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Method for 96-Well M13 DNA Template Preparations for Large-Scale Sequencing

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ABSTRACT

Efficient preparation of DNA templates is an important step in large-scale DNA sequencing. To ensure high-quality sequence data, we have prepared M13 phage DNA templates using a glass fiber-filtration method. We present the adaptation of this protocol to a 96-well format using commercially available filter plates. Two variations are described: one using polyethylene glycol precipitation and a second where the phage particles are disrupted before filtration, thus eliminating the need for precipitation. Using either of these protocols, 96

templates can be prepared in less than 2 h. Sufficient DNA for 1–2 dye primer sequencing reactions is routinely obtained from 1 mL of culture, and the resulting sequence data are of high quality.

INTRODUCTION

“Shotgun” methods have been favored in many laboratories for large-scale DNA sequencing, primarily because of the suitability of the procedure for automation. A rate-limiting step has been DNA template preparation, and several attempts have been made to design reliable protocols with high throughput. We have consistently utilized the M13 filamentous phage as the sequencing vector because of the superior quality of the resulting data. While manual preparation of M13 templates is straightforward and reliable, previous attempts at developing a high-throughput format were cumbersome, unreli-

able or produced impure DNA that limited the options for the subsequent sequencing.

Several M13 template preparation methods have been based on the protocol of Eperon (5), who replaced phenol extraction of precipitated phage particles (13,19) with phage lysis using detergent and heating followed by ethanol-precipitation to obtain pure phage DNA. Subsequent variations used a 96-well format and, in some, alternative chaotropic agents and magnetic particles (1,6,8,12,16,17). In general, this group of methods is attractive because it is inexpensive, but it is currently not widely used for large-scale sequencing. Attempts have been made to increase the template preparation throughput by directly using the phage lysates for sequencing after dilution (3,7,11). These protocols are very rapid and efficient, but the resulting DNA is distinguished by low purity, since

sequence is reliably generated only by certain DNA polymerases (11).

Kristensen et al. (10) described M13 DNA purification by immobilization of acetic acid-precipitated phage particles on glass fiber filters, followed by washing with a chaotropic agent to remove phage proteins and treatment with 70% ethanol to precipitate and purify the M13 DNA. An advantage of this approach was the relatively few transfer steps involved, and it was shown to be automatable in Reference 18. The procedure was proposed to be improved by adaption to a 96-well format (10). Previously in our laboratory, M13 templates were prepared using a modification of this protocol in a 24-well aluminum block. This procedure, although too slow for the high throughput required in genome scale sequencing, reliably produced DNA of high quality.

Approximately 500 kb of human sequence has been determined using these templates. Kolner et al. (9) described a related procedure using liquid lysis of the phage particles and washing them in a 96-well format using a cell harvester.

In this study, we present two protocols that utilize glass fiber-filter capture for preparation of M13 templates entirely in a 96-well format. To minimize the labor required, we have used commercially available 96-well filter plates. One protocol utilizes polyethylene glycol (PEG) precipitation of phage particles, while the second uses liquid lysis of phage particles followed by direct capture on the filter. These methods reliably produce M13 DNA of sufficient quality for *Taq* cycle sequencing (4), and they contain fewer steps and less handling than previous protocols.

Figure 1. Outline of the M13 template DNA preparation protocols presented in this study. The arrow between each step indicates a transfer to a new 96-well plate. The first two steps are carried out in 96-deep-well plates, while step III is carried out in a filter plate on a vacuum manifold, and in step IV the M13 DNA is eluted into any 96-well format plate.

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MATERIALS AND METHODS

Vectors and Cell Lines

Cosmids were obtained from the human X chromosome flow-sorted library prepared at Lawrence Livermore National Laboratories (Livermore, CA, USA). M13mp19 RF DNA was obtained from Pharmacia Biotech (Piscataway, NJ, USA). *E. coli* TG1 cells were obtained from Amersham (Arlington Heights, IL, USA), and supercompetent *E. coli* XL-1 blue cells were obtained from Stratagene (La Jolla, CA, USA).

M13 Cultures

M13 libraries were prepared using a previously described uracil DNA glycosylase (UDG) cloning-based method (2). Plaques were picked into 4 mL 2× YT medium in a 15-mL microcentrifuge tube with 20 µL of an overnight TG1 culture added. The cultures were incubated for 6–8 h at 37°C with vigorous shaking. The cultures were stored at 4°C until DNA preparation, and a portion of each culture was transferred to a 1.5-mL microcentrifuge tube for long-term storage at 4°C.

M13 Template Preparations

Two milliliters of each M13 culture were transferred to each well of a 96-square, deep-well titer plate (Part No. 140504; Beckman Instruments, Fullerton, CA, USA), which was centrifuged at 3000 rpm (1750× *g* in a Model GR422 centrifuge using an M4 swing-out rotor; Jouan, Winchester, VA, USA) for 15 min to remove cells and debris. Approximately 800 µL of each supernatant were transferred to a 96-deep-well titer plate (Part No. 267007; Beckman Instruments) using an 8-channel pipet (impact, 15–1250 µL; Matrix, Lowell, MA, USA). In Protocol 1, where PEG precipitation was used, 125 µL 20% PEG 8000 were added to each well.

Each sample was mixed using a multichannel pipet and incubated at room temperature for 20 min. The precipitated phage was pelleted by centrifugation for 20 min at 3000 rpm. After removal of all but approximately 50 µL of the supernatants, the phage pel-

lets were resuspended using an 8-channel pipet and transferred to a 96-well, glass fiber-filter Type C plate (Multi-Screen®-BC, Model MABCN1210; Millipore, Bedford, MA, USA). In Protocol 2, 300 µL of 8 M NaClO₄ were added to 1 mL of phage supernatant after removal of the cells, and the samples were mixed using a multichannel pipet. After mixing, the plate was incubated for 15 min at 65°C followed by transfer of the entire volume to the 96-well, glass fiber-filter plate.

In both protocols, vacuum was applied to the filter plate using a Model MAVM 096.01 vacuum manifold (Millipore) until all the fluid was drained from the filters. In Protocol 2, the flow rate was kept relatively slow during the transfer of the NaClO₄-treated phage supernatant. In both protocols, the filters were washed with 3 mL of 4 M NaClO₄ in TE buffer (10 mM Tris, pH 8.0, 0.1 mM EDTA) per well using an 8-channel pipet to add 3–400 µL of solution at a time, under continuous vacuum. Next, the filters were washed with 5–10 mL of 75% ethanol. After this, the filter plate was taken off the vacuum manifold, and the excess ethanol was immediately removed by replacing the filter plate on a 96-well microplate and centrifuging for 10 min at 3000 rpm, followed by drying in a vacuum chamber for 1 min. The DNA was eluted by addition of 35 µL of TE buffer per well, followed by 10-min centrifugation at 3000 rpm over a clean microplate. One microliter of the eluate was subsequently analyzed using agarose gel electrophoresis to quantitate the DNA (14).

DNA Sequencing and Sequence Assembly

DNA sequencing was performed using the fluorescent automated approach (15) and Applied Biosystems DNA sequencers (Perkin-Elmer/Applied Biosystems Division [PE/ABI], Foster City, CA, USA). Editing and assembly of the sequence reads were performed using a combination of software, including the Staden XDAP package, Sequencher™ (GeneCodes, Ann Arbor, MI, USA), and software developed at the Molecular Biology Computational Resource Center at Baylor College of Medicine, Houston, TX, USA.

RESULTS

The 96-well protocols described here (Figure 1) were compared with the results from using the modified Kristensen et al. (10) glass fiber-filter method described above. Overall, both Protocols 1 and 2 provided equal or better performance, provided that care was taken with particular steps.

In the filter plates, the glass fiber-filters are supported by a porous polyvinylidene difluoride (PVDF) membrane. These filters are very consistent in quality, and they do not burst or leak when vacuum is applied. However, they did initially show low flow rate, which reduced the efficiency of the method. These plates have now been improved by the manufacturer by using PVDF membranes with bigger pore size, which allows a flow rate suitable for efficient washing of the M13 DNA.

Figure 2. Agarose gel electrophoresis of 14 single-stranded M13 templates prepared using 96-well glass fiber filtration (Protocol 1). Approximately 1/30 of each preparation was electrophoresed. All clones except those in lanes 10 and 11 contain 1.5–2.5-kb inserts. The lower molecular weight bands seen contain bacterial RNA, which has been found to not disturb the sequencing reactions.

There was little variation between individual wells using these filter plates. When a series of preparations were performed from the same M13 culture, there was very little variation in yield, indicating that the filters are uniform in quality.

The most time-consuming parts of Protocol 1 were the initial steps. To make this part of the procedure less cumbersome, Protocol 2 was developed, where the PEG precipitation of phage and centrifugation to pellet the precipitate were replaced by the liquid lysis procedure (9). In this procedure, the *E. coli* cells are removed by centrifugation as before, the supernatants transferred to a second deep-well plate and the phage particles lysed by addition of 8 M NaClO₄. The lysates are immediately transferred to the 96-well filter plate using a multichannel pipet. The DNA yields from this procedure were equivalent to the yields using Protocol 1. The yield from 1 mL of culture was routinely 3–5 µg of DNA, which is sufficient for 1–2 reactions using dye primer and 2–4 reactions using dye terminators in *Taq* cycle sequencing. Agarose gel electrophoresis of DNA preparations obtained using Protocol 1 is shown in Figure 2.

Several factors of importance for maintaining high-quality sequence data were investigated. The step of loading the precipitated or lysed phage onto the filter has been found to be particularly important. In Protocol 1, the phage pellet should not be resuspended too vigorously, since this can result in some of

the phage being lost through the filters. Similarly, in Protocol 2, the lysed phage mixture should be pulled through the filter slowly to ensure that a high proportion of the DNA binds to the glass fiber filters.

It was also found that in Protocol 2 it was necessary to wash the filters with 4 M NaClO₄, in spite of the NaClO₄ treatment before filtration, and omission of this step resulted in reduced sequence quality. The concentration of the ethanol solution used for washing the filters was increased from 70% to 75% because of loss of DNA observed from some preparations using the 70% solution. The volume of washes with 75% ethanol was found to be crucial for sequence quality. Usually, 5 mL per well were sufficient for both protocols. However, to ensure high-quality sequence data from all samples, we routinely use 7 mL of 75% ethanol per well.

In Protocol 2, it was found that a 10-min incubation at 65°C immediately following the addition of the 8 M NaClO₄ resulted in more consistent DNA yields. No effect was found in either protocol from changing the conditions of the elution of the template DNA from the glass fiber filter by introducing an incubation step, by changing the temperature of the solution added or by changing the time of the centrifugation to transfer the dissolved DNA to a microplate. The best elution conditions were to add TE buffer at room temperature, followed by immediate centrifugation for 10 min.

Figure 3. Chromatogram showing the quality of the sequence data obtained from M13 templates prepared using the 96-well, glass fiber-filter protocols presented in this study. Bases 436–460 are shown, from a *Taq* dye-primer reaction analyzed using an Applied Biosystems Model 373 Stretch sequencer (PE/ABI).

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A preparation of 96 M13 templates can be prepared in this way in less than 2 h. The preparation times per template can be shortened further by using up to four glass fiber-filter plates simultaneously, which is practical due to the configuration of the centrifuge rotors used. In this way, a single technician can prepare between 768 and 1152 templates in a single workday.

DISCUSSION

This study describes the development of methods for M13 template preparation that utilize 96-well, glass fiber-filter plates. The plates used were shown to be reliable and even in quality, also between individual wells in a plate. In the modified plates, the washing flow rate could be set to achieve efficient DNA purification.

The most important single factor de-

termining the quality of the sequence from templates prepared by these protocols, using standard *Taq* dye primer cycle sequencing, was found to be the extent of washing of the DNA on the filters with 75% ethanol. A low wash volume tends to result in higher background in the sequencing reactions. We routinely wash with 7 mL of 75% ethanol, which ensures high-quality sequence data from all samples (Figure 3).

These methods compare favorably to previously described protocols. Since the DNA is immobilized on filters, it can be easily purified by washing with relatively large volumes. The method described by Kolner et al. (9) using the same chemistry as Protocol 2, has the advantage that the washes are more rapid, but it has the disadvantage that the drying of the filters and elution of the DNA requires a transfer step that is not needed in the current protocols. Also compared with previous protocols

using thermal lysis and precipitation of DNA or magnetic beads for purification (1,5,6,8,12,16,17), which require several purification steps and transfers, the current method is at least equivalent or more efficient. The current methods also compare favorably to the more efficient thermal preparation protocols using the DNA directly after phage lysis (3,7,11) because of the greater flexibility in the use of sequencing reagents of the glass fiber-filter preparations.

Both Protocols 1 and 2 work well. Protocol 2 is faster, since PEG precipitation of phage particles is eliminated, which saves centrifugation and removal of the supernatants. While PEG precipitation of M13 phage and pelleting of the precipitate is a very robust procedure, liquid lysis of the phage particles is slightly more sensitive and needs to be performed carefully. In addition, more care needs to be taken while transferring the lysed phage suspension

to the glass fiber filters compared to the PEG-precipitated phage particles. Therefore, Protocol 1 is probably the more robust one, at least in the hands of inexperienced users.

In conclusion, the glass fiber-filter protocols for purification of high-quality M13 DNA templates presented here have relatively few steps, and they are performed entirely in a 96-well format with few transfers. This makes these methods highly suitable for use in high-throughput sequencing.

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