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Magnetic Bead Purification of Labeled DNA Fragments for High-Throughput Capillary Electrophoresis Sequencing

ABSTRACT

We have developed an automated purification method for terminator sequencing products based on a magnetic bead technology. This 384-well protocol generates labeled DNA fragments that are essentially free of contaminates for less than $0.005 per reaction. In comparison to laborious ethanol precipitation protocols, this method increases the phred20 read length by forty bases with various DNA templates such as PCR fragments, Plasmids, Cosmids and RCA products. Our method eliminates centrifugation and is compatible with both the MegaBACE 1000 and ABI Prism 3700 capillary instruments. As of September 2001, this method has produced over 1.6 million samples with 93% averaging 620 phred20 bases as part of Joint Genome Institutes Production Process.

INTRODUCTION

In the late 1990's, high-throughput capillary electrophoresis instruments were introduced to meet the demands of large scale sequencing labs working on the Human Genome Project. While these instruments provided faster turnaround times, they also created a need for large numbers of high purity DNA samples. The typical approach was to scale up and automate molecular biology methods designed for small-scale bench work. Since these methods often contained laborious and irreproducible steps like centrifugation, we decided to design a high-throughput method tailored for robotic platforms and capillary electrophoresis instruments.

MATERIAL AND METHODS

See Table 1 for purification protocol.

RESULTS AND DISCUSSION

In developing a high-throughput method to purify DNA sequencing fragments, we began with a magnetic bead technology previously used at our facility to purify template DNA for sequencing(1). This method bound crude DNA to carboxylated

Table 1. Sequencing Fragment Purification Protocol

1. Sequence RCA generated template with Amersham Pharmacia ET Terminator Kit (part# US81095) according to instructions except; reduce final volume to 5ul and process in a 384 well PCR plate.
2. Add 10uL of BET solution to each well with 384-well Multidrop.
3. Verify solution is mixed thoroughly. Mix by pipetting or vortex as needed.
4. Incubate at room temperature for 15 minutes.
5. Place 384 well plate on a plate magnet for 1 minute.
6. Place 384 well plate/magnet assembly on 384 Hydra and aspirate solution.
7. Add 15ul of 70% ethanol solution to each well with 384 well Multidrop
8. Place 384 well plate/magnet assembly on 384 Hydra and aspirate solution.
9. Air-dry samples for 10 minutes or continue to step 10.
10. Dispense 15uL of deionized water to each plate.
11. Mix by pipetting or vortex until beads are resuspended
12. Incubate 10 minutes at room temperature.
13. Place 384 well plate on plate magnet for 2 minutes.
14. Transfer 10ul of water solution to suitable plate for electrokinetic injection
15. Cover and store at -20°C or load onto MegaBACE 1000 or ABI 3700
16. MegaBACE 1000 (Inject -1.6KV/36 seconds, Run - 7KV/170 minutes)

Reagent/Stock Preparation

BET Solution (processes twenty 384-well plates)

Ethanol(100%)	64.0 mls
Deionized Water	7.0 mls
Tetra Ethylene Glycol	6.4 mls
Carboxylated Beads (5% solids.0.8um dia.)	2.0 mls

Common Issues

Trapped Air Bubbles Can Prevent Mixing
Evaporation During Thermal Cycling Causes Low Read-Length
Improper Mixing Causes Dropouts and Loss of Trace Resolution

magnetic particles with a solution of polyethylene glycol and sodium chloride. The beads were washed multiple times with 70% ethanol and pure DNA was eluted with water. While this method met the requirements listed above, we needed a technique that worked in 384-well PCR plates and produced extremely pure DNA.

Our first design consideration focused on replacing the polyethylene glycol and sodium chloride binding buffer. Sodium chloride inhibits electrokinetic injection and is significant source of variability in capillary electrophoresis(2). Polyethylene glycol (PEG) contamination reduces sequencing trace quality and requires several washes to remove from the sample(3). These extra washes combined with PEG's high viscosity create problems with bead mixing and cause significant loss labeled DNA during purification. Therefore, we were searching for a low viscosity, highly soluble binding buffer that had a negligible impact on electrophoresis trace quality.

It is well known that 70% ethanol can desalt and precipitate DNA. While this can be an excellent binding buffer, left over dNTP dyes from terminator sequencing also bind to the beads in this solution. These dyes elute with the labeled DNA fragments and inhibit electrokinetic injection. Lowering the ethanol concentration does solubilize the hydrophilic dyes(4) but the labeled hydrophobic ssDNA also tends to solubilize resulting in low yields. Therefore, we began searching for an additive that would force the ssDNA to bind to the beads at low ethanol concentrations while keeping the rhodamine based dyes in solution.
Flock et al(5,6) recently described

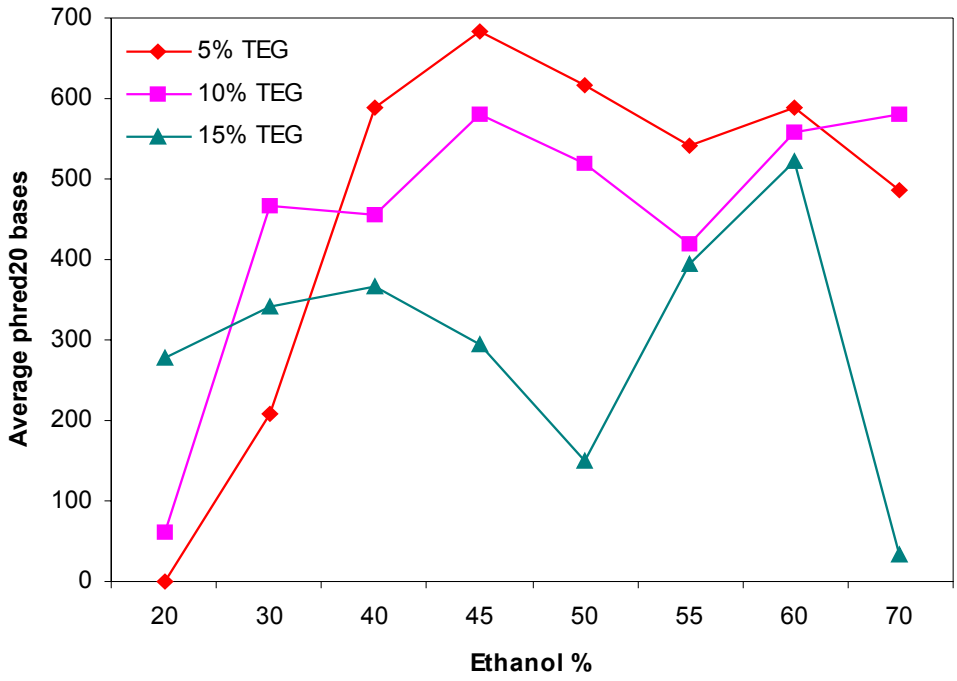


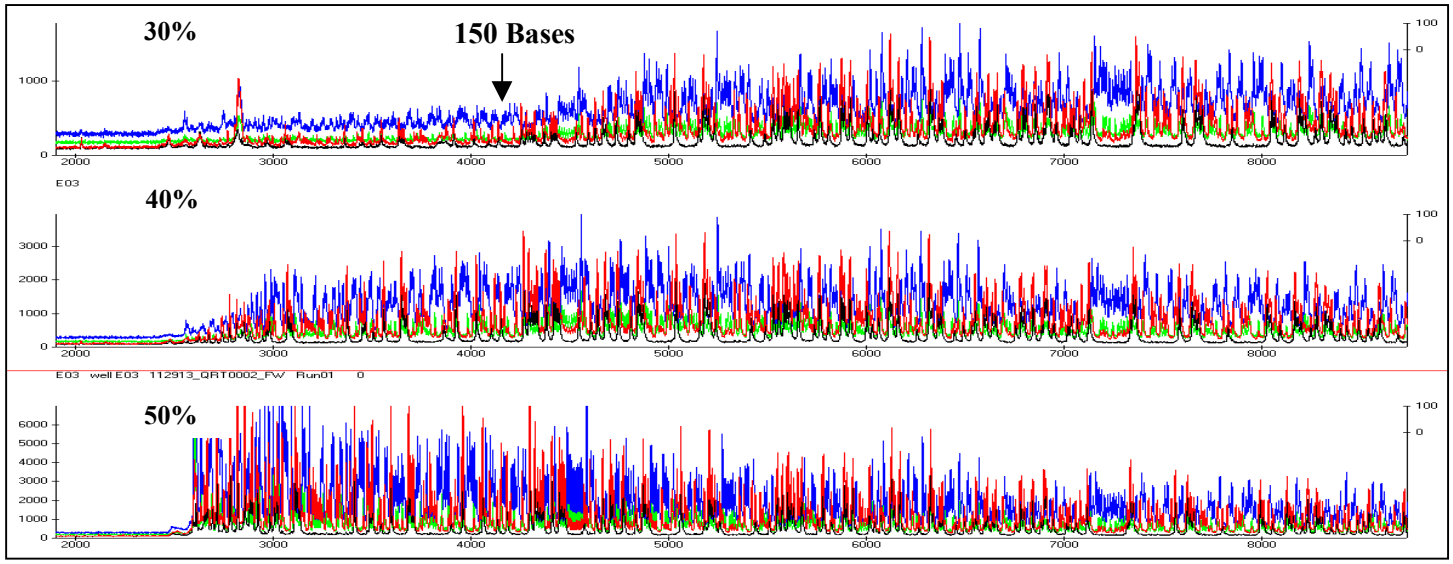
Figure 1 Comparison of average phred 20 read lengths for 5%, 10% and 15% TEG at ethanol concentrations from 20% to 70%. Each point is an average of twenty four samples and individual ethanol concentrations consisted of duplicate samples.

DNA precipitation through charge neutralization with salts or changes in the solution dielectric constant. Since we wanted to eliminate salt, we focused on low dielectric constant additives. To solubilze the dyes we needed a highly polar substance that could be easily washed out with both water and ethanol. Other desirable properties included a low viscosity, neutral charge, liquid phase at room temperature, solution density greater than water to encourage mixing, low toxicity and high stability. While several substances were discovered, Tetra Ethylene Glycol(TEG) best fit our criteria.
Various combinations of TEG and ethanol were tested for labeled ssDNA yield and sequencing trace quality. The optimal range was quite large at 50 $\pm$10% ethanol with 5% TEG as compared to 70 $\pm$3% range for ethanol precipitation (figure 1). Interestingly we discovered that the yield of ssDNA fragment below 150 bases could be controlled by adjusting the ethanol concentration as shown in figure 2. This allowed the leftover sequencing primers to be purified out increasing the reliability of electrokinetic injection.

To improve data quality further, we resuspended our purified samples in water. Salas-Solano et al(7) extensively characterized capillary injection and found that injection from water gives better peak resolution than injections from typical formamide and EDTA solutions. Water injections require samples that are virtually free of salts, dyes and template. Since our magnetic bead method eliminated salts and dyes, we explored methods that eliminated leftover template DNA.

Chen et al (8,9,10) found that heating plasmid template for fifteen minutes

Figure 2 Unprocessed Electropherograms obtained with a MegaBACE 1000. Traces show the effect of decreasing the ethanol concentration in the bead binding buffer at a constant 5% TEG Concentration. At 30% ethanol, the yield of fragments below 150 bases is reduced relative to larger fragments.



at 95^0C allowed the sequencing enzyme to degrade the plasmid during thermal cycling. Therefore, we used this method of template removal in our initial studies. Later, when RCA template generation was discovered by Amersham Pharmacia Biotech, we found that the large RCA template bound almost irreversibly to beads (Figure 3). This resulted in an essentially pure sample for our capillary electrophoresis.

The first phase of the project was optimized for 96-well PCR plates. The higher volume to surface area of these plates made bead handling much easier during washing. In phase two, we began reducing volumes and wash steps to prepare for the smaller 384-wells. We found that a single ethanol wash gave sufficient ssDNA purity were as multiple washes increased the dropout rate while providing no increase in sequencing quality or sample purity as measured by electrical conductivity. Interestingly, we observed that drying the beads to remove residual ethanol before water resuspension resulted in no increase in sequencing quality. Actually, the dried beads turned into a fine powder that was easily lost during processing.

Phase three began the transition to purification in 384-well PCR plates. Our major concern was to keep the small amount of magnetic beads in the plate wells during aspiration and washing. Engineers from Berkley National Labs designed a custom magnetic plate holder for the 384-well plates (Figure 4). Magnet plates consisting of neodymium plates were arranged to maximize the both field strength and orientation. This magnet coupled with a Robbins Scientific 384

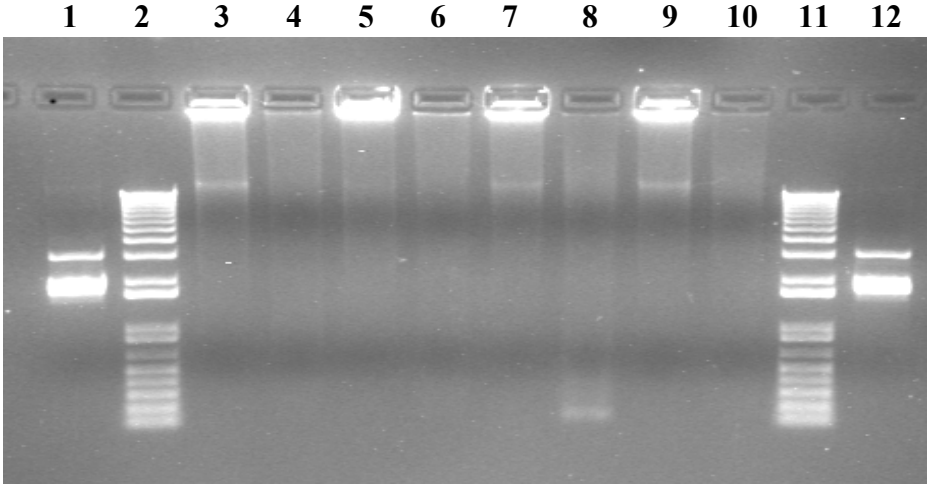


Figure 3 Lanes 1,12 (150ng pUC18); Lanes 2,11 1 kb-Plus DNA ladder(Gibco-10787-026); Lanes 3,5,7,9 (5 ul RCA template post sequencing reaction); Lanes 4,6,8,10 (5 ul BET purified sequencing reaction). Adjacent lanes 3 to 10 are duplicate samples.

syringe hydra has resulted in an excellent manual process that has produced over 800,000 samples with 91% averaging 605 phred20 bases (Table 2), thus far.

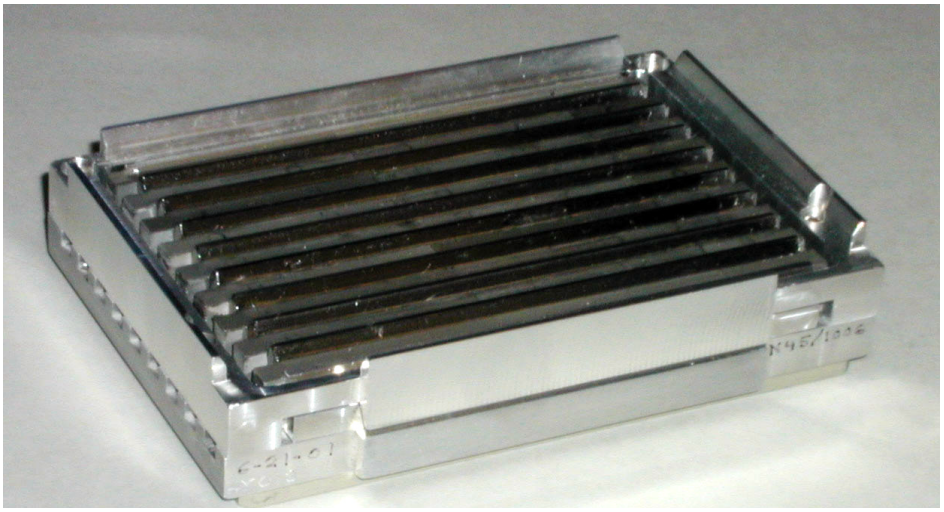
Phase four transferred the protocol to the BioMek FX robotic platform manufactured by Beckman Coulter. Initially, we tested another robotic platforms with steel tips and discovered that the plate magnet induced a magnetic field in the tips which resulted in bead loss and subsequent low yields of labeled ssDNA. Therefore, we moved to polypropylene tips that could be washed and reused. To eliminate the use of plate seals, we optimized the TEG ethanol concentrations to minimize evaporation effects associated with plates remaining uncovered for up to one hour. We also employed pipette mixing to eliminate vorterxing and plate movement steps. These automated systems eliminated 75% of the labor required for ethanol precipitation while maintaining reagent costs at $0.005 per sample. We also noted a forty base increase in our phred20 average read-lengths (Table 3).

Safety was another important design criteria for our automated method. Elimination of centrifugation reduced the risk of ergonomic injuries resulting from the loading and unloading of centrifuges. The substitution of water for formamide buffer eliminated the exposure to this teratogen toxin and ethanol consumption was reduced 400% eliminating fire hazards and waste disposal issues.

CONCLUSION

In August of 2001, the Joint Genome's entire production line was converted to our automated method which has produced over 800,000 samples with 93% averaging 620 phred20 bases (table 1). This method combined with the RCA process is a highly reliable 384-well method that is safer, less expensive and more suited to industrial scale molecular biology.

Figure 4 Custom magnetic plate holder for 384-well plates. The plate was constructed with neodymium magnet plates that were positioned based on computer models to maximize field strength and orientation. Patent Pending.



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	Number of Samples	Pass Rate (phred 20) >50 bases	Read Length (phred 20) >50 bases
AUTOMATED	**872,448**	**91.8%**	**621**
MANUAL	**802,944**	**93.2%**	**618**
OVERALL	**1,675,392**	**92.5%**	**620**

Table 2 Production results using both the manual and automated version of the BET protocol. Average Pass rates and read lengths of lanes producing more than 50 phred20 bases are shown.

96-Well	TEG/Bead/Ethanol	Ammonium Acetate
Plate Name	Average phred20 Bases	
MUN0009_FW	654	570
MUN0010_FW	660	567
MUN0011_FW	649	594
MUN0012_FW	648	598
MXL0026_FW	638	602
MXL0025_FW	646	630
MXL0027_FW	655	642
MBF2253_FW	618	626
MBF2254_FW	629	588
MBF2256_FW	638	623
AVERAGE	***644***	***604***

Table 3 Comparison of our custom bead purification method to the Ammonium Acetate Ethanol Precipitation method recommended by Amersham Pharmacia for dye terminator sequencing reactions. Plates were duplicate templates run on the same MegaBACE1000 Instrument. See text for method conditions

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