An automated method for DNA preparation from thousands of YAC clones

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ABSTRACT

We describe an automated method for the preparation of yeast genomic DNA capable of preparing thousands of DNAs in parallel from a YAC library. Briefly, the protocol involves four steps: (1) Yeast clones are grown in the wells of 96-well microtiter plates with filter (rather than plastic) well-bottoms, which are embedded in solid growth media; (2) These yeast cultures are resuspended and their concentrations determined by optical density measurement; (3) Equal numbers of cells from each well are embedded in low-melting temperature agarose blocks in fresh 96-well plates, again with filter bottoms; and (4) DNA is prepared in the agarose blocks by a protocol similar to that used for preparing DNA for pulsed-field gels, with the reagents being dialyzed through the (filter) bottoms of the microtiter plate. The DNA produced by this method is suitable for pulsed-field gel electrophoresis, for restriction enzyme digestion, and for the polymerase chain reaction (PCR). Using this protocol, we produced 3000 YAC strain DNAs in three weeks. This automated procedure should be extremely useful in many genomic mapping projects.

INTRODUCTION

Yeast artificial chromosomes (YACs) are rapidly becoming an essential tool in the genomic analysis of eukaryotic genomes, including in such applications as PCR screening, restriction enzyme digestion and fingerprinting, pulsed-field gel electrophoresis, or library hybridization. Although it is straightforward to prepare DNA from a small number of YAC clones, the analysis of a mammalian genome will typically require the study of 50,000 YAC clones. Such applications require a YAC DNA preparation method with a much higher throughput. Here we describe the first automated, high-throughput method for YAC-strain DNA preparation.

To develop a method that has high throughput and that is economical with respect to both effort and cost, we made a number of fundamental choices: To facilitate handling of large numbers of clones, the protocol uses 96-well microtiter plates. To minimize effort and maximize accuracy, the protocol employs

the Biomek 1000, a common laboratory workstation. To avoid the need for phase separations and centrifugations in the DNA extraction, the protocol involves embedding cells in agarose blocks and dialyzing the blocks against various reagents—as is done for preparing very high molecular weight DNA for pulsed-field gels.

Although the DNA preparation method is slow (requiring almost two weeks from start to finish), the procedure can be easily applied to large numbers of microtiter plates—making it possible to prepare thousands of YAC DNAs simultaneously. The method is also quite economical, requiring only a few inexpensive and easily constructed apparatuses in addition to off-the-shelf supplies and equipment.

MATERIALS AND METHODS

Overview of Method

The method involves two fundamental steps: (i) growing yeast to sufficiently high density in microtiter plates and (ii) extracting DNA from the yeast in microtiter plates. For both steps, a key component is the use of microtiter plates with 0.22 μ m membranes sealed to the bottoms of the wells (rather than having plastic well bottoms).

(i) Growing yeast in standard (plastic bottom) microtiter plates produces too few cells for practical purposes. YAC clones grown in 150 μ l liquid culture in 96-well plates typically yielded only 10^7 cells (less than 0.2 μ g DNA) per well—presumably due to insufficient nutrients. We therefore designed a procedure in which YAC clones were grown in the wells of a filter-bottom 96-well plate embedded in solid growth media (Fig. 1). The filter bottom on each well allows the yeast access to nutrients outside the well, but contains the cells within the well. Using these plates, we are able to achieve a ten-fold higher yeast density compared to standard 96-well plates. The 3 μ g of DNA produced from each clone is sufficient for multiple experiments.

(ii) The DNA preparation procedure was a modification of a standard agarose block protocol used for preparing DNA for pulsed-field gels (1). Because this protocol involves dialyzing reagents into and out of agarose blocks, it avoids the phase separations and centrifugations involved in most DNA preparation procedures. Using the Biomek, the agarose blocks are cast in

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filter bottom microtiter plates. Because these filters are porous, many plates can be dialyzed in parallel by placing them into a simple flooding chamber (Fig. 2). As in the standard agarose block DNA preparation protocol, the resulting DNA is very clean because low molecular weight impurities are removed by extensive dialysis.

The protocol is diagrammed in Fig. 3. The Biomek performs all of the pipetting operations through the cell embedding step; the later incubation steps are performed in a custom flooding chamber that supports four 96-well plates in a pool of the appropriate solution (Fig. 2).

Briefly, the protocol proceeds as follows: Initially, YAC clones are stored as frozen stocks in a 96-well plate. These are used to inoculate 96-well liquid cultures. The liquid cultures are then used to inoculate the solid growth plates. (These two phases of growth lead to substantially more uniform yield.) Once the solid plates have grown up, the cells are resuspended and a small aliquot is transferred from each well to a second microtiter plate for optical density (OD) reading. An automated reader records the OD readings on a diskette; a computer program then uses this data to calculate the volume of cell suspension that contains 1.5×10^8 cells (approximately 3 μg DNA). This program then modifies the Biomek program so that it will transfer the appropriate volume of cell suspension out of each well and into the block-forming plate (a fresh 96-well filter-bottom plate). This plate is briefly centrifuged to remove the liquid, the cells are resuspended in a zymolyase/EDTA solution, and an equal volume of low-gelling-temperature agarose solution is added. After mixing, the newly formed blocks are allowed to solidify. These blocks are then processed as in the standard agarose block DNA preparation protocol: they are incubated in a betamercapto-ethanol/EDTA solution to produce spheroplasts, then in a Proteinase K/Sarkosyl solution to lyse the cells and degrade proteins, and finally in PMSF and TE solutions to inactivate the Proteinase K and prepare the blocks for use.

For pulsed-field gel electrophoresis, the DNAs can be used at this point (or even without PMSF treatment) after normal equilibration with electrophoresis buffer. For restriction enzyme digestion, the agarose blocks are melted and digested with restriction enzyme, ribonuclease and agarase. After incubation, the digested DNA solution is free of contaminating large RNA molecules and is in liquid at room temperature (because of the agarase digestion).

Figure 1. Diagram of a solid growth plate. The plate consists of a Multiscreen 96-well filter-bottom plate (with flexible base removed) embedded in solid growth media in the lid from a 96-tip 'yellow tip' rack. The media covers the bottom 1/4 of each well. The notch in the base can be used to add small amounts of liquid media during incubation.

A complete protocol is given below.

High Throughput Protocol—YAC Clone Growth

The clones are all manipulated in the 96-well plate format. Long-term storage of clones is at -70°C in freezing media ($1 \times$ growth media, 15% glycerol) in 96-well plates ($1 \times$ growth media = $1 \times$ yeast ura- and trp- dropout media with 3.5% glucose prepared essentially as described in (2)). Our clones consisted of mouse DNA cloned into the pYAC4 vector in the AB1380 yeast strain (3).

Growth plates are inoculated in a two-step process: 1) (liquid growth plates) $10\mu\text{l}$ of thawed frozen stock of each clone is inoculated by Biomek 1000 (Beckman, Palo Alto, CA) into $140\mu\text{l}$ of $1.5 \times$ growth media in a 96-well flat-bottom plate, and incubated four days in a humidified 30°C incubator; 2) (solid growth plates) $20\mu\text{l}$ of the resuspended liquid growth plate culture is inoculated into a solid growth plate containing $30\mu\text{l}$ of $1.5 \times$ growth media in its wells, again using the Biomek. This plate is then incubated four to five days in the 30°C incubator.

A solid growth plate (Fig. 1) consists of the lid from a 96-tip 'yellow tip' $200\mu\text{l}$ pipette tip rack (USA Scientific Plastics, Ocala, FL), filled with about 135ml of $1.5 \times$ growth media and 0.7% SeaKem GTG agarose (FMC, Rockland, ME), and with a filter-bottom 96-well plate on top (Multiscreen GV 0.22 μ filter-bottom 96-well plate; Millipore, Bedford, MA), with its flexible base removed, embedded in the media to a depth of 1/4 the well depth. Prior to embedding the Multiscreen plate in the molten growth media, it is removed from its flexible base and shaken in a tray of $1.5 \times$ growth media in order to thoroughly wet the bottom of the plate. If necessary, during incubation, $1.5 \times$ growth media should be added on top of the solid growth media to maintain contact between the filter bottoms and the media. The Multiscreen plates used for yeast growth were sterilized by 0.5 Mrad of gamma-irradiation. A simple plastic carrier (Owl Scientific Plastics, Cambridge, MA) allows the Biomek to manipulate the solid growth plate.

High Throughput Protocol—DNA Preparation

Agarose Block Formation

After growth, the Biomek adds $100\mu\text{l}$ of 50mM EDTA, pH 7.5 to each well. The Biomek then resuspends the cells and transfers a $2\mu\text{l}$ sample to a 96-well plate (containing $198\mu\text{l}$ water in each well) for optical density reading (OD measurements were performed on an MR600 microplate reader attached to an IBM PC; Dynatech, Alexandria, VA). Based on the OD₅₇₀ reading, the Biomek transfers a volume of cells containing 1.5×10^8 cells to a fresh Multiscreen plate (the block-forming plate).

We use an automatic microtiter plate reader to read the OD of the wells, leaving well H12 empty as a blank for the reader. The data is collected by a computer. A computer program then

Figure 2. Diagram of a 'flooding chamber.' The solution treatment steps of the DNA preparation protocol are more easily performed by dialysis. The flooding chamber is filled with 325 mls to immerse the wells of the plates half way in liquid. Ports allow easy drainage. The internal dimensions are 11.3 by 33.2 cm with an internal width of 12.5 cm above the ledge.

modifies the Biomek program to transfer the appropriate fixed volume for each well.

The sole centrifugation step in the protocol is then used to remove all the liquid from the cells in the block-forming plate: 1000 rpm 1.3 minutes in a Beckman JS4.2 rotor with microtiter plate carriers. Using the Biomek, we then add 20 μ l of zymolyase/EDTA solution to each well (5 μ l of 3 mg/ml zymolyase 100T (Seikagaku America, St. Petersburg, FL) in zymolyase buffer + 15 μ l of 50mM EDTA, pH 7.5; zymolyase buffer = 1M sorbitol, 100mM NaCitrate, 60mM EDTA pH5.6). The plate is warmed to 40°C on the Biomek (5 minutes using the Biomek Heating-Cooling Block) and 20 μ l of agarose solution is added; each well is mixed thoroughly, and the plate kept at 4°C for 10 minutes to solidify the agarose (agarose solution = 2% InCert agarose (FMC) in 125mM EDTA, pH 7.5).

Agarose Block Processing

The block plates are immersed in 50mM EDTA, pH7.5, 10% betamercaptoethanol at 37°C for 48 hours. They are then immersed in 1 mg/ml Proteinase K (Boehringer Mannheim, Indianapolis, IN), 450mM EDTA pH 9, 1% Sarkosyl for 24 hours at 50°C. The Proteinase K solution is replaced, and the plates are again incubated 24 hours at 50°C. The DNA preparation is then complete.

In order to prepare the DNA blocks for further use, Proteinase K is inactivated by immersing the block trays in 40 μ g/ml PMSF

in TE, pH 7.6, at 50°C for 90 minutes. After 15 minutes at 4°C, the PMSF treatment is repeated. Finally, after two immersions in TE at 4°C for 3 hours and one immersion overnight, the DNA blocks are ready for further manipulation.

We perform these block processing steps in simple 'flooding chambers' (Fig. 2) that contain four plates at a time. Successive processing solutions can easily be poured into the chamber and onto the plates so that they can dialyze through the well bottoms, and later drain through the side ports.

High Throughput Protocol—Restriction Enzyme Digestion

When preparing the DNA for digestion, we liquify the blocks by heating them to 68°C. Since the blocks are still in the filter-bottom wells, we first seal the bottoms of the wells by embedding the whole plate in molten paraffin (AC-7015; Anachemia, Rouses Point, NY) on an aluminum carrier plate (a plate-sized thin block of aluminum with 96 shallow machined wells to hold the bottoms of the plate wells; Owl Scientific Plastics), then cooling to solidify the paraffin. The paraffin melts and resolidifies during the block melting step, but does not infiltrate the wells. The digestion is described in more detail below.

Restriction enzyme (RE) digestion with *Hind*III (20U/ μ l, New England Biolabs (NEB), Beverly, MA) or *Ban*I (20U/ μ l, NEB) was performed as follows: the block forming plate wells were sealed on the bottom by embedding the plate in molten paraffin at 58°C and cooling. Digestion was then performed by removing all but 55 μ l of liquid from each well, adding 6.6 μ l of 10 $\times$ RE buffer (10 $\times$ RE buffer = 250mM NaCl, 250mM Tris-HCl pH 7.6, 100mM MgCl₂, 100mM dithiothreitol, 10mM spermidine) and melting the blocks and solution at 68°C for 10 minutes. The plate was then cooled to 40°C (so that the agarose remained liquid) and 3 μ l of enzyme solution was added (enzyme solution = 1 μ l RE, 1 μ l 2.5U/ μ l agarase (Sigma, St. Louis, MO), 1 μ l 1mg/ml RNase A (Sigma)). The plate was digested 18 hours at 37°C in a humidified incubator. After digestion, internal marker DNA and sample loading buffer were added before loading the samples onto the gel.

Figure 3. Diagram of DNA preparation protocol.

Figure 4. Yeast growth in solid growth plates. Two solid growth plates were inoculated directly from the same frozen stock plate and grown according to our protocol. After growth, the yeast were resuspended, and the total number of yeast in each well calculated on the basis of OD measurements.

Figure 5. *Hind*III-digested YAC DNA. A single solid growth plate was inoculated from a frozen stock plate and its DNA was prepared and digested according to the protocol given in the text. *A*: One Southern blot containing 29 DNA samples from the plate was hybridized with a YAC vector, as well as an internal marker. The YAC vector probe should produce a single band for each clone, the size varying due to the presence of unknown insert sequence. The migration distances of external size standards are indicated along the side of the figure; the very light common top and bottom bands in the YAC lanes are internal markers with sizes 36 kb and 434 bp, respectively. The wells are at the top of the figure. *B*: The same Southern blot was then reprobbed with a yeast *CUP1* probe specific for yeast genomic DNA.

Characterization of YAC-Strain DNA—Agarose Gel Electrophoresis, Blotting, and Hybridization

To characterize the YAC strain DNA prepared with our method, we digested the DNAs with *Hind*III as described above, added internal markers (which result in 36kb and 434bp bands when probed with an Adenovirus-2 fragment), electrophoresed the digests on a standard agarose gel, and transferred the DNA from gels to filters. We probed these filters with both a yeast genome-specific probe and a YAC vector-specific probe in order to evaluate the quantity and quality of the DNA.

Samples were electrophoresed on a 0.8% ME agarose gel (FMC) in $0.5 \times$ TBE buffer at 1.65 V/cm for 20 hours. The gels were acid-nicked and denatured (4), then pressure-blotted in 0.4 N NaOH for 60 minutes at 75mm Hg using a Posiblot (Stratagene, La Jolla, CA) onto Genescreen Plus membranes (DuPont/NEN, Boston, MA), neutralized, UV crosslinked using Stratalinker autolink setting (Stratagene), and hybridized as described in (5). Probes were labelled by random-primed labelling (6) and used at 7.5×10^5 dpm/ml Church's hybridization solution. The internal marker probe was the Adenovirus-2 434 bp *Nae*I fragment. The pYAC4 vector probe was the 346 bp *Bam*HI/*Hind*III fragment of YIP5. The yeast genomic probe was three 1.9 kb *CUP1 Kpn*I fragments of the pJW6 genomic clone (7). Filters were washed four times for 15 minutes at 65°C in

$5 \times$ SSC/0.1% SDS and exposed as described previously (8), or quantitated on a Betascope 603 (Betagen, Waltham, MA).

Characterization of YAC-Strain DNA—CHEF Electrophoresis and PCR

In order to further evaluate the quality of the YAC strain DNA, we electrophoresed undigested DNA on a pulsed-field gel system to verify the presence of intact chromosomes. We also performed PCR on eight *Hind*III digested DNA samples with internal YAC vector primers to verify the suitability of this DNA for PCR.

Pulsed-field gel electrophoresis was performed as described (9) for 28 hours in a 6 V/cm field with a ramped 15 to 65 second reorientation time (0.05 second increment) using a 0.8% Seakem ME agarose (FMC) gel. Lambda concatemer size standards were purchased from FMC. The gel was blotted and hybridized as above, except that the standard capillary transfer technique was used for the blotting itself.

PCR was performed as described (10, 11) for 30 cycles with a 50°C annealing temperature, using 1/15 of a DNA block digested with *Hind*III, agarase, and RNase as above. The primers used (pYACprox-1 = 5' cag gcc tca acg gcc atg gcg acc aca ccc gtc c 3' and pYACinv-1 = 5' tag gcc ccg ccg gcc acc aac ttg gct acc gag ag 3') amplify a 194 bp fragment of the pYAC4 vector flanking the cloning site on the centromeric side.

Figure 6. CHEF gel electrophoresis of YAC DNA. 7 YAC DNAs produced by our method were electrophoresed on a CHEF gel as described in the text. *A*: Ethidium bromide-stained gel. The left lane contains lambda concatemer size standards. The next 7 lanes show DNA from 7 different YAC clones; from left to right, the approximate sizes of the YAC chromosomes are 245 kb, 145 kb, 135 kb, 200 kb, 150 kb, 150 kb, and 100 kb. The YAC chromosome in the first clone comigrates with a host chromosome, as verified by the YAC vector probing of the same gel (*B*).

RESULTS AND DISCUSSION

DNA Preparation

We reasoned that it would be most efficient to process clones in 96-sample plates, using a protocol which allowed processing many plates in parallel. We also chose to use an easily available laboratory robot, the Beckman Biomek 1000, to allow rapid and reliable pipetting. Since the Biomek 1000 cannot perform centrifugation and does not perform phase separations well, we selected a modified agarose block DNA preparation protocol which works by dialyzing reagents into and out of the plates. Our protocol thus avoids phase separations and contains only a single short centrifugation.

Using our protocol, a single DNA preparation takes almost two weeks. However, the effort expended per clone is small; after the agarose blocks are formed, it is in fact trivial. The inoculation and growth phases each require approximately 15 minutes of work per plate, block forming requires approximately 1 hour of work per plate, and block processing requires about 10 minutes of work per plate. Thus, it is possible to process 30 plates in parallel with this protocol (with the block forming step performed over two days). To demonstrate the feasibility and practicality of this protocol, we used it to prepare approximately 3000 YAC DNAs in 3 weeks of part-time work. We now describe the results of experiments testing the quantity and quality of the DNA produced by our method.

Yeast Growth, DNA Yield and DNA Quality

The growth of YAC clones in our solid growth plates is reproducible and uniform. Fig. 4 shows the number of yeast in each well of two solid growth plates (as calculated on the basis of OD readings). Although the two plates each contain the same 96 YAC clones in the same positions, they were separately inoculated directly from a frozen stock plate. Every well contains at least 1.5×10^8 cells, and almost all contain no more than twice that number of cells.

The yield of DNA produced by our protocol is also reproducible and uniform. We inoculated two growth plates from the same frozen stock plate of YAC clones (95 clones per plate) and processed them separately according to our protocol. Both plates of DNA were digested with *Hind*III, electrophoresed, and blotted separately as described above.

One filter was hybridized with a yeast *CUP1* probe specific for yeast genomic DNA (Fig. 5B). The result of quantitating the signal from this filter is a mean intensity of 27590 (number of decays counted) with a sample standard deviation of 5990 over 29 samples. In other words, DNA yields were typically within 22% of the mean.

The filters were hybridized with a probe specific for the YAC vector (Fig 5A). This filter exhibits a fairly even band intensity and a band pattern identical to that of another filter with DNA from the same YAC clones that was prepared separately by our method (data not shown). There is greater variation in band intensity with the YAC vector probe than with the genomic probe—indicating that the yield of YAC chromosomes varies reproducibly for biological reasons.

These hybridizations also demonstrate that the DNA produced by this method is also suitable for restriction enzyme digestion. Fig. 5A also shows that digestion is complete. A complete digest for a single YAC should yield exactly one band spanning the junction of the vector and the insert, which is seen in almost all lanes. (A few lanes reproducibly contained two discrete bands. These are probably due to multiple YAC clones within the strains, since the bands are widely separated and always seen in separate DNA preparations from the same clones. The YAC library from which the clones are taken is known to contain a small proportion of such wells.)

DNA produced by the method is suitable for pulsed-field gel electrophoresis; chromosomes are preserved intact, as shown by electrophoresing the DNA on a CHEF gel (Fig. 6).

Finally, the DNA is suitable for the polymerase chain reaction (PCR). We successfully used PCR to amplify a 194 bp YAC

vector region in 8 out of 8 samples tested (data not shown). The fact that the DNA preparations are suitable substrates for such enzymatic manipulations as restriction digestion and PCR is not surprising: in our protocol, the DNAs are extensively dialyzed to remove impurities.

CONCLUSION

We have developed a high-throughput yeast DNA preparation protocol that is easily automated and allows us to prepare thousands of DNA samples per person per month. It produces high quality DNA that can be used for electrophoresis, restriction enzyme digestion, and PCR. Thus, it can be used for both preparative purposes and for analytical purposes such as fingerprinting and walking. Its high throughput and low cost make it feasible to use this method to prepare all the YAC clone DNA necessary to physically map a mammalian genome.

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