Nucleic Acids Research, 1994, Vol. 22, No. 11 2173–2175

High-throughput detergent extraction of M13 subclones for fluorescent DNA sequencing

Elaine R.Mardis
Washington University Genome Sequencing Center, Washington University School of Medicine, Department of Genetics, 4444 Forest Park Boulevard, St Louis, MO 63108, USA

Received April 6, 1994; Accepted May 3, 1994

Large-scale genome sequencing efforts that utilize an M13 shotgun approach, such as the *C.elegans* project, require simple yet reproducible template preparation methods. These templates are subjected to DNA sequencing reactions that produce the data necessary to reconstruct the sequence of a given cosmid. To date, the most reliable template purification method for M13 subclones involves polyethylene glycol (PEG)-aided precipitation of viral particles, followed by phenol extraction to remove coat proteins and yield single-stranded DNA, which is concentrated by ethanol precipitation (1). Several alternative procedures have been developed, including glass fiber filter purification (2), detergent extraction (3,4) and magnetic bead purifications (5–8) but all have suffered from low yield or variable quality. This paper describes a high-throughput modification of the detergent extraction method of Mardis and Roe (4) which is amenable to use with multiple channel pipettors (8 or 12 channels), and eliminates both the phenol extraction and ethanol precipitation steps.

The method described here differs in its use of a nonionic extraction buffer coupled with heating to replace the chaotropic action of phenol in denaturing the M13 protein coat. We use 96-tube boxes (Beckman #20901–019) that hold strips of 12, 1.2 ml tubes (Beckman #20901–017), each filled with 800 μl of JM101 cells diluted in 2×YT growth medium (1:100 dilution of an overnight culture). Clear plaques are picked from transformation plates using sterile toothpicks, each of which is dropped into a single tube of the 96 tube box. When each tube contains a toothpick, all are removed and discarded, and the box is placed, with the lid taped on securely, in a 37°C incubator-shaker. Incubation is for 12–16 hours, with shaking at 300 rpm. Cells are removed from culture by centrifugation at 3500 rpm for 15 minutes in a Beckman GPR centrifuge, using a special 'Microplus' carrier (Beckman #362349).

A second 96 tube box is prepared during centrifugation by placing 120 μl of 20% PEG/2.5 M NaCl solution into each tube using a 12 channel pipettor. Once the centrifugation of M13 cultures is complete, 600 μl of the resulting phage supernatant is added to the PEG-containing tubes. A 96 tube cap (Beckman #267002) is securely placed over all of the tubes and the box is inverted several times to thoroughly mix the two solutions. Precipitation of phage particles occurs during a 15 minute incubation at room temperature and pelleting of phage is achieved by centrifugation of the 96 tube box at 3500 rpm for 15 minutes in the Beckman GPR centrifuge. The resulting supernatant is decanted by inverting the 96 tube box over a sink and the tubes are left, inverted, to drain onto a paper towel for 1–2 minutes. A paper towel is placed inside the lid of the 96 tube box, used to cover the tubes, and the entire box is centrifuged in an inverted position at 200–250 rpm for 2–3 minutes to completely remove the residual PEG from the tube walls. Phage pellets are resuspended by adding 20 μl of Triton-TE extraction buffer (0.5% Triton X-100 (Sigma), 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, pH 8.0) to each tube, covering all tubes with a piece of 3 M silver tape (R.S.Hughes Company, #425–3), and vortexing on a floor model vortexer (VWR #58816–115) for 30–45 seconds with pulsing between vortex speed levels 2 and 6. A brief centrifugation of the 96 tube box may be necessary to bring all of the phage solution down from the tube walls after vortexing. The bottom of the 96 tube box is removed to ensure good contact with the water bath, and to visually inspect the bottoms of the tubes for thorough resuspension of the phage pellet. The 96 tube box then is placed into an 80°C water bath for 10 minutes to achieve phage lysis and again is centrifuged briefly to bring down condensation. The silver tape is removed and the resulting solution is transferred to a Corning 96 well microtiter plate (#25850). Twenty to 40 μl of sterile double-distilled water is added to each well to further dilute the DNA, the amount added depending upon the average size of phage pellets resulting from the PEG precipitation step. The microtiter plate wells are covered by a piece of 3 M silver tape and lastly by the plate lid prior to storage at −20°C.

Cycle sequencing reactions are performed using 1 μl of each Triton-extracted DNA for A and C reactions and 2 μl for G and T reactions, and using Sequi-therm™ DNA Polymerase (Epicentre Technologies, Madison, WI), according to previously published procedures (9, 10). Temperature cycling was performed on the Perkin-Elmer PE9600 thermal cycler using a short cycling procedure developed in our lab (10). Although Sequitherm polymerase gave excellent results in terms of read length and fragment length uniformity, AmpliTaq™ polymerase did not yield good results with Triton-extracted M13 DNA in fluorescent primer cycle sequencing reactions. In an attempt to improve the AmpliTaq results, reduced amounts of Triton were tested in the M13 preparation. Although these reduced Triton percentages did not yield readable sequence trace data for AmpliTaq-catalyzed reactions, Figure 1 compares the Sequitherm generated fluorescent sequencing data resulting from M13 subclones purified using 0.5%, 0.25% and 0.1% Triton X-100. In addition, the substitution of other nonionic detergents such as NP-40 and Tween 20 at similar concentrations, have produced DNA that exhibits excellent sequence reaction data with Sequitherm, but not with AmpliTaq polymerase. Figure 2 shows

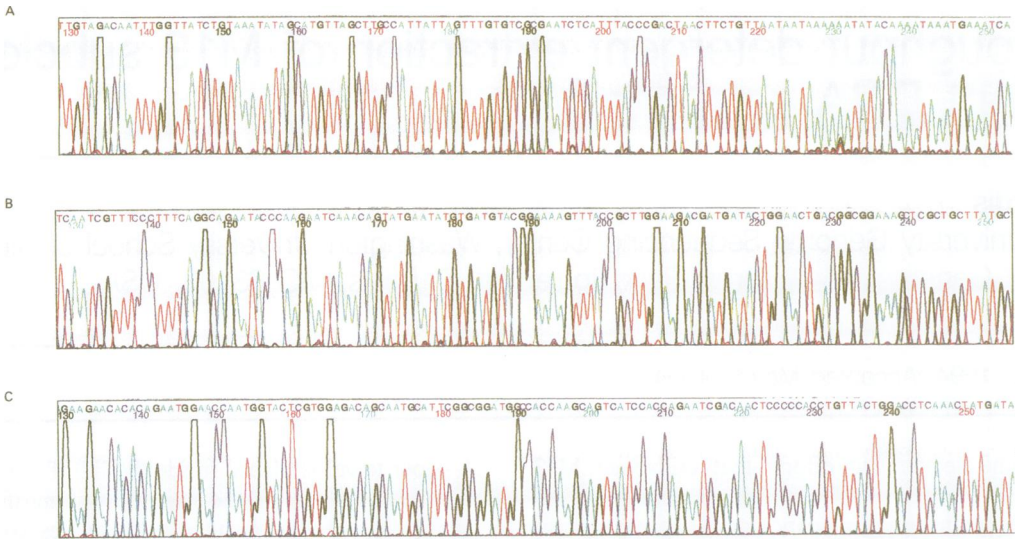

Figure 1. Representative sequence patterns derived from *C.elegans* M13 subclones purified with the method described in the text using **a)** 0.5% Triton-TE buffer, **b)** 0.25% Triton-TE buffer, **c)** 0.1% Triton-TE buffer. All sequencing reactions were performed as described. Electrophoresis and fragment separation were achieved by 5.6% polyacrylamide/7.5 M urea gels with 1×TBE buffer run at 30 W (constant power) on the ABI 373A DNA Sequencer.

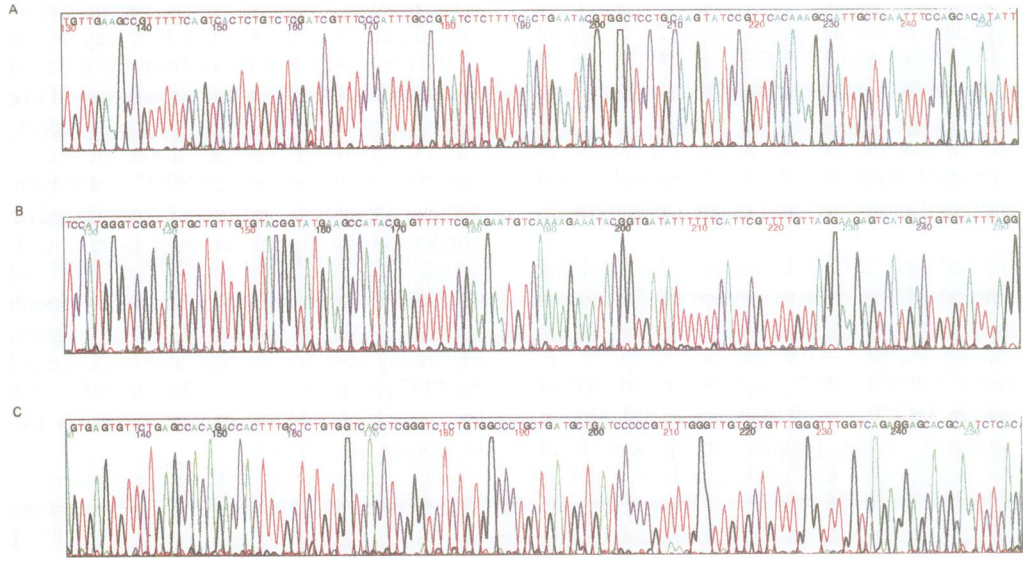

Figure 2. Sequence patterns derived from **a)** *C.elegans*, **b)** yeast and **c)** human M13 subclones purified using the method described in the text. Sequence fragments were produced by cycled sequencing reactions using Sequi-therm™ DNA polymerase, as described elsewhere (10). Electrophoresis and detection were on the ABI 373A DNA Sequencer as described in Figure 1.

trace data from the ABI 373A fluorescent sequencing instrument, for *C.elegans*, yeast and human M13 subclones purified using this method.

Currently, our production sequencing groups use the above procedure to produce up to 5000 M13 subclones per week, with 75–80% of the sequenced clones entering the database (about 10% of the remaining 20–25% includes removed M13 and cosmid vector sequences) using a modified database entry program (S.Dear, R.Staden and L.Hillier, unpublished). The success rate of database entry, template purity and sequence read length are all similar to what we have experienced using the PEG–phenol extraction method, while the thermal extraction method is more rapid and less labor-intensive. Thus, we have virtually eliminated the use of phenol for M13 template preparation, as well as the need to ethanol precipitate or wash the resulting DNA.

ACKNOWLEDGEMENTS

The author wishes to thank Dr Robert Waterston, Dr Richard Wilson and Dr Jennifer Couch for their critical reading of the manuscript, Lori Weinstock for technical assistance, and Drs

Ellson Chen and Massimo Zollo for their input regarding use of the 96 tube boxes for phage growth and DNA preparation (*BioTechniques* **16**, 370–372).

REFERENCES

1. Messing,J. (1983) *Methods Enzymol.* **101**, 20–78.
2. Zimmerman,J., Voss,H., Kristensen,T., Schwager,C., Stegeman,J., Erfle,H. and Ansorge,W. (1989) *Methods Mol. Cell. Biol.* **1**, 29–34.
3. Eperon,I.C. (1986) *Anal. Biochem.* **156**, 406–412.
4. Mardis,E.R. and Roe,B.A. (1989) *BioTechniques* **7**, 840–850.
5. Alderton,R.P., Eccleston,L.M., Howe,R.P., Read,C.A., Reeve,M.A. and Beck,S. (1992) *Anal. Biochem.* **201**, 166–169.
6. Fry,G., Lachenmeier,E., Mayrand,E., Giusti,B., Fisher,J., Johnston-Dow,L., Cathcart,R., Finne,E. and Kilaas,L. (1992) *BioTechniques* **13**, 124–131.
7. Hawkins,T. (1992) *DNA Seq.* **3**, 65–69.
8. Wilson,R.K. (1993) *BioTechniques* **15**, 414–422.
9. Halloran,N., Du,Z. and Wilson,R.K. (1992) In Griffin,H.G. and Griffin,A.M. (eds), *Methods in Molecular Biology. DNA Sequencing: Laboratory Protocols*. The Humana Press, Clifton, NJ., Vol. 10, pp. 297–315.
10. Fulton,L. and Wilson,R.K. Manuscript submitted.