Short Communication

A simple protocol of DNA sequencing with 10% formamide for dissolving G/C compression

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Abstract

Formamide has been widely used in urea/polyacrylamide gel to solve the compression problems that are occasionally found during the DNA sequencing of G/C rich regions. In this study, however, 10% formamide was added in annealing solution in stead of adding to the gel. The compressions were unfolded efficiently with a more rapid annealing reaction on ice in the presence of 10% formamide.

Key words: formamide, DNA sequencing, T7 polymerase

Introduction

DNA sequencing has become a major tool for the analysis of genes. Reliable DNA sequencing systems that allow the determination of a fragment of DNA up to about 400 nucleotides are available (Sanger and Coulson, 1978). One of the problems encountered in sequencing is band compressions in the G/C-rich region. Sometimes formamide of at least 10% was used to improve its denaturing capability in urea/polyacrylamide sequencing gels (Rochelear *et al.*, 1992). Formamide is known to lower the melting temperature of nucleic acid duplexes by weakening hydrogen bonds. Therefore it is widely applied to Southern and Northern blottings (Beyer, 1991), nuclease protection assays (Mironov *et al.*, 1995), S1 mapping (Murray, 1986) and *in situ* hybridization (Hubbell, *et al.*, 1976). To read the compressed sequences, two overlapping DNA strands are sequenced in both directions with 5'- and 3'-primers. However, it requires a lot of additional works like sub-cloning and primer preparation. Alternatively, substituting dGTP by dITP (Mills and Krammer, 1979) or 7-deaza-dGTP (Mizusawa *et al.*, 1986) in the dideoxy sequencing reaction considerably reduced band compression by decreasing stacking energy of the folded structure. In

this paper we described a simpler method to solve G/C-compressions by involving 10% (v/v) formamide in annealing reaction.

Materials and Methods

Two ml of XL-1 Blue *E. coli* harboring plasmid had been cultured overnight. The plasmid DNA was miniprepared according to conventional protocol and redissolved in 20 μ l of deionized water. 5 μ l was used for DNA sequencing.

Denaturation of the double-stranded DNA

Up to 10 ml of miniprep DNA can be added to a 1.5-ml microcentrifuge tube containing the mixture of 4 ml of 2 M NaOH, 4 μ l of 2 mM EDTA, pH 8.0 and 22 μ l of H₂O, which was prepared prior to use. The tube was vortexed gently and incubated at 37°C for 20 min. Four μ l of 2 M ammonium acetate, pH 4.5 was added and mixed gently. After 110 μ l of ethanol was added, denatured DNA was precipitated at -20°C for 30 min or for at -70°C for 15 min. The salt-precipitated DNA was centrifuged at 3,000 rpm for 10 min in a microcentrifuge. The supernatant was removed carefully. The pellet was rinsed gently with ice-cold 70% ethanol and air-dried briefly.

Annealing of primer to template

The pellet was dissolved in a total volume of 14 μ l of the annealing mixture containing 10% (v/v) formamide. Annealing mixture was composed of 2 μ l of annealing buffer (280 mM Tris-HCl, pH 7.5, 100 mM MgCl₂, and 350 mM NaCl), 2 μ l of 1 pmol of primers, 8.6 μ l of H₂O, and 1.4 μ l of formamide. The solution stuck to wall of the centrifuge tube was collected by centrifugation briefly and heated at 95°C for 2 min (carefully so that its cap is not opened during boiling). The hot tube was transferred immediately to ice and cooled for 10 min. The evaporated solution was centrifuged to the bottom of tube. While cooling is in progress, 2.5 μ l of each termination mix (G, A, T, C) was dispensed to a microsample plate. It was kept covered on ice until used.

Labeling

Labeling mixture was prepared by mixing the following components: 1.6 μ l of deionized water, 0.4 ml of labeling mix (7.5 μ M dGTP, 7.5 μ M dCTP, and 7.5 μ M dTTP), 1 μ l of 0.1 M DTT, 1.6 μ l of enzyme dilution solution which was supplied along with T7 polymerase (20 mM Tris-HCl, pH 7.5, 5 mM DTT, 100 μ g BSA/ml and 5% glycerol), 1

Figure 1. Nucleotide sequencing using the dideoxy-chain termination protocol. Inserts in vector Bluescript were sequenced with universal primers using T7 polymerase in the presence (+) or absence (-) of 10% (v/v) formamide in annealing solution. Several compressions are obvious in the sequencing ladder in the absence of formamide (left panels in A, B, and C). Compressions are indicated by arrows in panels A and B and a bracket in C. Electrophoresis was done on 5% urea/acrylamide sequencing gel at 60 W constant power. The gel was dried on a 3 MM filter paper and exposed to a Hyperfilm (Amersham) overnight without intensifying screen. Parts of sequence of protein tyrosine phosphatase BDP1 above 230 bases and 120 bases from primer were shown in panels A and B, respectively. Band compression in high GC region in BDP1, which is bracketed in panel C, was also resolved. The electrophoresis was done until xylene cyanol FF reached the bottom in panel A and C and two thirds of the gel in panel B.

μ l of [α - 35 S]dATP (1,000 Ci/mmol, Amersham, Buckinghamshire, UK), and 0.4 μ l of T7 polymerase (8-12 U/ μ l, Pharmacia, Uppsala, Sweden). Labeling mixture was added and mixed with annealed solution by gentle pipetting without forming air bubbles. Labeling was performed for 4 min at room temperature.

Termination

Each aliquot of 4.5 μ l radio-labeled solution was mixed with each G, A, T, and C termination mixes on ice by gentle pipetting. Termination was done for 5 min at 42°C. After termination, 5 μ l of stop solution was added to each well and mixed. Stop solution was made by mixing 95% formamide, 20 mM EDTA, pH 8.0, 0.03% BPB, and 0.03% xylene cyanol FF. Before loading a sample on sequencing gel, it was heated at 95°C for 2 min and cooled on ice quickly. 2-3 ml of each sample was applied to a well of sequencing gel. The remaining solution could be stored at -20°C for 1 month. If further aliquots are to be loaded, samples can be done without heating.

Results and Discussion

To eliminate band compression 10% (v/v) formamide was added to the annealing buffer. Annealing between sequencing primer and DNA template was carried out on ice for 5 min after 2-min denaturation of template DNA at 95°C. 10% formamide in annealing step was diluted to 7% in labeling mixture and 4.8% in termination mixture. Application of this protocol to DNA sequencing of protein tyrosine phosphatase BDP1 (Kim *et al.*, 1996) gave better results than without formamide as shown in Figure 1. In

addition, time could be saved because of rapid annealing as well. Usually cooling was done slowly for 30 min or longer for annealing after incubation at 65°C. This method could be applied to the known protocol such as those including single-stranded DNA with slight modification in annealing step. However, this could not be used when DTT is used in annealing buffer, because DTT is unstable on heat denaturation at 95°C. T7 polymerase was used as described in 'Materials and Methods', its activity was not affected significantly up to 10 % formamide (data not shown). However, formamide concentration of higher than 10% decreased the activity of T7 polymerase.

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