Chapter 142
Evaluation of a Whole Genome Amplification Method Based on Improved Ligation-Mediated PCR

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Abstract DNA sequencing, genotyping, detection, and SNPs analysis usually require large amount of DNA. In the case that only tiny amount of DNA can be collected from samples, the whole genome amplification (WGA) technique is a good choice for its high efficiency. As a WGA method, ligation-mediated PCR (LM-PCR) has the shortcoming that strict conditions are required to avoid biased DNA amplification. Here, we used TspR I endonuclease to digest the target DNA into fragments with 9-nt-long (NNCASTGNN) sticky ends in which 4–5 different bases from other fragments are present. After ligation of these fragments to a universal adaptor, all the fragments will have ends with the same sequence for PCR, and the outstanding WGA was obtained by using only a uniform primer. The efficiency and sensitivity of this technique were evaluated and its application for DNA detection was also discussed.

Keywords Ligation-mediated PCR · High sensitivity · Whole genome amplification · TspR I endonuclease

142.1 Introduction

In many genetic studies such as DNA sequencing, genotyping, and detection of SNPs large quantities of DNA are required. However, in many cases only a small amount of target DNA can be collected because the quantity or quality of samples were not good enough, e.g., formalin-fixed paraffin-embedded tissues [1, 2]. Thus, the whole genome amplification (WGA) technique was employed to overcome this limitation. As a powerful tool, WGA plays an important role in various

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high-throughput genetic areas because it can provide sufficient DNA for large-scale genetic studies. Among the WGA methods, multiple displacement amplification (MDA) and ligation-mediated polymerase chain reaction (LM-PCR) were frequently used.

The MDA method, which relies on the strong strand displacement activity of the φ29 DNA polymerase, is an isothermal amplification approach based on rolling cycle amplification [3, 4]. In MDA, a primer with random sequence (e.g., a pool of all the hexamer primers) is usually used. LM-PCR is based on the PCR technique and inherited its high efficiency. LM-PCR was originally introduced in the field of footprinting for studying interactions of proteins with DNA in vivo [5–8]. By some modifications it can also be applied to perform methylation analysis and study of human diseases [9, 10].

For WGA, balanced amplification of all sequences is the basic performance. The application of traditional LM-PCR is limited, because it usually shows some deviation during amplification. The key procedure of LM-PCR is to prepare the fragments with a universal sequence at both ends. After digestion by a restriction enzyme, a universal adaptor with the same sticky end is ligated to all the fragments. One of the reasons of deviation during amplification is that ligation between digested fragments cannot be avoided because restriction enzyme digestion usually produce a 4–6-nt sticky end with palindromic sequence (e.g., AATT for EcoRI). Obviously, the unexpected ligation can also occur between the sticky ends of the adaptor. In this study, for improving the uniformity of LM-PCR, an improved approach using TspR I endonuclease as the restriction enzyme is developed. TspR I can digest DNA into fragments with 9-nt-long sticky ends, which can be used to decrease the self-ligation between fragments. A single-stranded adaptor is used and the self-ligation of adaptor molecules is avoided.

142.2 Materials and Methods

142.2.1 Materials

The 2686-bp-long pUC18 plasmid was extracted from bacteria culture solution. The extraction and purification procedures were referred to Molecular Cloning: A Laboratory Manual (the 3rd edition). Briefly, 500 mL of culture medium was centrifuged under 4 °C for 15 min to collect the cell pellets, resuspended the pellets with 200 mL of STE buffer (10 mM Tris–HCl, 0.1 mM NaCl, 1.0 mM EDTA, pH 8.0) and centrifuged again, then added 18 mL of lysis buffer I (50 mM glucose, 25 mM Tris–HCl, 10 mM EDTA, pH 8.0) and 20 mg lysozyme, added 40 mL of lysis buffer II (0.2 M NaOH, 1 % SDS) and stored at room temperature for 5–10 min. Then 20 mL of lysis buffer III (5.0 M potassium acetate 60.0 mL, acetic acid 11.5 mL, H_2O 28.5 mL) was added and kept on ice for 10 min. After that, samples were centrifuged for 30 min, liquid supernatant was collected, 0.6

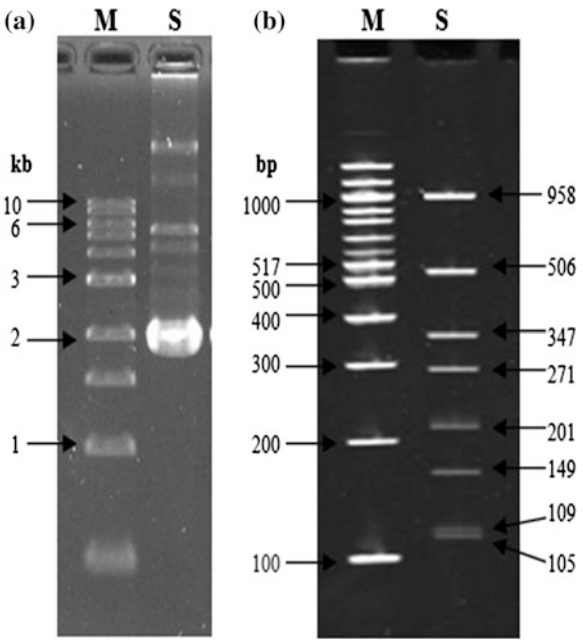

Fig. 142.1 Purification **a** and digestion **b** results of pUC18 plasmid

times volume of isopropanol was added to precipitate the DNA for 10 min at room temperature. After another centrifuge, precipitate was washed with 70 % ethanol, and resolved with 3 mL of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). After extraction step, polyethylene glycol (PEG) precipitation method was used to purify the plasmid [11]. The concentration of pUC18 was determined by Nanodrop 2000 (Thermo Scientific). An amount of 2 μL of purification product was electrophoresed on 1.2 % (w/v) agarose with TBE buffer and stained in 0.5 μg/mL ethidium bromide for 20 min. Then 1 μg pUC18 plasmid was digested by TspR I ednonuclease (New England Biolabs, Inc.). The reaction mixture contains 50 mM KAc, 20 mM Tris-Ac, 10 mM $Mg(Ac)_2$, 1 mM DTT, 100 μg/mL BSA, the total mixture was incubated at 65 °C for 2 h and 2 μL of digestion product was electrophoresed on 8 % (w/v) polyacrylamide gel electrophoresis. The purification and digestion results were shown in Fig. 142.1.

The adapter and primer were synthesized by IDT (Integrated DNA Technologies, Inc.). Adapter: CTCACTCTCACCAACGTCGACAGCTTNNCASTGNN, Primer: CTCACTCTCACCAACGTCGACAGCTT [12].

142.2.2 Impact of Different DNA Ligase

The ligation reaction was the key procedure in the whole experiment and the ligation results mainly depended on the efficiency of DNA ligase. Here two

different ligase, T4 DNA ligase and Taq ligase were used to evaluate their effect to the Improved LM-PCR.

The ligation reaction was performed under the manufacture's protocol. The reaction mixture contains 5 pg pUC18 plasmid fragments, 0.2 pmol adapter, and 5.0 U DNA liagse. The total volume was 20 μL and the reaction with T4 DNA ligase (Fermentas) was incubated at 16 °C overnight while the reaction with Taq ligase (New England Biolabs, Inc.) was incubated at 45 °C, respectively. Use ddH_2O instead of DNA ligase for negative control. After ligation, PCRs that used ligation product as template were performed under uniform conditions: 1.0 μL of ligation product added to 19 μL of PCR mixture [10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH 8.3, 1 U DreamTaq DNA polymerase (Fermentas), 0.25 mM dNTPs, 0.75 μM primer], the mixture was incubated at 72 °C for 5 min for the 3′-end filling reaction. PCRs were performed as follows: initial denaturation at 94 °C for 3 min and 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and primer extension at 72 °C for 1 min [13, 14]. The samples were incubated at 72 °C for another 5 min and 2 μL of PCR product was electrophoresed on polyacrylamide gel electrophoresis.

142.2.3 Impact of Ligation Time and Temperature

The high efficiency of T4 DNA ligase depends on the ligation temperature and time. Two sets of experiments were done respectively to evaluate the effect of ligation time and temperature. The ligation and PCR reactions were referred to 2.2. The ligation time used were 4, 8, 12, 16 h and the ligation temperature were 16, 25, and 37 °C, respectively.

142.2.4 Impact of Primer Annealing Temperature

The primer annealing temperature used above was calculated by primer premier 6.0 (PREMIER Biosoft international, Palo Alto, CA) and maybe not the optimal temperature in actual experiment. Gradient annealing PCR were performed. The annealing temperatures were set up as 57, 60, and 63 °C, respectively. All other parameters were the same as mentioned in 2.3.

142.2.5 Improved LM-PCR Performed on E. coli Genome

Various concentrations of *E. coli* genome digestion product was used as template and the reaction conditions were the same as the conditions mentioned above.

142.3 Results

142.3.1 Principle of Improved LM-PCR

Similar to traditional LM-PCR, the Improved LM-PCR can be mainly divided into 3 steps: (1) preparation of DNA fragments by TspR I: double-strand DNA was digested by TspR I endonuclease into numerous fragments with different length and the same sticky end of NNCASTGNN. (2) ligation of adapter to both ends of the fragments: fragments were ligated with adapter which comprised of a primer sequence and a linker sequence. The linker sequence NNCASTGNN ligated to the 3′ sticky end produced by TspR I and the primer sequence served as template for primer extension thus created a substrate for PCR with uniform primer bonding site at each end. (3) amplification of the newly formed fragments by PCR: after PCR with universal primer, all of the fragments with the same end sequence could be amplified (Fig. 142.2).

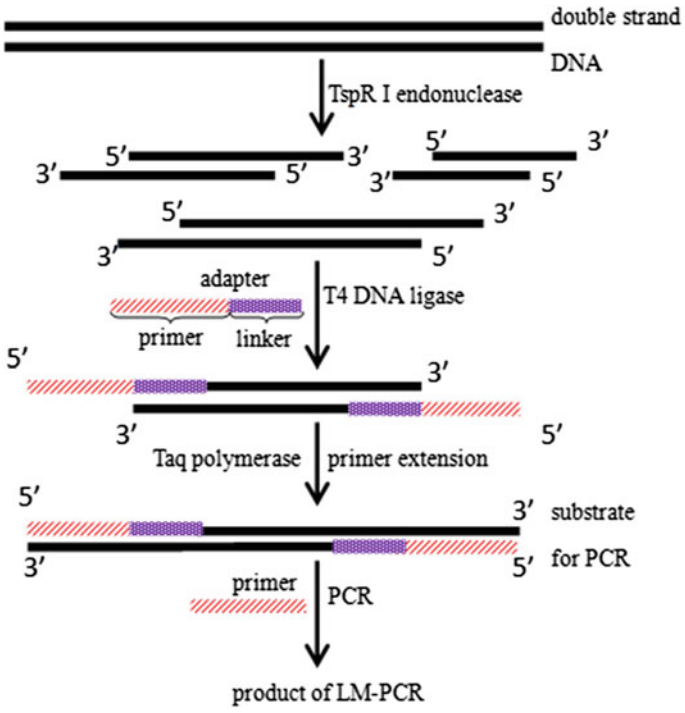

Fig. 142.2 Flowchart of improved LM-PCR

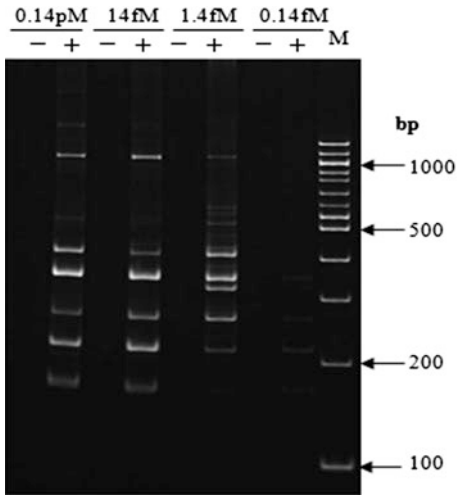

Fig. 142.3 Results of improved LM-PCR. "−" means negative control without DNA ligase. Ligation condition: 0.2 pmol adapter, 16 °C, 12 h

142.3.2 Results of Improved LM-PCR

The effect of Improved LM-PCR performed on pUC18 plasmid was shown in Fig. 142.3. The sensitivity was also evaluated by using 10-fold serial diluted template. The results showed that the Improved LM-PCR amplified the whole sequence of pUC18 plasmid successfully and the sensitivity was very high. When the concentration of template was 1.4 fM (∼50 fg), all of the eight fragments could be amplified; when the concentration lowered to 0.14 fM (∼5 fg), amplification of five of them could still be detected.

142.3.3 Results of Different DNA Ligase

T4 DNA ligase and Taq ligase were two mainly used ligases in genetic studies. The effect of the two ligases was shown in Fig. 142.4. When using T4 DNA ligase, all of the eight target bands could be seen, but less were obtained by Taq ligase. The results showed that the efficiency of T4 ligase was higher than Taq ligase.

142.3.4 Results of Different Ligation Temperature and Time

The high efficiency of T4 DNA ligase depends on the optimal reaction temperature and reaction time. At a higher temperature of 37 °C the band was indistinct and smaller fragments were not obtained. At lower temperature of 25 and 16 °C the number of target bands obtained was seven and eight, which means that 16 °C was better (Fig. 142.5a). The result of reaction time experiment showed that 12 h was the optimal reaction time (Fig. 142.5b).

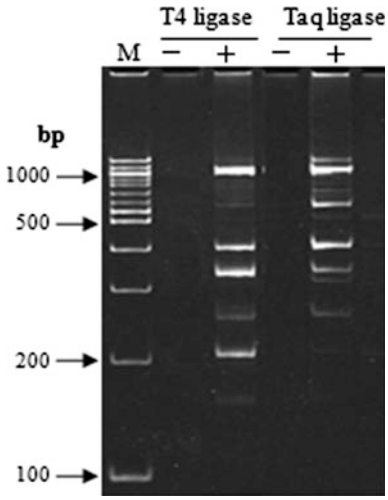

Fig. 142.4 Results of different DNA ligase. Ligation with T4 ligase was at 16 and 45 °C for Taq ligase

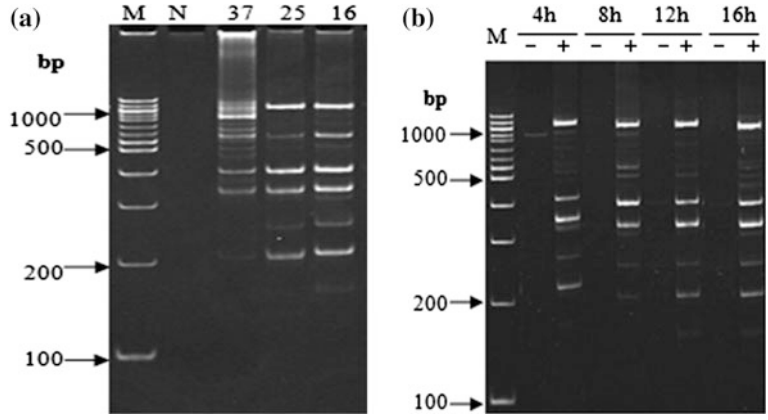

Fig. 142.5 Different ligation temperature and reaction time with T4 DNA ligase. **a** Ligation at various temperatures. **b** Ligation with various times

142.3.5 Results of Different Annealing Temperature

The annealing temperature used previously was calculated by software theoretically and need to be verified in actual experiment. The results indicated that with annealing temperature of 57, 60, and 63 °C the amplification efficiency was almost the same and 60 °C was slightly better (Fig. 142.6). Compared to other factors, the annealing temperature had less impact on this experiment.

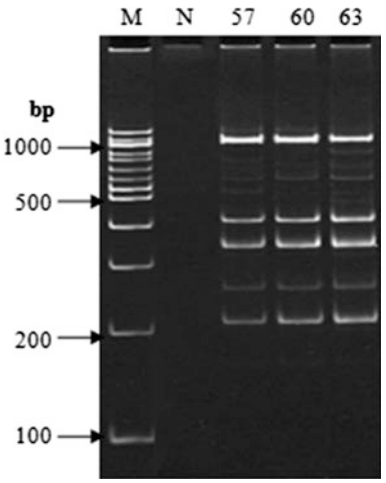

Fig. 142.6 Impact of annealing temperature. N: negative control without DNA ligase. Gradient annealing temperature was set on S1000 thermal cycler (BIO-RAD) with 57, 60 and 63 °C

142.3.6 Effect of Improved LM-PCR Performed on E. coli Genome

The continuous bands in Fig. 142.7 indicated that large amount of *E. coli* genome fragments were amplified. Plenty of bands could be seen even the concentration of genome was 8 aM. Most of the fragments ranged from 100 to 1000 bp and larger fragments that more than 1000 bp could also be amplified (Fig. 142.7). The results showed that Improved LM-PCR could amplify the whole genome with high sensitivity.

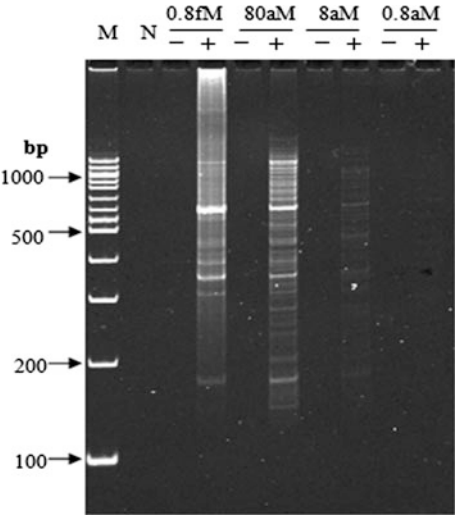

Fig. 142.7 Results of amplify the whole genome of *E. coli* with improved LM-PCR. N: negative control that no templates were added

142.4 Discussion

Theoretically, pUC18 plasmid could be cut into ten fragments by TspR I endonuclease but only eight of them could be examined on polyacrylamide gel electrophoresis either because the amount of the other two fragments (27 and 13 bp) run too fast in the gel or they were too little to be stained by ethidium bromide, e.g., the 13 bp fragment only owe 4 bp double strand and the 9 bp long was single strand. Thus our aim was to amplify the eight fragments obtained by digestion. The final LM-PCR products were 61 bp longer than the original fragments due to the ligation of adapter on both ends.

Compared to others work that used double-strand adapter, the adapter we used was single strand. As described before single strand is easier to operate [15]. The 9-nt-long sticky end produced by TspR I promised the ligation efficiency. Traditional LM-PCR that employed only one endonuclease tends to cause the self-ligation between fragments because all the fragments had the same end. But TspR I endonuclease could resolve this problem due to its sticky end was NNCASTGNN. This sticky end makes all of the fragments have at most 5 bases different to each other which will reduce the probability of self-ligation. Before ligation reaction, digestion product was serial diluted to even 10^7 times. Thus the effect of TspR I endonuclease that still existed in the sample could be ignored, and the loss of DNA during the removal of endonuclease procedure could also be avoided. The amplification efficiency may be affected by varieties of factors and among them the DNA polymerase was one of the key factors, some reports suggested that combination of *pfu* exo$^-$ and Taq polymerase at primer extension step and amplification step could improve the efficiency [16].

The sensitivity of WGA was especially important when there was not sufficient sample, thus, the LM-PCR technique based on high sensitivity PCR could play an important role in such conditions. Compared to other PCR-based WGA technique, degenerate oligonucleotide primer PCR (DOP-PCR) and primer extension pre-amplification PCR (PEP-PCR) also have high sensitivity that even one single cell could be used to perform WGA, but the main challenges were fidelity and balanced amplification [17–21]. LM-PCR was performed under strict conditions to avoid the biased amplification and our results showed that 50 fg plasmid in 20 μL of ligation reaction solution can still obtain all the eight target bands, and five of them could be obtained when only 5 fg plasmid was used, which indicated the high sensitivity and balanced amplification of Improved LM-PCR. Compared to the genome of *E. coli*, the pUC18 plasmid was small and simple. The result of LM-PCR performed on *E. coli* genome indicated that LM-PCR could amplify the whole genome well but more work is needed to evaluate the cover rate of LM-PCR used on larger genome DNA.

142.5 Conclusion

As a WGA method, the efficiency of Improved LM-PCR was evaluated in our study. The results showed that Improved LM-PCR may overcome the problem existed in the traditional LM-PCR. The effect of different reaction conditions was also evaluated. Under the optimal conditions, 1.4 fM plasmid in the ligation reaction could be amplified completely which showed that Improved LM-PCR has high sensitivity, and the results of LM-PCR performed on *E. coli* genome showed that Improved LM-PCR could also be used to perform WGA on larger genome with very low template concentration. All of the data obtained above indicated that improved LM-PCR could be a very useful tool in a variety of genetic analyses.

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