kit (Tiangen Biotech (Beijing) Co., Ltd.). The amplified fragment does not have any SmaI-recognized site.

Ligation of the blunt-end PCR products to the SmaI-cut vector

Two ligation reactions, A and B, were set up. Reaction A was: 0.62 μ g of SmaI-cut pUC19, 0.257 μ g of the AtDPBF4 PCR products, 1.5 μ l of 10 x T4 DNA Ligase Buffer (660 mM Tris-HCl, pH 7.6, 66 mM MgCl₂, 100 mM DTT, 1 mM ATP), 1 μ l of T4 DNA ligase (350 u/ μ l, TaKaRa Biotechnology (Dalian) Co., Ltd.), 0.5 μ l of SmaI (10 u/ μ l), deionized water up to 15 μ l. The mixture was incubated at 16°C overnight. The components of the reaction B are same with the reaction A except that SmaI is absent in the reaction B. 15 μ l of the above ligation reactions were used for transformation to 200 μ l of *Escherichia coli* DH5 α competent cells (Sambrook and Russell, 2001). After transformation, 200 μ l of the total 1000 μ l of the transformed bacterium culture were spread onto LB plate containing 100 μ g/ml ampicillin, 40 μ g/ml X-Gal and 24 μ g/ml isopropyl- β -D-thio-galactoside (IPTG). Numbers of white and blue colonies were counted after the plates were incubated at 37°C overnight.

Identification of recombinant clones

The randomly chosen white and blue colonies on the reaction A plate were inoculated into 2 ml of liquid LB with 100 μ g/ml ampicillin, respectively, and grew at 37°C overnight for plasmid

preparation by the Alkaline Lysis method (Sambrook and Russell, 2001) and then for checked by PCR amplification and restriction enzyme digestion.

Activity determination of restriction endonucleases in the 1x buffer of T4 DNA ligase

The restriction endonucleases, SacII, SacI, Sall, HindIII, XbaI, PstI, SmaI, EcoRI, EcoRV, NcoI and BamHI, were bought from the TaKaRa Biotechnology (Dalian) Co., Ltd. Two digestion reactions were set up for assaying these endonucleases activities; 37°C reaction: the 1 x ligation buffer (66 mM Tris-HCl, pH 7.6, 6.6 mM MgCl₂, 10 mM DTT, 0.1 mM ATP), 0.45 μ g pUC19 DNA, 5u restriction enzyme (SacII, SacI, Sall, HindIII, XbaI, PstI, SmaI, EcoRV, or NcoI, respectively), at 37°C for 3 h; 16°C reaction: the 1 x ligation buffer same as the 37°C reaction, 0.35 μ g pUC19 DNA, 5u restriction enzyme (BamHI, EcoRI, Sall, HindIII, XbaI, PstI or SmaI, respectively), at 16°C overnight.

RESULTS

The transformation results of the ligation reactions A and B are presented in Figure 1 and Table 1. Total colonies are 577 on the plate of the reaction A transformed *E. coli* DH5 α , and 1325 on the reaction B plate, respectively. 86.8% of the colonies on the reaction A plate are white and the rest are blue. Only 1.8% of the total colonies are white on the reaction B plate (Figure 1 and Table 1). The ratio of white colonies to total colonies on the reaction A plate is far higher than that on the reaction B plate. Liu

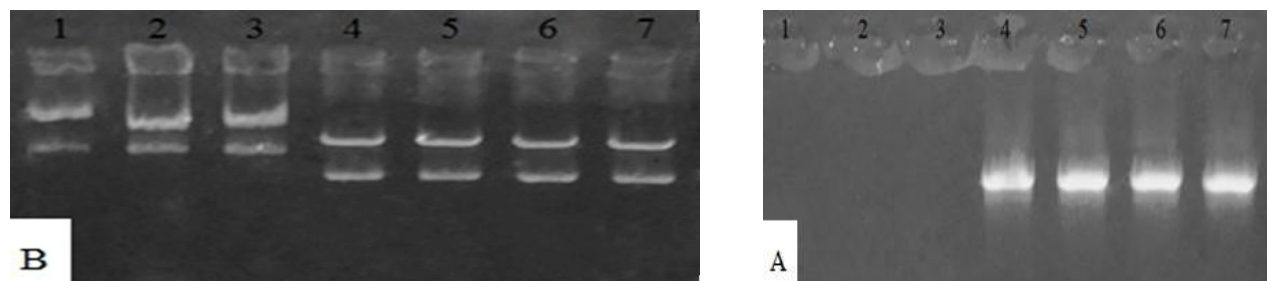


Figure 2. Plasmids were prepared from the colonies on the reaction A plate. Plasmids numbered from 1 to 3 were from blue colonies and 4 to 7 from white colonies. These plasmids were either amplified by PCR (A) or digested with the restriction enzyme NotI (B).

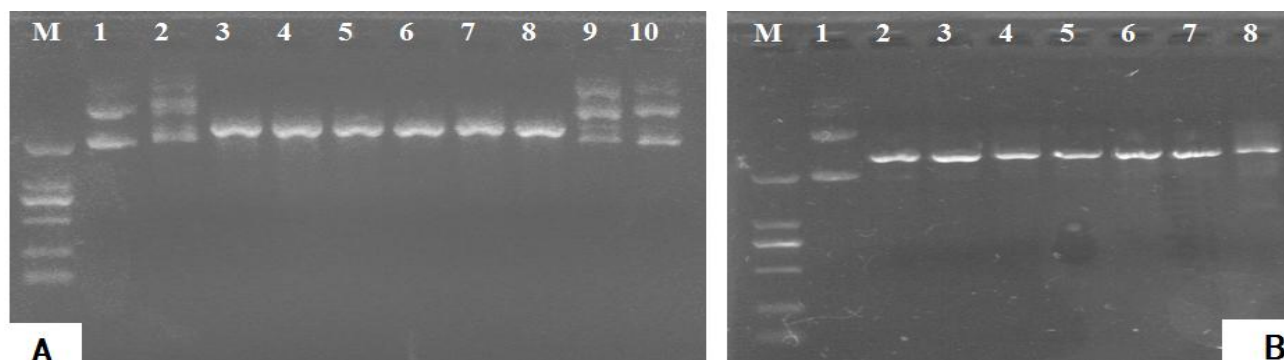


Figure 3. Digestion results of pUC19 DNA with the restriction endonucleases in the 1 x T4 DNA ligase buffer. A was at 37°C for 3 h. The digestion reactions from 1 to 10 were with no endonuclease, SacII, Sacl, Sall, HindIII, XbaI, PstI, SmaI, EcoRV and NcoI. B was at 16°C overnight. The digestion reactions from 1 to 8 were with no endonuclease, BamHI, EcoRI, Sall, HindIII, XbaI, PstI and SmaI. M is a molecular marker.

and Schwartz (1992) reported that the ratio of white colonies to total colonies was 1.2% if SmaI was not added to the ligation reaction. This result is almost same as ours (1.8%). However, the ratio in the experiment of Liu and Schwartz (1992) in the presence of SmaI (30%) is much lower than ours (86.8%). At present, we are not sure if the difference is because of the incubation temperature, the incubation time, or both temperature and time. Nonetheless, both results suggested that non-dephosphorylated blunt-end plasmid is much easier to be self-ligated than to be ligated with another fragment. The added restriction enzyme, SmaI, can dramatically increase the recombinant ratio of the vector and the cloned fragment.

To make sure that white colonies are recombinant, we randomly picked up 3 blue colonies and 4 white colonies on the reaction A plate for further assaying. These plasmids from these colonies were prepared, then amplified with the AtDPBF4 gene primers or digested by the restriction enzyme, NotI, and its recognized site is not present in the pUC19 plasmid but was inserted into the both ends of the amplified AtDPBF4 gene. The plasmids from the 3 blue colonies cannot be amplified or digested by NotI (Figure 2). The plasmids from the 4 white colo-

nies can be either amplified or digested by NotI (Figure 2). These results confirmed that the white colonies from the reaction A plate are recombinant.

The restriction endonuclease used in this procedure is not limited to SmaI. Other restriction endonucleases could be utilized if their recognition sites are not regenerated in recombinant molecules (Liu and Schwartz, 1992). Moreover, activities of restriction endonucleases in a ligation buffer are necessary. Here, digestion activities of some often-used endonucleases were assayed in the 1 x buffer of T4 DNA ligase (Figure 3). The results showed that Sacl, Sall, HindIII, XbaI, PstI, SmaI, BamHI and EcoRI could cut pUC19 DNA molecules to a linear form. However, SacII, EcoRV and NcoI cannot digest the same DNA molecules because they do not have any recognized sites on pUC19 DNA (Figure 3). Most restriction endonucleases need magnesium as their co-factors as well as T4 DNA ligase (Withers and Dunbar, 1995). Our results also proved that all the tested endonucleases work at both 37 and 16°C even if they might decrease their activities. This result suggested that the above endonucleases could also be used in the T4 DNA ligase reaction in order to increase recombinant molecules.

DISCUSSION

In this paper, the restriction endonuclease *Sma*I in addition to T4 DNA ligase reaction was proven to efficiently protect a non-dephosphorylated vector from self-ligation. When compared with common ligation protocols, this procedure skipped a dephosphorylation step of linearized vectors and a phosphorylation step of PCR products or primers. Therefore, it is simpler, faster and more economical. In the case of *Sma*I added into the T4 DNA ligase reaction, the ratios of recombinant molecules were dramatically increased from 1.2 to 30% (Liu and Schwartz, 1992) or from 1.8 to 86.6% in this paper. The higher recombinant ratio in this paper could have resulted from either the lower incubation temperature or the longer incubation time. Since *Sma*I-recognized site does not exist in the recombinant molecules, the longer incubation time must favor the more recombinant molecules. Other restriction endo-nucleases whose recognition sites are not regenerated in recombinant molecules, could also be used in the procedure (Liu and Schwartz, 1992). Of course, they must keep their activities in a ligation reaction. Some often-used endonucleases were assayed in the 1 x buffer of T4 DNA ligase. The results showed that the 8 tested endonucleases could keep their activities in that buffer. According to the producer's data (TaKaRa Biotechnology (Dalian) Co., Ltd.), T4 DNA ligase does not need other salts besides $MgCl_2$ in its reaction. *Eco*RI will increase its activity in a no-salt reaction, but *Sal*I will lose its activity. The other 6 endonucleases would decrease, to a different extent, their activity if there are no salts in their reactions. However, in our test, *Sal*I digested pUC19 DNA to a linear form at both 37 and 16°C. The presented activity of *Sal*I in the no-salt T4 DNA ligase buffer might be brought about by the *Sal*I storage buffer in which the concentration of KCl is as high as 400 mM.

ACKNOWLEDGEMENTS

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