Benchmarks

PCR-Based Setup for High-Throughput cDNA Library Sequencing on the ABI 3700™ Automated DNA Sequencer

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Many large-scale sequencing projects are now being conducted or considered that rely on the latest capillary electrophoresis-based automated sequencing system from Applied Biosystems, the ABI 3700™ (PE Biosystems, Foster City, CA, USA). Because of the electrokinetic injection protocol and the physical properties of the capillaries and polymer used, reaction setups that have been optimized for the gel-based ABI 377™ systems (PE Biosystems) do not necessarily perform to expectations in the capillary system. We found this to be especially so when sequencing PCR products. We describe a rapid, inexpensive and reliable method for preparing template and sequencing reactions for application to high-throughput sequencing on the ABI 3700 instrument.

The overall goal of the procedure was to produce a sequence product with little contamination by salt or unincorporated fluorescent dyes while minimizing cost and effort. We had previously developed a robust protocol similar to the one described here, having a high (80%–90%) overall success rate on our ABI 377 systems. However, our initial attempts to use the same protocol on the ABI 3700 produced only short read lengths, primarily because the sequencing reactions were too successful, that is, they were producing more product than could be handled by electrokinetic injection. Also of concern was the observation that sequence quality dropped from the first run of a plate to the fourth, apparently because of degradation of the reaction in the formamide or water loading solution while the plate sat at room temperature on the instrument. A third concern was the possibility that fragments of the bacterial cells used as templates for the reaction might decrease the lifetime of the capillary array by blocking the capillaries. Several adaptations were nec-

essary to reliably produce high-quality sequence reads on the ABI 3700.

The starting materials for the sequencing reactions were arrayed, normalized cDNA libraries in 384-well plates. The libraries were constructed in the pSPORT6™ vector (Life Technologies, Rockville, MD, USA) in which the cloning site is flanked by sequences corresponding to the standard M13, T3, T7 and SP6 sequencing primers. The average insert size in these normalized libraries was approximately 1000 bp, with a range from 100 bp to 4 kb. The individual clones were stored frozen in LB broth supplemented with 10% glycerol and 50 mg/L ampicillin.

All manipulation steps on individual wells were carried out on a Biomek® 2000 robotic workstation (Beckman Coulter, Fullerton, CA, USA) using MP20 and MP200 pipetting heads. Before amplification, plates were defrosted and replicated in a fresh 384-well growth plate (Marsh Biomedical Products, Rochester, NY, USA) containing 50 µL LB broth containing 50 mg/L ampicillin using a 384-pin high-density replicating tool (Beckman Coulter). We found the use of fresh cells, rather than glycerol stock cells, substantially increased the rate of successful amplification. The clones were grown overnight at 37°C while being shaken at 225 rpm, and a 10-µL aliquot transferred to a 384-well PCR plate. Plates were centrifuged at 2000× *g* for 20 min to pellet the cells. The medium was removed by inverting the plates and shaking them. Cells were resuspended in 50 µL 10 mM Tris (pH 8.0) by vortexing, and 1 µL transferred to a 384-well PCR plate containing 10 µL PCR mixture [0.1 mM each dNTP, 0.2 U *Taq* DNA polymerase (Amersham Pharmacia Biotech, Piscataway, NJ, USA)], 1.1 µL 10× *Taq* buffer and 0.5 µM each M13 forward and reverse sequencing primers. Plates were subjected to 30 cycles of amplification (94°C for 30 s, 60°C for 30 s and 72°C for 2 min), followed by centrifugation at 2000× *g* for 10 min to pellet remaining bacterial debris. To remove the remaining amplification primers from the sequencing mixture, a 2.5-µL aliquot of the reaction was transferred to a fresh 384-well sequencing reaction plate (PE Biosystems) containing 0.2 U exonuclease I (New England Biolabs,

Beverly, MA, USA) in 7.5 µL water, mixed and incubated for 1 h at 37°C. The exonuclease was inactivated by incubation at 65°C for 20 min, and the amplification products were precipitated by the addition of 20 µL 100% ethanol and centrifugation at 1700× *g* for 30 min. Residual ethanol was removed by inverting the plate and centrifuging at 50× *g* for 2 min.

Sequencing was initiated by resuspending the amplification products in 5 µL sequencing reaction mixture [0.5 µL BigDye™ Terminator Ready Reaction Mix (PE Biosystems), 1.5 µL half-BD™ sequencing reagent (Sigma, St. Louis, MO, USA) and 3 µL 1 pM SP6 sequencing primer] and subjected to 25 cycles of 96°C for 30 s, 50°C for 1 min and 60°C for 4 min. Sequencing reactions were precipitated by the addition of 20 µL 70% isopropanol with mixing and centrifugation at 1700× *g* for 30 min at room temperature. Residual isopropanol was removed as described above. Pellets were washed with 20 µL 70% ethanol, with another cycle of centrifugation and removal of residual ethanol. Plates were air dried for 5–10 min and stored in a desiccator at -20°C. Immediately before being placed in the loading chamber of the sequencer, 30 µL ultrapure formamide (PE Biosystems) were added, and the plates were spun at 670× *g* for 2 min at room temperature. We did not find it necessary to heat denature the samples before loading them onto the sequencer. Parameters for running the instrument with POP-6 polymer are shown in Table 1.

We found that replacing the growth media with fresh buffer significantly increased the success of the clone amplification step for our normalized libraries. It is likely that this is an effect of the host strain used for normalization of the library because a separate, commercial, non-normalized bovine lymph node library (Stratagene, La Jolla, CA, USA) did not show the same effect and this step was unnecessary. However, the media replacement step is inexpensive and does not significantly increase the time required for the process. The procedure described here has an amplification success rate of approximately 85%–95%, which is not significantly different than the success rate for template preparation by automated mini-

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Table 1. ABI 3700 Running Parameters

Run Temperature	42°C
Cuvette Temperature	30°C
Max Current	800
PreRun Voltage	6000
PreRun Time	300 s
Sample Volume	25 µL
Wash Volume	200 µL
Injection Voltage	2500
Injection Time	30 s
Run Voltage	5250
Sheath Flow Volume	12 000
Sheath Flow Period	942
Run Time	10 000 s

prep and costs substantially less than commercially available automated miniprep kits. After thermocycling, the additional centrifugation step was implemented to reduce the amount of bacterial debris that might be transferred to the sequencing plate and potentially block the capillaries. We have not tested the necessity of this step, but our capillary arrays typically last over 350 runs with no measurable decrease in the sequence quality of the standard provided by PE Biosystems.

The quality of the sequences generated was substantially increased by the use of a nested sequencing primer (which was also true for reaction setups for gel-based systems). The reactions show minimal sensitivity to the variation in template concentration that occurs during the amplification of the clone inserts. A significant cost savings was obtained by reducing the size of the sequencing reaction to 5 µL and the volume of BigDye reagent to 0.5 µL per reaction. In combination with the less expensive halfBD reagent as described, these sequencing reactions produce more than enough product for visualizing on the ABI 3700. The 5-µL reaction volumes were the minimum used for the Biomek 2000, but it is likely that this volume could be successfully reduced even further. A variety of sequence reaction clean-up protocols were tried, including several commercial silica binding matrices and gel fil-

tration columns, but these more expensive techniques had relatively little impact on sequence quality when compared to precipitation combined with washing of the pellet.

Reaction success was dramatically improved by using four volumes of 70% isopropanol for precipitation to a final concentration of 56% compared to the addition of 100% isopropanol to a final concentration of 60%–70%. The use of diluted isopropanol tended to reduce the precipitation of unincorporated dyes, and the lower final concentration decreased the amount of short sequencing product in the pellet. The end result was more uniform signal intensity along the capillary because of a reduction in “front-loading,” an artifact that results from the preferential injection of short fragments and small molecules such as unincorporated nucleotides. Front-loading was also decreased by including the 70% ethanol wash of the pelleted sequence material, which further reduced the amount of salt and dyes in the injection volume. Finally, the greatest success was achieved by resuspending the sequencing reactions in 30 µL formamide immediately before placing the plate on the sequencer because prolonged storage in formamide either at –20°C or at room temperature decreased resolution and resulted in other sequencing artifacts. The use of 30 µL formamide rather than the minimum 10 µL resulted in substantially improved resolution and decreased generation of artifacts when comparing the first and last runs from a 384-well plate. However, decreased resolution was observed whenever the reactions were exposed to formamide for more than 16 h and became equivalent to the resolution observed when using water rather than formamide to resuspend the pellet.

Using this approach, a single robotic workstation, four 384-well thermocyclers and a single technician are sufficient to produce twelve 384-well plates of sequencing reactions, which represents the maximum weekly throughput of the instrument (48 runs or approximately 4800 sequencing reactions, assuming normal instrument downtime for changing the polymer and reagents and clearing the database). This is accomplished during a five-day work week, except that the formamide

addition, brief plate spin and loading onto the instrument are performed on weekends, normally requiring less than 30 min unless a new bottle of polymer must be loaded. Average read lengths are 550 ± 120 bp, with signal intensities of approximately 800–1200 U as defined by the ABI-prism software.

This basic setup has been successful in generating expressed sequence tag data on the coding strand. We have produced approximately 180 000 sequences using this method, with an overall success rate of 75%–80%. This approach does not work for sequencing the noncoding strand using vector primers, presumably because of “slippage” of the polymerase during amplification of the clones, which leads to variation in the length of the polyT region at the end of the clone. However, this limitation can be circumvented using anchored polyT primers to generate 3' end sequence. On a per-sequence basis, the total cost of all materials and reagents roughly equals that of commercially available automated miniprep kits. Therefore, the cost of the sequencing reaction represents the approximate savings over using a PCR-based approach. In summary, the protocol described is a low-cost, efficient and reliable approach to high-throughput sequencing on the ABI 3700.

Mention of product names is necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the same by USDA implies no approval of the product to the exclusion of others that may also be suitable. The authors would like to thank Sherry Kluver for manuscript preparation. Address correspondence to Dr. Timothy P.L. Smith, USDA, ARS, U.S. Meat Animal Research Center, Spur 18D, PO Box 166, Clay Center, NE 68933, USA. e-mail: smith@email.marc.usda.gov

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