One step DNA sequencing of single-stranded DNA with reverse dye-primer

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DNA sequencing can be performed much faster and more easily by an automated DNA sequencer, which can process a large quantity of samples at one time. We use an Applied Biosystems DNA sequencer, Model 373A. Among several methods applicable to the sequencer, the dye-primer method of single-stranded template with Taq DNA polymerase gives the most reliable results. If both dye-primers (forward dye-primer (−21M13) and reverse dye-primer (M13RP1)) are available, the dye-primer method will be able to give at least 400 nucleotide sequences from each end. However, only the forward dye-primer is available for a single-stranded template. For use of a reverse dye-primer, two methods are available. 1) The insert can be recloned into another vector. 2) A double-stranded fragment can be produced by PCR with both primers. In both cases, an additional step is required, providing a large obstacle to extensive DNA sequencing.

In this report, we describe a one-step DNA sequencing of reverse dye-primer method of Taq DNA polymerase with single-stranded DNA template. The condition for the dye-primer method of Taq DNA polymerase is 15 cycles at (30 sec at 95°C, 30 sec at 55°C and 1 min at 70°C), which is close to the normal PCR reaction. If one uses the mix of unlabeled forward-primer of M13 vector (M4) and reverse dye-primer (M13RP1, ABI) instead of forward dye-primer (−21M13, ABI) for single-stranded template, a DNA sequencing reaction by PCR may directly give DNA sequences from the other end of the insertion. Figure 1 presents DNA sequencing profiles of double-stranded (Figure 1(A)) and single-stranded (Figure 1(B)) templates of M13mp18 containing 1.6 kb insertion of Chinese hamster RNA polymerase II cDNA with a reverse dye-primer by Taq DNA polymerase. Single-stranded templates gave a more explicit profile and a reliable nucleotide sequence than the double-stranded template. In this case, we added M4 primer up to one third molar ratio against reverse dye-primer. To check the reproducibility of this method, we have applied the present method to a different template of single-stranded DNA containing 459 base yeast DNA. Figure 2(B) shows an explicit sequence profile obtained by the reverse dye-primer (M13RP1) as well as that obtained by forward dye-primer (−21M13, Figure 2(A)). For the reverse dye-primer, we also added M4 primer up to 1/3 molar ratio. The complementary sequences obtained by both dye-primers are shown by solid lines in Figures 2(A) and 2(B), indicating that the present method of reverse dye-primer for single-stranded template can offer a reliable and explicit sequence profile as well as normal forward dye-primer method.

We examined the optimum quantity of M4 primer by changing the molar ratio of M4/reverse dye-primer from 1/10 to 1/1. The optimum result was obtained with 1/3 molar ratio, though all cases from 1/10 to 1/1 ratio gave fairly good profiles (data not shown). We also examined other clones having 100 base to 2,000 base insertions, which showed that the present method worked very well in most cases examined. A serious problem occurred only when the template DNA was contaminated by other ss DNA(s) containing shorter insertions since the PCR reaction preferred a shorter sequence to a longer sequence. Here, we report a simple one-step procedure for single-stranded DNA sequencing with reverse dye-primer by Taq DNA polymerase.

1. Prepare A, C, G and T reaction mixtures with manufacture protocol of Taq Reverse Dye Primer Cycle Sequencing Kit (ABI) except that 5 µl of 100 ng/µl ss DNA template was mixed with 2 µl of 0.5 pmole/µl unlabeled forward primer (M13 primer M4, Takara Shuzo Co.) and used as template, that corresponds to one third molar of reverse dye-primer.

2. PCR; 15 cycles at (30 sec at 95°C, 30 sec at 55°C and 1 min at 70°C) followed by 15 cycles at (30 sec at 95°C and 1 min at 70°C).

3. Combine the PCR reactions from the bottom of each of the four tubes into ethanol mixture (80 µl ethanol + 1.5 µl 3 M sodium acetate, pH 5.3). After standing on ice for 10 min, collect the PCR products by microfuge for 10 min and wash once with 70% ethanol.Dry the pellet for 2 min under vacuum.

4. Load on DNA sequencing gel with usual procedure.

The peak resolution and background of the sequence profile obtained by this procedure is comparable to that of an ordinary forward dye-primer. One can get more than 400 nucleotide sequences from each end of insertion concurrently by this simple one-step reaction, that is, more than 800 nucleotides of sequence as a total.

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Figure 1. DNA sequencing profiles of double-stranded (A) and single-stranded (B) DNA templates with reverse dye-primer method. DNA template of M13 mp18 contains 1.6 kb insertion of Chinese hamster RNA polymerase II cDNA at EcoRI site. (A) The concentration of ds DNA was 200 ng/µl. The ds DNA was purified by equilibrium centrifugation in CsCl gradient (1). (B) 5 µl of 100 ng/µl ds DNA template was mixed with 2 µl of 0.5 pmole/µl unlabeled M13 primer, M4, and used as template. The single-stranded M13 DNA was prepared by small-scale preparation (1). Reaction mixtures were prepared by manufacture protocol of Taq Reverse Dye Primer Cycle Sequencing Kit (ABI, #401120). PCR reaction is described in the text.

Figure 2. DNA sequencing profiles of single-stranded DNA template with forward dye-primer (A) and reverse dye-primer (B). DNA template of M13 mp18 contains 459 base insertion of yeast (S. pombe) cDNA at Smal site. (A) The concentration of ss DNA was 100 ng/µl. (B) 5 µl of 100 ng/µl ss DNA template was mixed with 2 µl of 0.5 pmole/µl unlabeled M13 primer, M4, and used as template. The ss M13 DNA was also prepared by small-scale preparation (1). Reaction mixtures were prepared by manufacture protocol of Taq Dye Primer Cycle Sequencing Kit (ABI). The PCR reaction is described in the text. Solid lines with arrow head indicate the complementary sequences obtained by the both methods.

REFERENCE