Plasmid sonication improves sequencing efficiency and quality in the Beckman Coulter CEQ system

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We report on an unexpectedly high rate of unreadable chromatograms from plasmid sequencing using Beckman Coulter’s protocols, chemistry, and CEQ8000 instrument. Failed or poor quality plasmid sequence chromatograms were accompanied by a sharp drop, fluctuation, or steady decline in the current and a corresponding delay in signal counts beyond the time of capillary injection. We observe a correlation between the presence of supercoiled DNA and these sequencing problems. Herein we demonstrate that plasmid sonication, which is known to fragment supercoiled DNA, is an effective way to improve sequence phred20 read lengths to the point that they are not significantly different from Beckman Coulter’s control template or enzymatically linearized plasmids.

Numerous attempts by our facility to sequence a diversity of plasmids using Beckman Coulter’s (BC; Fullerton, CA, USA) dye terminator cycle sequencing (DT1CS) and the CEQ8000 system produced an unexpectedly high rate of unreadable sequence chromatograms. The raw data signals for many failed or poor quality plasmid sequences were accompanied by either a drop or anomaly in the current and a delay in signal beyond the 20 min injection point. We hypothesize that these results are caused by the presence of supercoiled DNA, which may interfere with the sequencing reaction and/or hinder electrophoretic mobility.

All forms of covalently closed circular DNA (ccDNA) exhibit some measure of supercoiling caused by twist, writhe, and excess turns (1,2). Solvent chemistry and temperature affect the physics of ccDNA isomers to form different supercoiled topologies, or topoisomers, that relax when one or both strands are nicked (3). Topoisomers and relaxed forms of ccDNA are distinguished by their unique electrophoretic mobility characteristics (4).

The pre-heat treatments prescribed by BC for plasmid DTCS are said to denature plasmid for improved signal strength and current stability. Although BC protocols prescribe three different pre-heat treatments for “certain plasmid DNA templates,” we observed no significant difference in sequence quality between the three pre-heat (PH) treatments. Plasmids are generally resistant to cleavage at 95°C, but rapidly degrade when temperatures reach 107°C (5). Unless PH treatments nick one or both strands of ccDNA into relaxed form, the denatured state will not necessarily be retained and topoisomers will be carried forward into both the cycle sequencing reaction and capillary injection phases.

Using prescribed BC methods, we report an average phred20 read length of 127 bp for readable sequences and complete sequencing failure for 27% of plasmids purified with the Qiagen QIAprep Spin Miniprep kit (Mississauga, ON, Canada). In contrast, we report >95% success rate for BC-supplied pUC18 control plasmid. Agarose gel electrophoresis reveals a higher ratio of nicked to supercoiled topoisomer in the BC-supplied pUC18 compared with pUC18 prepared with the Qiagen Miniprep kit (abbreviated pUC18-Q herein; Figure 1, lanes 1 and 2). To test whether the presence of a high ratio of supercoiled topoisomers leads to the reported sequencing problems, we digested plasmids with restriction enzymes prior to sequencing (Figure 1, lane 3). Restriction digestion (RD) improved both read length and current values that are equivalent with pUC18 control and linear template sequencing. While RD improves sequencing quality, this is not a practical solution due to the extra expense, time, and handling, which, importantly, are not needed for other manufacturers’ sequencing systems.

We investigated sonication as an alternative method. Sonication is used for nicking and shearing DNA in large-scale shotgun sequencing projects and for the construction of microsatellite libraries (6,7). Empirically, we determined that sonication of PH-treated plasmids for 2 s at power setting 2 (abbreviated PHS herein) using a sonic dismembrator (Model 100; Fisher Scientific, Ottawa, ON, Canada) fragments most supercoiled DNA (Figure 1, lane 4).

To assess the quality of our sequencing results from a variety of plasmids (Table 1), we compiled phred20 scores (8) for 386 sequence trace files generated from plasmid templates by our sequencing facility from August 2005 until November 2007. During this time period, plasmids received one of the three treatments (PH, n = 125; PHS, n = 171; RD, n = 90) prior to sequencing. The pUC18 control template provided by BC (n = 23) received no treatment. The BC PH treatment shows significantly smaller mean phred20 scores compared with PHS, RD, or the pUC18 control (Figure 2).

Our results clearly show that PHS or RD of supercoiled topoisomers eliminate our plasmid sequencing problems. Current anomalies are observed during electrophoresis when sequencing both untreated plasmids, other than the

Figure 1. Sonication efficiently linearizes plasmid DNA. Equal molar amounts of pUC18 plasmid DNA from two sources were electrophoresed on a 1% agarose gel: control pUC18 from Beckman Coulter (untreated, lane 1) and pUC18 prepared with a Qiagen QIAprep Spin Miniprep kit (untreated, lane 2; EcoRI-digested, lane 3; and sonicated, lane 4). Supercoiled forms are observed at ∼2 kb in lanes 1, 2, and 4. A bright nicked form is observed in lane 1 at ∼5 kb.
Benchmarks

pUC18 control, and PCR templates contaminated with plasmids through colony PCR (9); anomalies are more pronounced (e.g., Figure 2, lower panel [PH]) for larger plasmids. Coupled with a delay in fluorescent signal counts, these anomalies support the hypothesis that supercoiled topoisomers inhibit electrophoretic mobility through the capillary. We tested this hypothesis further by adding supercoiled plasmid to BC pUC18 control reactions. Completed cycle sequencing reactions \((n = 8)\) were each divided into three; one control and two treatments, to which were added 50 fmol of pUC18-Q (3 kb) or pCR-TOPO (5 kb) plasmid. Paired \(t\)-tests showed that only the addition of the larger pCR-TOPO plasmid caused a significant reduction in phred20 scores (Mean difference \(= 50.2, P = 0.002\)). Although current fluctuations were not observed in this experiment, similar tests with the pCR-TOPO and similar-sized vectors showed current abnormalities in 43% of plasmid additions \((n = 13)\).

Template accessibility during the sequencing reaction may also be improved by relieving supercoiling. As noted, the addition of pUC18-Q plasmids to sequenced BC pUC18 controls does not significantly affect read length. Sequencing the pUC18-Q plasmid with only a PH treatment (i.e., not PHS or RD), however, leads to significantly lower phred20 scores (Mean = 209, \(P = 0.001\)) coupled with peak resolution problems (irregular spacing and peak shape, and a high background), which are also observed when sequencing other PH-treated plasmids. In contrast, the addition of larger plasmids is associated with reduced read lengths and current anomalies, however, it does not affect peak shapes, spacing, or levels of background. We speculate that a high ratio of supercoiled plasmid also interferes with DTCS efficiency, causing peak resolution problems.

The improvements to sequencing plasmids that we report herein will have immediate benefits for researchers using CEQ instruments. Although extreme care is required to obtain high ratios of supercoiled DNA using the traditional plasmid mini-prep technique (6), plasmid DNA obtained from the Qiagen Miniprep kit consistently yields the vast majority of the plasmid in the undamaged supercoiled form (Figure 1). Other commercially available

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**Figure 2. Template sonication results in significantly longer sequence read lengths.** Two representative sequence runs showing raw fluorescent signal and current levels for plasmid receiving the pre-heat plus sonication (PHS) treatment (PHS, upper panel) and the pre-heat (PH) treatment (lower panel). A time delay in raw data signal to 33 min corresponds with a marked drop in current levels. The inset shows a histogram with standard error bars and a significant increase \(GT2\) test \(P < 0.01^{*}\) in mean phred20 read length (failed sequences were not included in these calculations) in all methods compared with the PH treatment; control refers to the Beckman Coulter (BC) pUC18 control sequence.
plasmid extraction kits use silica membrane technology (e.g., Promega’s “Wizard,” Invitrogen’s “Purelink,” and Mo Bio’s “UltraClean”) optimized for yielding high ratios of supercoiled DNA. Extraction kits are standard in most molecular genetic laboratories and are the basis of automation techniques. The PHS is a simple, fast, and cost-effective method that can easily be incorporated into any laboratory. Removing the supercoiled structure improves sequencing efficiency while ensuring optimal phred20 read lengths.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

REFERENCES


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