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Automated polymerase chain reaction product sample preparation for capillary electrophoresis analysis

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Abstract

The analysis of crude polymerase chain reaction (PCR) products by capillary electrophoresis (CE) is often compromised due to the presence of a high concentration of salt. Salt interferes with the electrokinetic injection and induces localized heating within the column; hence, PCR products must be desalted or cleaned-up prior to CE analysis. A variety of commercial clean-up systems are available that have been traditionally used to prepare PCR products for cloning, sequencing and digestion with restriction enzymes. These systems were tested for their effectiveness in preparing PCR products for CE analysis and were evaluated based on CE resolution, salt removal, DNA recovery, processing time and cost. One particularly effective clean-up system, membrane dialysis, was automated using a robotic workstation.

Keywords: Polymerase chain reaction; Enzymes

1. Introduction

Capillary electrophoresis (CE) provides fast, high-resolution separation of DNA restriction fragments and polymerase chain reaction (PCR) products. Unfortunately, PCR product analysis by CE using electrokinetic injection is impeded by the presence of salt in the reaction mix. Typical PCR reactions contain 50 m*M* of KCl and 1.5 m*M* of $MgCl_2$, which cause irreproducible injections and migration times as a result of the chloride ions competing with the DNA for migration into the capillary [1,2] (Fig. 1A). An alternative mode of injection, termed pressure injection, eliminates the problems induced by salt when using electrokinetic injection; however, it can produce significant band broadening which reduces resolution, and is therefore not typically used for DNA analysis [3–12].

Several techniques exist to purify the PCR product from the other components in the reaction mix. These include anion–exchange chromatography or high-performance liquid chromatography (HPLC) [13], adsorption of DNA onto Glass Milk [14], selective precipitation of DNA by ammonium acetate–2-propanol [15] or polyethylene glycol [16], gel permeation [17] and slab gel electrophoresis via acrylamide or agarose. Unfortunately, these methods can be time consuming, labor intensive, expensive and/or result in low recovery of PCR product.

We have examined alternative approaches for PCR product preparation. These simple and fast techniques included (1); dialysis using MF-Millipore membrane filters (Millipore, Bedford, MA, USA), (2); spin filtration through a thin, cellulose mem-

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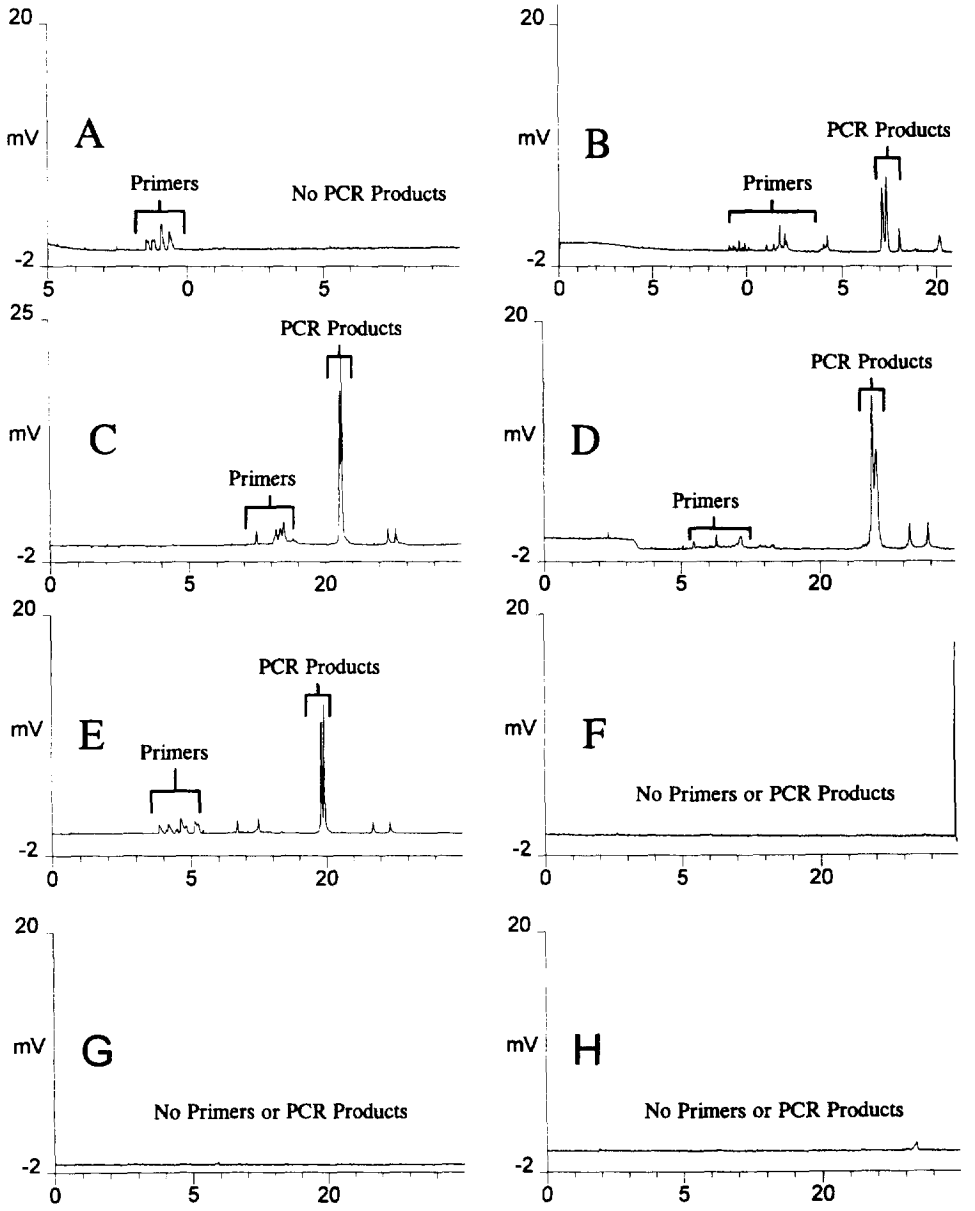

Fig. 1. CE analysis of unpurified and purified HUMTHO1 PCR products. Each analysis was performed at 30°C with a run voltage of −11 kV and a wavelength absorbance of 260 nm. The capillary had an effective length of 40 cm×75 mm I.D. and contained a 5% (w/w) polymer (Applied Biosystems). PCR products were either (A) unpurified, or purified using (B) a MF-Millipore Membrane Filter (Millipore), (C) a Microcon 100 (Amicon), (D) a UF30 Spin Filter (Integrated Separation Systems), (E) a Spin-X UF-100 (Costar Scientific), (F) a Chroma Spin-100 (Clontech), (G) a Bio Spin-30 (Bio-Rad) or (H) a QIAquick (Quiagen). PCR products were not detected in panels F, G and H due to the high concentration of salt in the sample that remained after spin column purification (see Table 1).

brane [Microcon 100 (Amicon, Beverly, MA, USA), Centrex UF-0.5 (Schleicher and Schuell, Keene, NH, USA), UF30 spin filter (Integrated Separation Systems, Natick, MA, USA), Ultrafree-MC (Millipore), Spin-X UF-100 (Costar Scientific, Cambridge, MA, USA)] and (3); passing the samples through a spin column containing a matrix [Chroma Spin-100 (Clontech Laboratories, Palo Alto, CA, USA), Bio

Spin-30 (Bio-Rad Laboratories, Hercules, CA, USA) and QIAquick (Qiagen, Chatsworth, CA, USA)]. Based on CE resolution, salt removal, DNA recovery, processing time and cost, the Spin-X UF-100 spin filters (Costar Scientific), the Microcon 100 (Amicon) spin filters and the MF–Millipore Membrane Filters (Millipore) were the most effective devices for preparing PCR products for CE analysis.

The membrane filters offered the distinct advantage of being amenable to automation since no centrifugation step was required. By using a Biomek 1000 robotic workstation (Beckmann Instruments, Fullerton, CA, USA) and 96-well membrane filter microplates (Millipore), high throughput sample desalting was achieved. The entire automation process involved transferring PCR products that were amplified in a 96-well microplate to a fabricated dialysis apparatus. When dialysis was complete, the samples were transferred to 0.5-ml microcentrifuge tubes for CE analysis.

2. Experimental

2.1. DNA Isolation

DNA isolation was performed using the Purgene' DNA isolation kit (Gentra Systems, Minneapolis, MI, USA).

2.2. PCR Amplification

PCR products were generated from the HUMTH01 locus of a heterozygous individual (the resultant PCR products were 191 and 199 base pairs) or from the ARSB locus (the resulting PCR product was 151 bp). Briefly, a 50-μl mixture containing 10–20 ng of DNA template, 0.5 *M* primer pair, 0.2 m*M* each of dATP, dGTP, dCTP and dTTP, 10 m*M* Tris–HCl, pH 8.3, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.1% Triton X-100, 2.5 U of Amplitaq DNA polymerase (Perkin-Elmer, Foster City, CA) was subjected to thermal cycling [4 min at 95°C, 10×(94°C 30 s, 64°C 30 s, 70°C 45 s) and 20×(90°C 30 s, 64°C 30 s, 70°C 45 s]. Amplifications were performed in either 0.2-ml MicroAmp reaction tubes using the Perkin-Elmer 9600 thermal cycler or 96-well PVC microplates (Falcon) using an MJ Research PTC-100 thermal cycler.

2.3. Manual sample preparation

The PCR products were cleaned-up using either an MF-Millipore membrane filter (Millipore,), a Microcon 100 (Amicon), a Centrex UF-0.5 (Schleicher and Schuell), a UF30 Spin Filter (Integrated Separation Systems), an Ultrafree-MC (Millipore), a Spin-X UF-100 (Costar Scientific), a Chroma Spin-100 (Clontech Laboratories), a Bio Spin-30 (Bio-Rad Laboratories) or a QIAquick (Qiagen) according to the manufacturer's instructions. Each procedure was performed thirty times. Twenty of the resulting samples were subjected to liquid chromatography (LC), whereas, ten were analyzed by CE.

2.4. Liquid chromatography

The non-metallic LC system was comprised of a Dionex DX-100 ion chromatograph (Dionex, Sunnyvale, CA) with the Dionex IonPac, AS4A analytical column (250×4 mm I.D.), an anion self-regenerating suppresser (4 mm) and Dionex AI450 software for data collection and analysis. The injection loop was 25 μl with an eluent system of 1.8 m*M* sodium carbonate–1.7 m*M* sodium bicarbonate and a flow-rate of 1.5 ml/1 min.

Three chloride standards with concentrations of 1, 5 and 10 parts per million respectively were prepared from the Dionex five-anion standard (fluoride 20 mg/l, chloride 30 mg/l, nitrate 100 mg/l, phosphate 150 mg/l and sulfate 150 mg/l) and were used to calculate the chloride concentration in the samples.

2.5. Capillary electrophoresis

The CE systems consisted of an Applied Biosystems Model 270A-HT (Applied Biosystems, Foster City, CA, USA) and a BioFocus 2000 capillary electrophoresis system (Bio-Rad, Hercules, CA, USA). All injections were performed electrokinetically at −7 kV for 7 s. Separations were performed at 30°C using fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) containing 5% polymer [2.9 ml ABI DNA fragment analysis reagent 7% (w/w) (Applied Biosystems), 0.7 ml 40%

urea and 0.4 ml DNA fragment analysis buffer (Applied Biosystems)]. Absorbance was measured at 260 nm.

2.6. DNA quantification

Double-stranded DNA was selectively stained with Hoechst 33258 [18] and quantified using the CytoFluor 2350 fluorescence measurement system (Millipore) with the Cytocalc Version 2.00.06 software for data analysis.

2.7. Automated sample preparation

PCR products were generated in a 96-well microplate using the MJ Research PTC-100 thermal cycler and then dialyzed by automation using a Biomek 1000 robotic workstation (Beckmann Instruments). To perform automated dialysis, the Biomek 1000 was fitted with an MP200 pippeting tool (5–200 μl capacity, eight tips), a 96-well microplate containing PCR product in each well, a dialysis apparatus and a mini-tube rack (Fig. 2). The dialysis apparatus was built by boring out a Biomek minitube rack, sealing the bottom of the rack with a plastic cover and filling the resultant chamber with distilled water. A 96-well membrane filter plate from the Millipore Mutiscreen filtration system (Millipore) was placed in the top of the holder, with the bottom of the wells below the water surface. Dialysis was accomplished by transferring 35 μl of PCR product from the 96-well plate to the 96-well dialysis apparatus. After 30 min, the samples were transferred to 0.5-ml microcentrifuge tubes in the minitube rack and loaded onto a CE system.

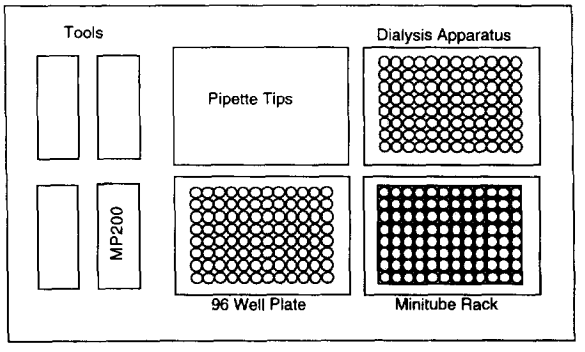

Fig. 2. Configuration of the Biomek 1000 platform to perform automated dialysis. A Biomek 1000 robotic workstation was fitted with an MP200 pippeting tool (5–200 ml capacity, eight tips), a 96-well microplate containing PCR product in each well, a dialysis apparatus and a mini-tube rack. Dialysis was accomplished by transferring 35 ml of PCR product from the 96-well microplate the 96-well dialysis apparatus. After 30 min the samples were transferred to 0.5-ml microcentrifuge tubes in the minitube rack and loaded onto a CE system. The dialysis apparatus consisted of a 96-well membrane filter plate (Millipore) placed in a holder that was filled with water.

3. Results and discussion

The Spin-X UF-100 spin filter, the Microcon 100 spin filter and the MF-Millipore membrane filters were found to be effective for PCR product preparation for CE analysis since they resulted in distinct products on the electropherograms (Fig. 1), eliminated over 99% of the salt (Table 1) and recovered at least 95% of the DNA (Table 1). The Centrex UF-0.5, UF30 Spin Filter, and the Ultrafree-MC recovered only 55%, 62% and 60% of the DNA, respectively.

To our surprise, the Chroma Spin-100 and Bio Spin-30 spin columns, both consisting of loose gel resins, actually increased the amount of salt in the PCR reaction by an average of 48% and recovered only 39% of the DNA. Thus, the corresponding electropherograms generated by CE revealed no primers or products (Fig. 1, panels F and G). The high levels of salt in the sample may have been a result of contamination from the storage buffer in which the gel resin is packed. However, washing the column extensively with deionized water prior to loading the sample had no effect (data not shown). The QIAquick PCR purification spin column was among the fastest of the clean-up methods (Table 1). However, the removal of 86% of the salt was not sufficient for CE analysis (Fig. 1, panel H) and only 36% of the PCR product was recovered (Table 1).

Although sample preparation using the membrane and spin filters is relatively fast when a few samples are desalted, they are still time-consuming when a large number of samples are processed. Therefore, the sample clean-up was automated using a Biomek 1000 robotic workstation. The only effective clean-up method that could be automated was dialysis using the MF-Millipore membrane filters, since the

Table 1
Chloride removal, DNA recovery and cost for each purification device

Device	Method	%Cl removed	%DNA recovered[a]	Time (min)	Cost per use ($)	MWCO[b] (kD)
Membrane filter	Dialysis	98.9±0.19	96.2±0.0	20	1.41	NA
Microcon 100	Spin filter	99.0±0.22	99.3±0.40	32	2.00	100
Centrex UF-0.5	Spin filter	98.8±0.44	54.6±1.60	12	2.20	100
UF30 spin filter	Spin filter	98.7±0.47	61.8±5.90	12	1.65	30
Ultrafree-MC	Spin filter	98.8±0.54	60.0±0.48	12	1.85	30
Spin-X UF-100	Spin filter	98.9±0.31	95.8±5.60	12	2.00	100
Chroma Spin-100	Spin column	−39.5±8.90	31.3±4.30	14	1.59	91
Bio Spin-30	Spin column	−57.1±6.50	46.9±7.307	10	2.96	40
Qiaquick	Spin column	85.5±8.60	35.7±7.20	10	1.20	NA

[a] The primers contributed no more than 1.0% of the DNA recovered since Hoechst stain has a 10-fold less affinity for ssDNA than that for dsDNA.
[b] Molecular mass cut-off.

Spin-X UF-100 and Microcon 100 spin filters required centrifugation. A routine for the Biomek 1000 was designed to automate membrane dialysis using a 96-well membrane filter plate (Millipore) placed in a custom holder (Fig. 2). PCR product amplified in a 96-well plate was transferred to the 96-well dialysis apparatus. When 30 min had elapsed, the samples were transferred to 0.5-ml microcentrifuge tubes in the minitube rack. The tubes were loaded into a CE system and analyzed. This automated dialysis method removed a sufficient amount of salt from the sample to achieve effective detection of the PCR product (Fig. 3). In addition, the dialysis apparatus could easily be used in other robotic workstations and would readily accommodate CE systems that can autoload from 96-well plates.

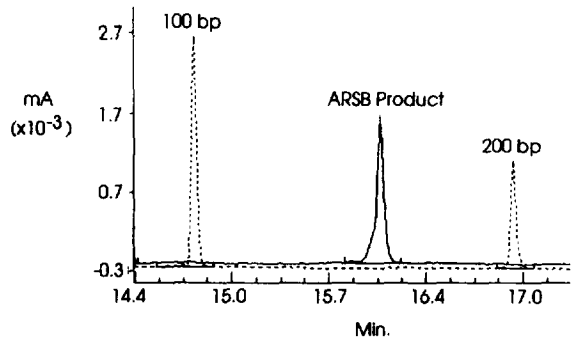

Fig. 3. Electropherogram of an ARSB PCR amplification. ARSB PCR products were subjected to automated dialysis using the Biomek 1000 and then analyzed by CE. Shown is a typical electropherogram with the ARSB product indicated. When the dialysis is omitted, there is no product detected (data not shown). mA, milliamps.

4. Disclaimer

The opinions or assertions herein are those of the authors and do not necessarily reflect the views of the Department of the Air Force, the Department of the Army or the Department of Defense.

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