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# Improved cycle sequencing of GC-rich DNA template

**Jong-Soon Choi[1], Jin-Sung Kim[2], Cheol-O Joe[3], Soohyun Kim[1], Kwon-Soo Ha[1] and Young-Mok Park[1, 4]**

1 Biomolecule Analysis Group, Korea Basic Science Institute, Taejon 305-333, Korea
2 Department of Biochemistry, Kon-Kuk University, Choongju 380-701, Korea
3 Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Taejon 305-701, Korea
4 Corresponding Author: Tel, 042-865-3420; FAX, 042-865-3419; E-mail, ympark@comp.kbsi.re.kr

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Abbreviation: DMSO, dimethyl sulfoxide; PCR, polymerase chain reaction; GC, guanine and cytosine; EDTA, ethylenediaminetetraacetic acid

## Abstract

**Even when DNA sequencing of purified DNA template failed under the optimal condition, it can be generally contributed to high GC content. GC-rich region of template causes a secondary structure to produce shorter readable sequence. To solve this problem, the sequencing reaction was modified by using dimethyl sulfoxide (DMSO). It was found that 5% (v/v) of DMSO in the reaction mixture recovers sequencing signal intensity with reduced frequency of ambiguous bases. When DMSO was added to sequencing reaction of DNA template with normal GC content, it did not show any adverse effect. Sequencing accuracy and unambiguous base frequency were significantly improved at concentration of 2% to 5% (v/v) DMSO in GC-rich DNA template. DMSO has been empirically introduced to enhance the efficiency of PCR in GC-rich templates. However, the underlying mechanism of improved cycle sequencing by DMSO is unknown. Thus, cycle sequencing reaction was remodified with other additives such as *N*-methyl imidazole, *N*-methyl-2-pyrrolidone, *N*-methyl-2-pyridone and glycerol, possessing the similar chemical properties as DMSO. Most of methyl nitrogen ring-containing chemicals did not improve sequencing accuracy, whereas only glycerol mimicked the positive effect of DMSO by the same extent. In the present study, we suggest that the treatment of DMSO improve cycle sequencing by the alteration of structural conformation of GC-rich DNA template.**

**Keywords:** cycle sequencing, high GC, dimethyl sulfoxide

## Introduction

With the advent of fluorescent cycle sequencing technique, the genomic information of many organisms have been greatly accumulated during the past decades. Cycle sequencing as a chain termination method is performed by heat stable *AmpliTaq* DNA polymerase, which synthesizes new DNA strand and terminates at the incorporation of fluorescent dye-labeled dideoxynucleoside triphosphate (Prober *et al.,* 1987). Except for poor quantity and quality of DNA template, the false sequencing can be attributed by the inherent sequence of template. Typically, templates which have long homopolymer regions, very AT-rich or GC-rich base compositions, or which contain long short tandem repeats, are all difficult to sequence. Among these, templates with GC content greater than 65% frequently give very weak signals when sequenced under standard conditions (Nanthakumar, 1993). Therefore, the modification for sequencing GC-rich templates is indispensable. Generally double-stranded templates containing high GC content can produce shorter readable sequences than other templates do. One of the possible reasons is that GC-rich templates generate secondary structure to prohibit the denaturation, annealing and extension step during PCR process, subsequently resulting in the inefficient DNA sequencing (Hengen, 1996). These problems can be solved to some extent by increasing the denaturation temperature to 98˚C, the amount of DNA polymerase from 4 to 8~16 units, and PCR cycling number to 30 or by performing a hot-start PCR to keep secondary structures from forming quickly. These works, however, are troublesome or laborious and rather time-consuming for best optimization. To determine the sequences of problematic DNA templates, we applied cycle sequencing of GC-rich templates with dimethyl sulfoxide (DMSO), known as a PCR facilitator (Pomp and Medrano,1991). In addition, we examined the effect of other cosolvents which have similar chemical properties as DMSO. Here, we introduce an improved cycle sequencing method using cosolvents.

## Materials and Methods

Plasmid DNA, pYHD was used as a sample source of high GC DNA template (71% GC content) from a derivative of double strand pGEM3Zf(+) containing 1.1 kb *Sal*I insert, coding superoxide dismutase from *Streptomyces fradiae*. As a control plasmid DNA (53% GC content), pABB was

constructed with pBluescriptSK(+)-*EcoO109*I-digested insert of 625 bp PCR product, covering 3'-flanking region of PsaA and 5'-flanking region of PsaB of photosystem I core protein in *Synechocystis* sp. PCC 6803. Plasmid DNA isolation and manipulation were carried out as described previously (Maniatis *et al*., 1982). Aliquots of stock DMSO solution (Sigma Co., ACS reagent) were diluted with deionized water and combined with sequencing reactions before PCR. *N*-methyl imidazole, *N*-methyl-2-pyrrolidone and *N*-methyl-2-pyridone as nonshared electron-protonating nitrogen compounds which have similar basicity and polarity as DMSO, were used as a cosolvent of cycle sequencing. Also we tested the effect of glycerol with high solubility and viscosity as DMSO. All solvents were ACS reagent grade supplied by Sigma company. Final concentration of test solvents were determined to 5% (v/v), based on the effective concentration of DMSO as previously cited. Plasmid DNA (1 μg) and -21M13 universal primer (3.2 pmol) were mixed with Terminator Premix in a total reaction volume of 20 μl. Terminator Premix per reaction tube (ABI, Foster City, CA) was adjusted to final concentrations of TACS buffer (80 mM Tris-HCl, 2 mM $MgCl_2$, 20 mM $(NH_4)_2SO_4$, pH 9.0), deoxynucleoside triphosphate mix (750 μM dITP, 150 μM dATP, 150 μM dTTP, 150 μM dCTP), DyeDeoxy Terminators (each 1 ml of G, A, T and C-specific fluore-scence tag, ABI) and *AmpliTaq* DNA polymerase (4 units/ reaction). Reaction mixtures were overlayed with 20 μl of sterile mineral oil and PCR was started in a model PTC-150 MiniCycler™ (MJ Research, Watertown, MA). Following an initial denaturation at 96˚C for 2 min, PCR was performed for 25 cycles at 96˚C for 30 sec, 50˚C for 15 sec, and 60˚C for 4 min and extended at 60˚C for an additional 5 min. Unincorporated nucleotides were re-moved from the amplified products using prehydrated Centri-Sep™ spin solumns (Princeton Separations, Adel-phia, NJ). And then the eluents were dried completely and resuspended in 6 μl of loading buffer (deionized formamide/ 50 mM EDTA, pH 8.0, 5:1, v/v). Prior to loading samples on 6% denaturing polyacrylamide gel, the samples were heated to 90˚C for 2 min and placed quickly on ice. After loading the sample, electrophoresis was carried out on a model 373A automated DNA sequencer (ABI Inc.) at a constant power of 30 W for 12 h, as suggested by the manufacturer. Automatic base calling was performed by the ABI sequence software, version 1.2.1 analysis soft-ware.

## Results and Discussion

High GC-containing templates generate frequently local secondary structure, leading to inefficient PCR (Filichkin and Gelvin, 1992). It has been reported that there are some organic solvents such as DMSO, formamide, tetramethyl ammonium chloride, nonionic detergents and glycerol to enhance PCR reaction (Varadaraj and Skinner, 1994). Formamide has been used to eliminate base compression in GC-rich template during DNA sequencing (Kang and Kim, 1997). Also, DMSO is known to affect the catalytic activity of DNA polymerase and change the structure of DNA template (Bookstein *et al.,* 1990; Chester and Marshak*,* 1993). Based on the previous reports, the effect of DMSO was examined in GC-rich template (pYHD), possessing 71% GC content. When 5% (v/v) DMSO was added to sequencing reaction, both signal strength and signal-to-noise ratio were significantly improved (Figure 1 and 4). Automatic base calling was observed as over 97% accuracy throughout the first 300 nucleotides. Analyzed sequence data contained fewer ambiguous

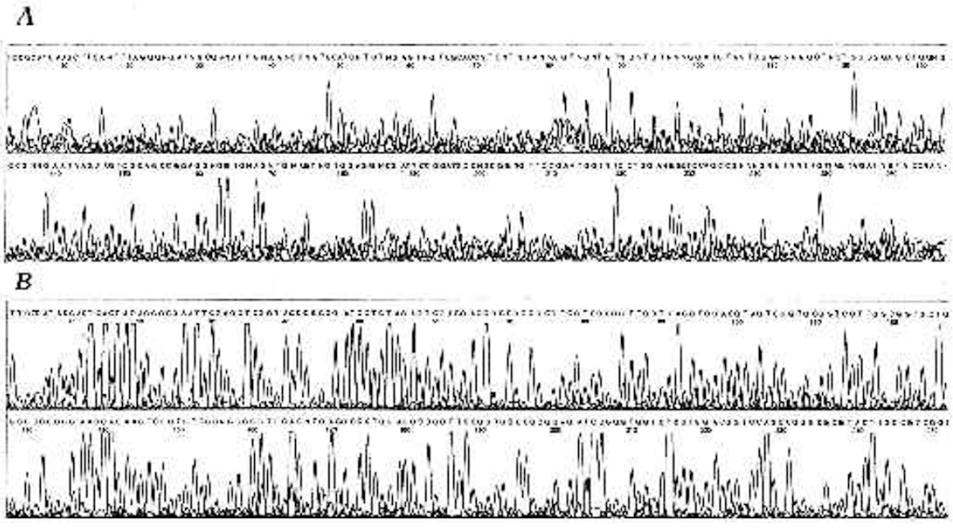


**Figure 1.** Automatic basecalling of fluorescent, cycle sequencing analysis in DNA template of 71% GC content (pYHD) without DMSO (A) and with 5% (v/v) DMSO (B).

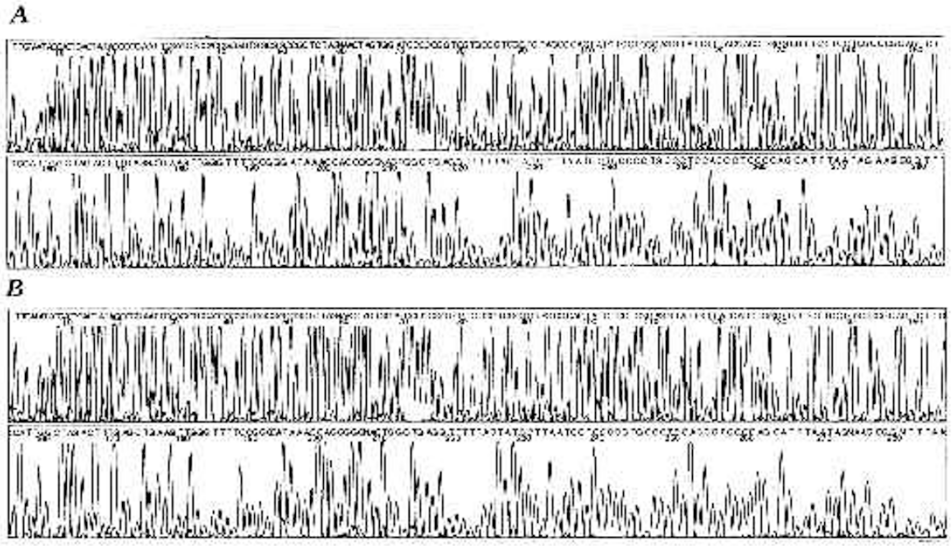


**Figure 2.** Automatic basecalling of fluorescent, cycle sequencing analysis in DNA template of 53% GC content (pABB) without DMSO (A) and with 5% (v/v) DMSO (B).

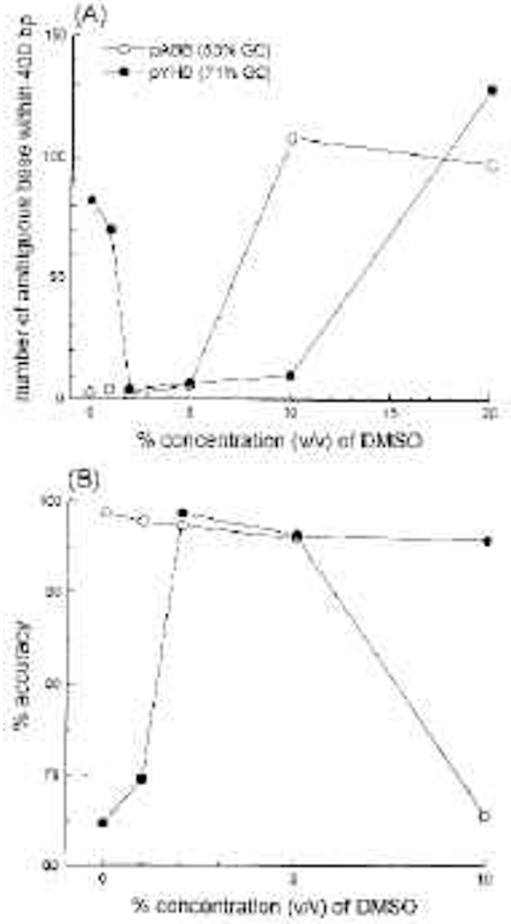


**Figure 3.** Effect of varying concentrations of DMSO on ambiguous base calling (A) and sequence accuracy (B) in 71% GC content-DNA (pYHD) and 53% GC content-DNA (pABB).

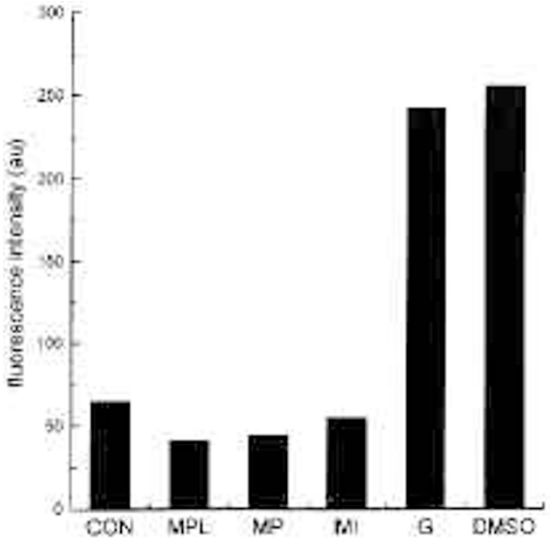


**Figure 4.** Effect of various cosolvents (CON, control; MPL, N-methyl-2-pyrrolidone; MP, N-methyl-2-pyridone; MI, N-methyl imidazole; G, glycerol; DMSO, dimethyl sulfoxide) on fluorescence intensity in 71% GC content-DNA (pYHD). The concentration of additives was determined at 5% (v/v) for qualitative analysis.

**Table 1.** Summarized data of chemical characteristics of cosolvents. Viscosity of test solvents was measured as final 5% (v/v) in aqueous solution by viscometer. (Schott Gerätt, Co., CT-050)

| Cosolvent | Polarity | Basicity | Viscosity | Chemical Structure |
|---|---|---|---|---|
| *N*-methyl-2-pyrrolidone | 0.45 | 1.10 | 1.170 |  |
| *N*-methyl-2-pyridone | 1.00 | 1.00 | 1.138 |  |
| *N*-methyl imidazole | 0.88 | 1.20 | 1.115 |  |
| Dimethyl sulfoxide | 1.00 | 1.42 | 1.118 |  |
| Glycerol | 0.85 | 0.52 | 1.166 |  |

basecalls (N's) than those obtained in the absence of DMSO. When DMSO of the same concentration was added to sequencing reaction in normal DNA template (pABB) of 53% GC content, DMSO did not show adverse effect on sequence base calling (Figure 2). Therefore, we confirmed the stability of DMSO as a cosolvent of cycle sequencing reaction, besides the improved effect of DMSO on cycle sequencing of GC-rich DNA as observed by other investigators (Winship, 1989; Sun *et al.,* 1993; Scheidl *et al.,* 1995).

To determine the working concentration of DMSO, irrespective of GC content, various concentrations of DMSO were pretreated in sequencing reaction. No significant differences were seen in the accuracy of automatic base calling and the number of ambiguous base in both types of DNA templates (Figure 3). At concentration above 10% (v/v) DMSO, enhanced sequencing data were not observed any longer in GC-rich DNA template, perhaps due to the inhibition to *AmpliTaq* DNA polymerase (Gelfand, 1989). For plasmid DNA with high GC content, enhanced sequencing signal became apparent at 2% (v/v) and maximal at 5% (v/v), and was maintained through 10% (v/v) DMSO. At 20% (v/v) or above concentration, however, cycle sequencing became less efficient and no sequencing data were detected. Therefore, cycle sequencing with DMSO was enhanced in common at concentrations of 2 to 5%, irrespective of GC content. The sequence accuracy of any type of template was observed over 96% at the same range of concentration of DMSO.

Based on the effect of DMSO, we examined other effective cosolvents, possessing similar chemical properties such as polarity, basicity and viscosity as DMSO, in GC-rich template. Cycle sequencing reaction was modified with the addition of some polar and basic solvents such as *N*-methyl imidazole, *N*-methyl-2-pyrrolidone, *N*-methyl-2-pyridone. And the influence of viscosity on cycle sequencing was also tested with glycerol. Chemical properties of test cosolvents are summarized in Table 1, according to an investigator's result (Tan *et al.,* 1994). Among test chemicals, polar and basic organic solvents did not improve cycle sequencing of high GC template at all (Figure 4). On contrary, these polar and basic chemicals inhibited the polymerization of complementary strand when added to sequencing reaction in DNA template of normal GC content (data not shown). We suggest that the chemical structure of N-methyl nitrogen ring compounds resembles the structure of pyrimidine base. Thus, newly incorporating bases at extension step during PCR may compete with these cosolvents, resulting in inefficient product. Glycerol, however, mimicked the positive effect of DMSO to the same extent, even though glycerol has no similar chemical property as DMSO except for the viscosity. Glycerol has high viscosity in itself, but can be easily solubilized in water solution. Thus, it is believed that glycerol may increase the enzyme activity of *AmpliTaq* DNA polymerase rather than the structural change of DNA template. According to the previous report, it is described that glycerol can improve the specificity of polymerizing enzyme while *N*-methyl-2-pyrrolidone inhibits the enzyme activity (Varadaraj and Skinner, 1994).

In present study, we investigated extensively the chemical effect of other cosolvents with similar properties to DMSO on cycle sequencing. The underlying mechanism of the enhancement of cycle sequencing by DMSO are unknown. It is suggested that DMSO may affect the melting temperature of the primer (Chester and Marshak, 1993)

and/or the thermal activity of *Taq* polymerase (Gelfand, 1989). It was postulated that DMSO decompresses GC-rich DNA (Lee *et al.,* 1981) by forming local hydrogen bonding with DNA bases. However, it is necessary to study the conformational transition of DNA template by cycle sequencing enhancers, since the postulation for the interaction between DMSO and DNA template is very tentative.

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