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Research Article

Cost-Effective Paramagnetic Bead Technique for Purification of Cycle Sequencing Products

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The quality of sequencing results depends greatly upon the quality and purity of the template as well as the purity of the fluorescently labeled products generated by cycle sequencing. Numerous approaches have been used for purification of cycle sequencing products, including alcohol precipitation, affinity-based chromatography, size exclusion chromatography, commercially-available proprietary methods, and paramagnetic bead technology. In this paper, we describe an affordable paramagnetic technology method using BioMag Carboxyl beads. Compared to other well-established, proprietary methods for purification of cycle sequencing products, this method produced consistently good results, with a very low reagent cost and short procedure time.

1. Introduction

Since the 1990's, the most widely used method of automated DNA sequencing has been the Sanger dye-termination enzymatic sequencing method, used in conjunction with gel-based or capillary array electrophoresis sequencers that use laser-induced fluorescence detection [1]. Although next-generation sequencing technologies [2, 3] are quickly gaining ground, Sanger sequencing remains the method of choice for many researchers for sequencing PCR amplicons and for filling in sequence gaps in genomic sequencing projects. For Sanger sequencing, most laboratories use BigDye Terminator v.3.1 Cycle Sequencing kits (Applied Biosystems, Foster City, CA, USA).

Two major factors influence the final quality of raw sequence data: (1) concentration, integrity, and purity of the DNA template, and (2) the effective removal of excess dye terminators and impurities from cycle sequenced products before analysis on sequencing instruments [4, 5]. Methods for purification of cycle sequencing products often can be costly, time-consuming, or both, and may result in partial or full loss of the original template. The choice of technique used for purification of cycle sequencing products can depend on several factors such as cost, hands-on time, need for high-throughput processing, sequence data quality, and the preference of the users. Numerous approaches are available for the purification of cycle sequencing products, including as alcohol precipitation [6], affinity-based chromatography [7], size exclusion chromatography techniques [8–10], proprietary kits, and use of the paramagnetic particles or beads [11, 12].

The use of magnetic beads for purification of cycle sequencing products was introduced in 1992 [12]. In this method, purification relies upon adsorption of DNA to magnetic beads in the presence of ethanol. Bead-bound cycle sequencing products are washed with ethanol and DNA is eluted off the magnetically-captured beads in water or other low ionic solutions. Several commercial magnetic beads kits for purification of cycle sequenced products became available in the last 10 years including the CleanSEQ (Agencourt Bioscience Corporation, Beverly, MA, USA), MagDTR Magnetic Resin (Edge Biosystems, Gaithersburg, MD, USA), MagSi-DNA clean^{FIX} (MagnaMedics, Geleen, Netherlands),

and Wizard MagneSil Sequencing Reaction Clean-Up System (Promega, Madison, WI, USA). Although these kits are convenient and typically perform well, their cost is high; the price per sample ranges from US $0.25 to $0.57. A low-cost alternative method, alcohol precipitation, requires a high degree of technical proficiency to yield consistently good quality data. Proprietary kits employing affinity or size-exclusion chromatography methods typically yield excellent results but are expensive. We have developed a method which uses inexpensive magnetic beads (BioMag Carboxyl beads, Bangs Laboratories, Inc., Fishers, IN, USA) at the fraction of the cost of the commercially available magnetic beads, kits (less than US $0.02 per sample). The method is fast (25 min per 96 samples) and has minimal potential for user error. In this paper, we present our method and demonstrate that the quality of sequence data obtained is comparable to that obtained using more expensive proprietary methods.

2. Materials and Methods

2.1. Preparation of BioMag Carboxyl Beads. Prior to use, the BioMag Carboxyl beads must be washed and resuspended in 5 M NaCl. Beads from a 1 mL volume of bead stock were captured using a 1.5 mL magnetic separator (Bangs Laboratories, Inc., Fishers, IN, USA) and then washed in 1 mL of 0.5 M EDTA. The beads were captured again using a magnetic separator and resuspended in 0.5 mL of 0.5 M EDTA. The bead suspension was then combined with 19.5 mL of 5.0 M NaCl. The washed bead suspension has a shelf life of 12 months when stored at 4°C.

2.2. Purification of Cycle Sequencing Products Using BioMag Carboxyl Beads. Amplicons of rotavirus VP4 and VP7 genes were generated by reverse transcription-polymerase chain reaction (RT-PCR) amplification of viral RNA as described [13–15]. Cycle sequencing was performed using BigDye Terminator v.3.1 Cycle Sequencing mix (10 or 20 μL reactions) in a 96-well reaction plate. Ten microliters of well-mixed bead suspension was added to each cycle sequencing reaction along with 42 μL (10 μL reaction) or 62 μL (20 μL reaction) of 85% ethanol and mixed by pipetting up and down. The reaction plate was inserted onto a SPRIPlate 96R ring magnetic plate (Agencourt Bioscience Corp., Beverly, MA, USA) and incubated for 5 minutes at room temperature. The reaction plate/magnetic plate assembly was then inverted onto a lint-free wipe and tapped gently a few times to remove any residual liquid. The reaction plate/magnetic plate assembly was then returned to an upright position and 200 μL of 85% ethanol was added into each well. After allowing the beads to settle for 30 sec, the reaction plate/magnetic plate assembly was inverted and tapped gently onto a lint-free wipe to remove any residual liquid. The 85% ethanol wash was then repeated once (2 washes total). The reaction plate/magnetic plate assembly was then inverted onto a lint-free wipe and centrifuged at 65 × g for 10 sec. The reaction plate/magnetic plate assembly was returned to an upright position and 40 μL of molecular biology grade water was added to each well. After placing a plate septum onto the 96-well reaction plate, it was removed from the SPRIPlate 96R ring magnetic plate and placed into a 96-well plate base into which a Direct Inject Magnet (Agencourt Bioscience Corp., Beverly, MA, USA) had been inserted. After applying a plate retainer, the reaction plate assembly was installed on an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), and sequencing products were analyzed using 3130 POP-7 Performance Optimized Polymer and 50 cm 3130xl and 3100 Capillary Array (Applied Biosystems, Foster City, CA, USA).

2.3. Production of Replicate Sequences Using Other Cycle Sequencing Purification Methods. Sequences obtained by using the paramagnetic bead technique were compared with those obtained when cycle sequencing products were purified by (1) alcohol precipitation; (2) a widely-used, proprietary centrifugal size-exclusion column designed to remove unincorporated dideoxynucleotide terminators from cycle sequencing reactions; (3) a proprietary in-well purification kit which captures unwanted cycle sequencing reaction components and then collects them at the bottom of each well through a centrifugation step. Amplicons of rotavirus VP4 and VP7 genes were generated by reverse transcription-polymerase chain reaction (RT-PCR) amplification of viral RNA and then subjected to cycle sequencing as described. For each of the purification techniques, three 10 μL volumes of cycle sequencing reaction were purified in triplicate using each technique. Purified cycle sequencing products were analyzed on an ABI Prism 3130xl Genetic Analyzer as described.

3. Results and Discussion

Cycle sequencing using BigDye Terminator v.3.1 Cycle Sequencing mix was carried out using 38–86 ng of RT-PCR product per reaction. Sequences obtained after purification of the cycle sequencing product with size exclusion columns, the in-well method, and the BioMag Carboxyl bead method averaged more than 800 bp of high quality (>95%) sequence (Table 1). The chromatography peaks were well resolved and symmetrical, and the peak height was consistent for all 3 methods (Figure 1). Use of the size exclusion column and in-well methods resulted in the best overall sequence quality and read length. The length of sequences produced using the BioMag Carboxyl beads were similar, and the quality of sequences was slightly lower but within the standard deviation ranges of the size-exclusion column and in-well methods (Table 1). Dye blobs were noted around base no. 70 in both the in-well and bead methods (Figure 1). While this artifact is seen occasionally in sequences prepared using this bead cleanup method (as well as the in-well method), the sequence typically can be read accurately in this region. The two main advantages of BioMag Carboxyl beads compared to the 2 proprietary methods are much lower cost per sample and reduced time necessary to prepare the fluorescent products for sequencing (Table 1). The alcohol precipitation method has the lowest cost per sample (Table 1) but yielded the poorest quality sequences (Table 1, Figure 1). We found that this method is very prone to user error when a 96-

Table 1: Summary of results of cycle sequencing purification methods examined in this study.

Technique	Sequence length/quality[a]	Cost, US $[b] (per sample)	Time needed[c] (per 96 samples)	Advantages	Disadvantages
BioMag Carboxyl beads	815 ± 1/97 ± 3	0.006–0.015	25 min	Low cost Quick Good results	Moderate potential for user error Occasional blobs
Proprietary size exclusion columns	816 ± 1/99 ± 1	2.2–2.6	3-4 h	Best results	Costly Time consuming Moderate potential for user error
Proprietary in-well technique	816 ± 1/99 ± 1*	1.94	37 min	Quick Good results Low potential for user error	Costly Short shelf life Occasional blobs
Ethanol precipitation	744 ± 35/87 ± 11**	~0.001	1 h 45 min	Lowest cost	Loss of amplicon Low quality results High potential for user error

[a]Sequence length and quality scores for overlapping fragments were obtained directly from Sequencher 4.8 software (Gene Codes Corporation, Inc., Ann Arbor, MI). Prior to analyzing the data, raw sequences were trimmed by aligning all of the sequences of one reaction and determining the unusable part of the longest sequence and cutting all sequences at that base. Measurements and standard deviation are calculated using triplicate results of 4 different reactions (primers used were con2, con3, VP7R and 9con1L). Measurement based on *1 and **2 fewer sequences due to low quality of final sequence.

[b]Cost per reaction was calculated by totaling the cost of consumables needed to clean one 10 μL cycle sequencing reaction and does not account for the cost of equipment.

[c]Time needed to purify 96 samples was measured from the moment the plate containing the cycle sequencing reactions was removed from the thermocycler after cycle sequencing to the time purification was completed and the 96-well septum was placed on the plate.

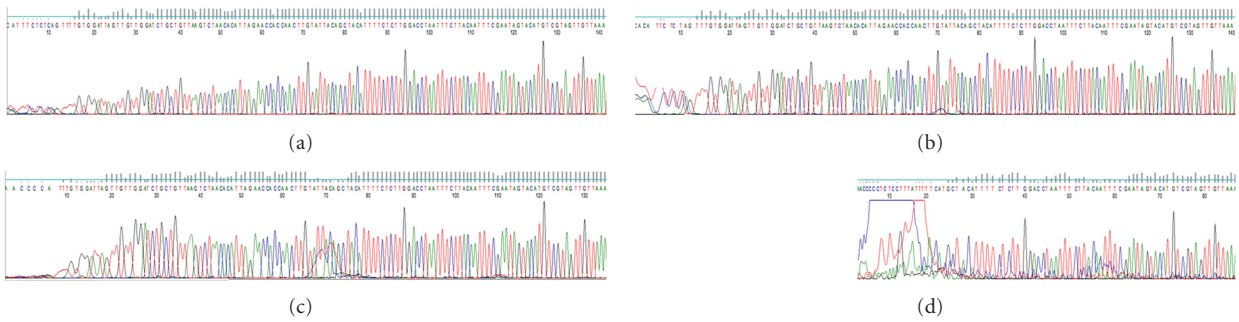

Figure 1: Representative chromatograms of the rotavirus Wa strain VP7 gene sequenced with VP7R primer and purified by proprietary size-exclusion columns (a), a proprietary in-well clean up kit (b), BioMag Carboxyl beads (c), and alcohol precipitation (d).

well plate is used, and the quality of sequencing results was highly dependent upon the user's technical skill. The potential for user error was lowest for the BioMag Carboxyl beads technique and the proprietary size exclusion columns. An automated version of BioMag Carboxyl beads technique has been used successfully to clean up hundreds of thousands of cycle sequencing reactions using both PCR products (500–15,000 bp in length generated using multiple brands of PCR enzymes) as well as plasmids. Sequences obtained using reactions cleaned up by this method have been analyzed on both the ABI Prism 3130xl Genetic Analyzer and the AB 3730xl DNA Analyzer.

In conclusion, BioMag Carboxyl beads produce results comparable to size-exclusion columns and the proprietary kit we tested, and were superior to alcohol precipitation. Cost is a clear advantage, with the BioMag Carboxyl bead technique being up to 200 times less expensive than the proprietary methods we evaluated and 20–40 times less expensive than other commercially available magnetic bead kits. BioMag Carboxyl bead purification offers the highest quality sequence at the lowest reagent cost and short procedure time, although there is an initial investment in reusable hardware.

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Conflict of Interests

The authors declare that they have no conflict of interests.

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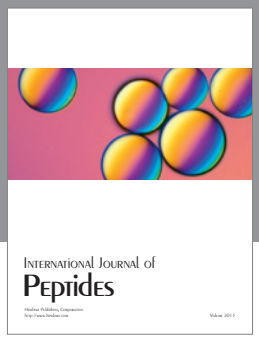

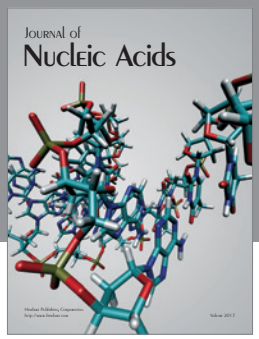

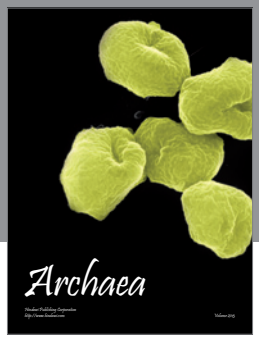

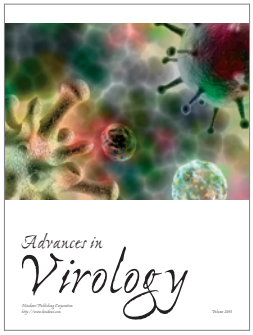

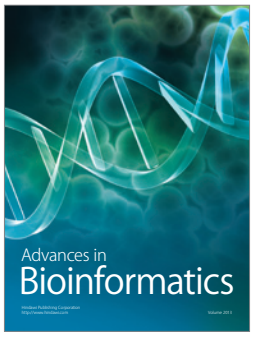

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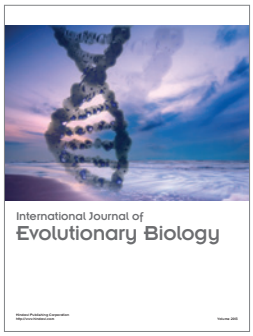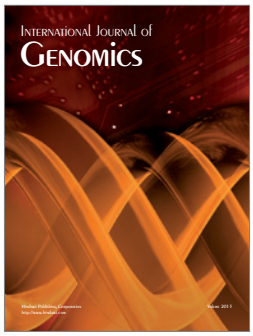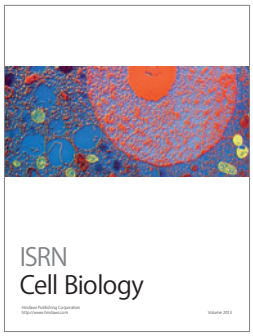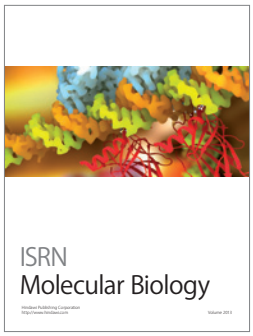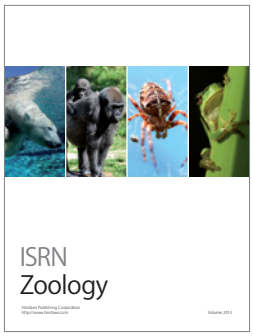
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