DNA Sequencing Using Ultra Small Amounts of Reagents and Template

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Abstract

One of the key points in the genome project is finding ways to reduce the running cost in DNA sequencing. One way is to use a highly-sensitive fluorescent DNA sequencer, where only trace amounts of template DNA and reagents are needed. An experimental protocol was optimized for the trace amounts of DNA analysis by using the hybridization rate of primers on template DNA, which was estimated to be $7.5 \times 10^5$ M$^{-1}$ sec$^{-1}$ at 37°C. One femtomole of template DNA with 0.001 unit of modified T7 DNA polymerase (Sequenase Ver. 2.0) and also 0.45 fmol of M13 template DNA with 0.01 unit of Taq DNA polymerase were enough to sequence DNA of up to 400 bases.

Key words: highly sensitive fluorescent DNA sequencer; hybridization reaction rate coefficient

1. Introduction

DNA sequencing is fundamental in life and biological sciences. A great deal of DNA sequence information has already been obtained in the human genome project, but the sequences accumulated so far are only a small fraction of the whole genome. Completion of the genome project depends on the development of a high-throughput automated DNA sequencer and the reduction of sequencing costs.1,2

One way to reduce sequencing costs may be utilization of a new method such as sequencing by hybridization.3 Another is the use of an extremely sensitive sequencing instrument that requires minimal amounts of reagents.4 A highly sensitive electrophoretic instrument which can detect a DNA band containing less than 0.1 amol of fragmented DNA by fluorescence detection has been reported.5 This instrument has made it possible to read DNA sequences using two orders of magnitude smaller amounts of DNA than that used in conventional DNA sequencing. This paper describes how sequencing conditions can be optimized to minimize the amounts of reagents needed.

2. Methods

2.1. Apparatus

The DNA sequencer used in this work has already been described in detail.5 Briefly, in the work reported here, the labeling fluorophore Sulforhodamine 101 was used and the excitation light is a He-Ne laser (594 nm, 5 mW). The laser irradiates a thin gel plate (0.3 mm thick) from the side, 25 cm apart from sample loading wells. A fluorescent line image was detected by a two-dimensional detector (cooled CCD camera with an image intensifier) through a cylindrical lens for increasing the collected fluorescence and a band-pass filter for selecting the detected wavelength.

2.2. Methods

The polymerase reactions were carried out by two different methods. The first reaction was designed to produce one short DNA species from the reaction for evaluation of the sensitivity of the measurement system. A labeled primer was hybridized on the target DNA and extension by DNA polymerase using dGTP and ddTTP generated the following short DNA fragment:

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Labeled primer

5' *-NH(CH2)6-GATGTGCTGCAAGGCGATTAAGTT

Extended four bases

-—CTACACGACGTTCCGCTAATTCAACCCATTGCG M13mp18

G : dGTP T : ddTTP * : Sulforhodamine 101
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Thus, the polymerase reaction incorporates three dGTP molecules before a terminator ddTTP was taken into the extended fragment. A four-base extension was adequate for distinguishing the extended primers from the non-extended primer by length. This allowed highly sensitive detection of the DNA fragments by electrophoresis because only one product peak appears at the position four bases away from the primer's peak. In this case, we used modified T7 DNA polymerase because it

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produces fewer artifact peaks than does Taq DNA polymerase. The preparation of the labeled primer was as follows: Amino-linked oligomer was synthesized using an ABI 392 synthesizer. Amino-linked oligomer (2×10^{-8} mol) dissolving in 400 μl of 0.1 M carbonate buffer (pH 9.0) was mixed with 2×10^{-6} mol of a sulforhodamine 101 dissolving in 100 μl of N,N-dimethylformamide. After heating at 37°C for 3 h, the reaction mixtures were neutralized by adding 12 μl of acetic acid. The labeled oligomer was purified by gel electrophoresis and an OPC cartridge (Perkin-Elmer Applied Biosystems, Foster City, CA, USA).

The polymerase reaction was carried out on a 1 μl scale as follows: One problem in detecting small amounts of DNA is the adsorption of DNA onto surfaces. As much as 2 fmol/mm² of DNA is adsorbed and this cannot be neglected when analyzing trace amounts of DNA. To prevent this adsorption, the detergent polyoxyethylene (20) sorbitan monolaurate (Tween20) was added to the reaction mixtures described before.6 M13mp18 DNA was used as a template DNA. A half microliter of annealing mixtures containing various amounts of template and primer in buffer solution [50 mM Tris-HCl (pH 7.5) containing 25 mM MgCl₂, 62.5 mM NaCl and 0.05% Tween20] was heated at 65°C for 10 min and then incubated at 37°C for 20 min. The annealing mixture was chilled on ice and 0.27 μl of diluted enzyme (Sequenase Ver. 2.0 (United States Biochemical, Cleveland, OH, USA), 0.88 unit/μl in 2.1 mM Tris-HCl buffer (pH 7.5) containing 28.7 mM dithiothreitol, 3.5 mM MgCl₂ and 5.2 mM isocitrate) and 0.23 μl of termination mixtures [0.1 mM dGTP and 0.1 mM dTTP in 20 mM Tris-HCl buffer (pH 7.5)] were added. Then the reaction mixture was heated up to 37°C immediately and kept at the same temperature for 1 min. The reaction was stopped by adding 0.5 μl of formamide. The reaction products were heated at 95°C for 2 min and then chilled on ice. One and a half microliters of each product was loaded onto the 5% polyacrylamide gels (acrylamide:bisacrylamide =19:1, containing 7M urea, 89 mM Tris-HCl, 2.5 mM EDTA-2Na and 89 mM boric acid).

The second reaction, which was designed for DNA sequencing, was carried out using M13mp18 DNA as a template and Sulforhodamine 101-labeled oligonucleotide as a primer. Its structure was:

5'-NH(CH₂)₆-GATGTGCTGCAAGGCGATTAAGTT³'

*: Sulforhodamine 101

Modified T7 DNA polymerase and Taq DNA Polymerase were used for sequencing.

The protocol for modified T7 DNA polymerase and Taq DNA Polymerase were used for sequencing.

The protocol for modified T7 DNA polymerase was as follows: Each half microliter of M13mp18 DNA (diluted in 0.1% Tween20), primer (diluted in 0.1% Tween20) and 5× buffer solution [200 mM Tris-HCl (pH 7.5) containing 100 mM MgCl₂ and 250 mM NaCl] were mixed. The mixture was heated at 65°C for 10 min and then annealed at 37°C for 20 min. After incubation, 0.7 μl of diluted modified T7 DNA polymerase [0.13 unit/μl in 2.1 mM Tris-HCl (pH 7.5), 28 mM dithiothreitol, 3.5 mM MgCl₂ and 5.2 mM isocitrate] was added to the mixture. The solution was divided into four aliquots and then a half microliter of termination mixtures [20 mM Tris-HCl (pH 7.5) containing 0.1 mM dNTP and 1 nM ddNTP or 5 nM ddNTP] were added. The extension reaction was carried out at 37°C for 10 min. The reaction was stopped by adding 0.5 μl of formamide. The reaction products were heated at 95°C for 2 min and then chilled on ice. One and a half microliters of each product was loaded onto the 5% polyacrylamide gels (acrylamide:bisacrylamide =19:1, containing 7M urea, 89 mM Tris-HCl, 2.5 mM EDTA-2Na and 89 mM boric acid).

Cycle sequencing reactions using Taq DNA polymerase were set up as follows: 4.5 μl of M13mp18 template DNA (0.1 fmol/μl containing 0.1% Tween20) was mixed with 1 μl of labeled primer (1 pmol/μl containing 0.1% Tween20), 3 μl of 5× buffer solution (50 mM Tris-HCl (pH 8.5) and 30 mM MgCl₂), and 2 μl of diluted Taq DNA polymerase (AmpliTaq Perkin-Elmer) 0.005 unit/μl in 25 mM Tris-HCl (pH 8.5), 0.1 mM EDTA, 0.15% Tween20, 0.15% Nonidet P40, then filled up to 15 μl with distilled water containing 0.05% Tween20. This mixture was divided into four aliquots of 3.5 μl. Then 1 μl of termination mixtures [180 μM ddGTP, 1000 μM ddATP, 1200 μM ddTTP and 500 μM dCTP containing 20 mM Tris-HCl (pH 7.5), 30 μM dATP, 30 μM dCTP, 60 μM 7-deaza-dGTP and 30 μM dTTP] were added to
Figure 2. Primer concentration dependence of primer extension. The hybridization reaction lasted for 1200 s at 37°C. The symbols, +, △, and ● are the experimental results obtained with 0.05 pmol/μl, 5 fmol/μl, and 0.5 fmol/μl of template, respectively. A hybridization reaction rate coefficient was estimated to be $k = 7.5 \times 10^5 \text{M}^{-1}\text{sec}^{-1}$. The lines, — — — — — —, and — — — — are the respective calculated curves using the hybridization coefficient.

the reaction tubes. Thermal cycling was performed on a model PJ 1000 thermal cycler (Perkin-Elmer) as follows: first 15 cycles (denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, extension at 72°C for 1 min), second 15 cycles (denaturation at 95°C for 30 sec, extension at 72°C for 1 min) and storage at 4°C. Reactions were stopped by adding 2.25 μl of formamide. The reaction products were heated and denatured at 95°C for 2 min and chilled on ice. Two microliters of the sample were loaded onto the gels.

3. Results and Discussion

3.1. Detection of trace amount of DNA

A typical electropherogram extended for DNA fragments obtained by extending primers by four bases with 0.5 amol, 1.5 amol and 5 amol of template and 100 fmol of primer, is shown in Fig. 1. The peaks at 28-mer appeared in the electropherogram increased with template amounts. The detection limit was about 0.1 amol/band with a S/N of about 2. This value is almost the same as reported previously in which labeled DNA fragments were prepared by ligation reactions. The results show that the amount of template DNA required for detecting DNA bands with an S/N of about 10 will be 0.3 fmol when the number of DNA fragment species being sequenced is as large as 500. This means that it would be possible to sequence DNA with only 0.3 fmol of template DNA, or three orders of magnitude lower than that required in the conventional DNA sequencing protocol.

3.2. Hybridization reaction coefficient

As demonstrated above, the use of a highly sensitive analyzer will reduce the amount of template DNA needed for sequencing. Although a high primer concentration is advantageous for increasing the hybridization efficiency, it is not good for lowering background signals. Therefore it is necessary to optimize the primer concentration. A critical factor for a sequencing reaction at a low reagent concentration is hybridization efficiency of primers on templates. We estimated the hybridization efficiency from the amount of DNA fragments produced under various conditions. A hybridization reaction is an equilibrium reaction, and a dissociation reaction (dehybridization) speed slows as the temperature goes down. As the reaction temperature, 37°C, is much lower than the denaturing temperature (about 70°C) of the primer hybridized to the target, the production speed of the hybrid depends only on a collision frequency of a primer with a template DNA. When the hybrid concentration at reaction time $t$ is denoted by $Y(t)$. $Y(t)$ can be represented as a function of primer concentration $P(t)$ and template DNA concentration $D(t)$:

$$\frac{dY(t)}{dt} = k \cdot P(t) \cdot D(t)$$  \hspace{1cm} (1)

where $k$ is the reaction coefficient to be evaluated. We can represent $Y(t)$ as,

$$Y(t) = D(0) - \frac{P(0) - D(0)}{P(0)} \cdot e^{-kP(0)t}, \hspace{1cm} (P(0) \gg D(0))$$  \hspace{1cm} (2)

or

$$Y(t) = D(0) - P(0)kt/(1 + kP(0)t), \hspace{1cm} (P(0) \approx D(0))$$  \hspace{1cm} (3)

The primer concentration dependence of the reaction product is shown in Fig. 2 for various template concentrations. The quantities of the reaction products were estimated by the fluorescence intensities in DNA fragment electropherogram. Using Eq. 3 we calculated the $k$ value to be $7.5 \times 10^5 \text{M}^{-1}\text{sec}^{-1}$ when $t=1200$ s, $P(0)=0.1$ nM, and $D(0)=0.1$ nM with $Y(t)$ volume (Fig. 2). Using the $k$ value, we can rewrite the relation $kP(0)t \gg 1$ as $P(0) \gg 0.2$ nM when $t=1200$ s. This suggests that sufficient hybridization will occur when the primer amount is larger than 1 fmol and the volume of the reaction mixture is 1 μl, which agrees with the experimental findings. In the following experiments, we used 0.01 pmol of primer.

$$Y(t) = D(0) - P(0)kt/(1 + kP(0)t), \hspace{1cm} (P(0) \approx D(0))$$  \hspace{1cm} (3)
3.3. Reduction of enzyme amount

The amount of enzyme required for a sequencing reaction depends on the amount of hybrid and the length of the extended fragment. To determine the minimum amount of enzyme required for a small scale reaction, we obtained DNA fragment electropherograms using various amounts of enzyme. The amount of products for various fragment sizes were unchanged by varying the quantity of enzyme when using 1 fmol of DNA as a template (Fig. 3). The figure shows that as little as 0.0001 units of enzyme is enough for sequencing 1 fmol of template DNA.

3.4. Sequencing trace amounts of DNA

The resultant DNA fragment electropherogram at different dNTP/ddNTP ratios were shown in Fig. 4 (dNTP/ddNTP ratio was 20) and Fig. 5 (dNTP/ddNTP ratio was 100). In DNA sequencing, the average length of extended DNA fragments varies with the dNTP/ddNTP ratio. When the ratio is low, the average length becomes short and the number of short DNA fragments increases. Therefore, less template DNA is required for
Figure 6. DNA sequencing electropherogram obtained from Taq cycle sequencing reactions using 0.45 fmol of M13mp18 as a template. Twenty-seven percent (0.13 fmol) of the reaction product was used for electrophoresis. DNA sequences of up to 400 bases can be determined.

reading short DNA fragment. This result suggests that only 0.2 fmol of template DNA is enough to read 100 bases and only 1.3 fmol of template DNA is sufficient to read 400 bases when using modified T7 DNA polymerase. Taq cycle sequencing gave more intense signals. As shown in Fig. 6, DNA sequences of up to 400 bases can be determined using 0.45 fmol of template DNA.

In our sequencing protocol, as described above, the amount of template DNA can be reduced to several-hundredths compared with conventional methods. The amount of enzyme can be also reduced to at least one-
hundredth.

4. Conclusion

It has been demonstrated that subfemtomole DNA templates can be used for sequencing, and reagent costs can be reduced dramatically. Highly sensitive sequencing also enables DNA sequencing directly from a colony without PCR amplification. It also enables direct sequencing from large templates such as cosmid or P1 clones. The critical factor in a sequencing reaction is not the total amount of template DNA and reagents but their concentration. If the reaction volume as well as DNA band volume in electrophoresis can be further reduced, the amount of reagents required for DNA sequencing could be small enough to neglect the reagent cost in sequencing. From this viewpoint, gel capillary electrophoresis is suitable for ultra-small scale DNA sequencing if some good sample loading method can be developed.

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