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Preparation and screening of an arrayed human genomic library generated with the P1 cloning system

(gene isolation/high molecular weight genomic DNA)

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ABSTRACT **We describe here the construction and initial characterization of a 3-fold coverage genomic library of the human haploid genome that was prepared using the bacteriophage P1 cloning system. The cloned DNA inserts were produced by size fractionation of a *Sau*3AI partial digest of high molecular weight genomic DNA isolated from primary cells of human foreskin fibroblasts. The inserts were cloned into the pAd10sacBII vector and packaged *in vitro* into P1 phage. These were used to generate recombinant bacterial clones, each of which was picked robotically from an agar plate into a well of a 96-well microtiter dish, grown overnight, and stored at −70°C. The resulting library, designated DMPC-HFF#1 series A, consists of ≈130,000–140,000 recombinant clones that were stored in 1500 microtiter dishes. To screen the library, clones were combined in a pooling strategy and specific loci were identified by PCR analysis. On average, the library contains two or three different clones for each locus screened. To date we have identified a total of 17 clones containing the hypoxanthine-guanine phosphoribosyltransferase, human serum albumin–human α-fetoprotein, p53, cyclooxygenase I, human apurinic endonuclease, β-polymerase, and DNA ligase I genes. The cloned inserts average 80 kb in size and range from 70 to 95 kb, with one 49-kb insert and one 62-kb insert.**

To facilitate the ongoing efforts to map and sequence the human genome, and to isolate intact genes that are larger than 40 kb in size, it is important to clone large unrearranged chromosomal fragments as single contiguous pieces of DNA. Cosmid and yeast artificial chromosome (YAC) systems have the potential to accomplish these goals but both have demonstrated deficiencies. Cosmid cloning systems have an upper cloning size limit of only 45 kb (1) and libraries generated in these systems often do not faithfully mimic the organization of the DNA in the genome. The YAC cloning system can accommodate genomic inserts that are up to 1 Mb in size and, therefore, have proven to be most useful for producing low-resolution chromosomal maps (2, 3). However, this system is plagued with a propensity to generate a large percentage of clones (up to 50%) that contain DNA segments from noncontiguous parts of the genome (chimeras). YAC cloning also appears prone to producing small deletions and/or rearrangements of the cloned insert (2, 3). Because of these problems, and because it is difficult to isolate microgram quantities of pure insert DNA from YAC clones, it is difficult to imagine that large YAC inserts will be the substrates for large-scale sequencing efforts. To deal with these issues two new bacterial-based cloning systems have been developed in recent years, the P1 system (4–7) and the bacterial artificial chromosome system (8). Advantages of the former include an ability to clone DNA fragments that are up to 95 kb in size in an *Escherichia coli* host, an ability easily to use those clones to isolate and manipulate microgram quantities of recombinant DNA, an ability to produce libraries with a minimal effort (compared to YACs), and an ability to produce libraries that faithfully represent the genomic source DNA (9–12). In addition, the P1 cloning vector, pAD10sacBII, contains a positive selection for cloning and the vector is organized such that cloned inserts are flanked by rare-cutting restriction enzymes as well as by T7 and Sp6 promoters (4).

To determine how well the P1 system is suited for genome mapping and sequencing it is important to produce and evaluate multicoverage P1 libraries. Toward this end a 3-fold coverage mouse library was constructed from cell line C127 (6) and recently a 4-fold coverage library was produced from ES cell line E14 (N.S., unpublished data). In this report we describe the production of an arrayed human genome library containing 130,000–140,000 clones whose insert sizes range primarily from 70 to 95 kb. This provides a theoretical genome coverage of 3- to 4-fold. PCR analysis of this library with unique sequence primers for various human genes indicates that each sequence analyzed is represented two or three times in the library.†

MATERIALS AND METHODS

Isolation and Size Fractionation of High Molecular Weight Human DNA. Human foreskin fibroblast (HFF) primary cells were isolated from a single individual and were obtained as passage no. 6 from Viromed Laboratories (Minnetonka, MN). They were shown to have a normal karyotype by Jaclyn Biegel (Cytogenetics Laboratory, Children's Hospital of Philadelphia). After a few passages, 6×10^7 cells were trypsinized and used for the preparation of high molecular weight DNA by our published proteinase K/SDS method (10, 13), with the following modification: after the lysed cells were incubated at 60°C for 2 hr, the solution was made 0.8 M NaCl and extensively dialyzed at 4°C against sterile TE buffer (10 mM Tris, pH 8/1 mM EDTA).

The appropriately sized DNA fragments for cloning were generated by partial digestion with *Sau*3AI and size fractionated once through a 10–40% linear sucrose gradient (10, 13). Fractions were spot dialyzed against TE buffer on Millipore VS 0.025-μm filters, concentrated 10-fold with butanol, dia-

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Abbreviations: YAC, yeast artificial chromosome; HPRT, hypoxanthine-guanine phosphoribosyltransferase; CHEF, clamped homogenous electric field; IPTG, isopropyl β-D-thiogalactoside; HSA, human serum albumin; AFP, human α-fetoprotein; HFF, human foreskin fibroblast.

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†The clones reported in this paper have been recorded in the Genome Data Base, Welch Medical Library, The Johns Hopkins University, Baltimore, MD.

lyzed again, and analyzed by clamped homogeneous electric field (CHEF) gel electrophoresis (10, 13). Fractions containing DNA with an average insert size between 60 and 110 kb were chosen for subsequent ligation to vector arms.

Preparation of Recombinant Clones. Vector arms containing *Bam*HI ends for the insertion of genomic fragments were prepared from the DNA of positive selection vector, pAd10sacBII (4), as described (5, 13). The ligation of vector arms to human DNA insert (2:1 molar ratio) was routinely performed with 0.3 μg of vector arms in a final volume of 16 μl using 400 units of T4 DNA ligase as specified by the vender (New England Biolabs). Before the addition of enzyme and buffer, the two DNAs were preincubated at 37°C for 30 min and then cooled on ice. Following overnight incubation at 16°C, the ligation reaction mixture was heated to 70°C for 10 min and then stored at 4°C for up to a week.

In vitro packaging of 2–4 μl of the ligation products was performed using bacterial extracts from DuPont/NEN (packase extract lot no. LA136 and head/tail extract lot no. PB093) as described (5, 7, 13). The packaged phage were stored without chloroform at 4°C and routinely used within 1 week. To produce 150–200 colonies per plate for the robotic arraying process, 3–15 μl of each phage lysate was used to infect 100 μl of the *E. coli* plating host NS3529 [*recA*−, *mcrA*−, Δ(*hsdR, hsdM, mcrB, mrr*) (λ*imm*λ*LPI*) (λ*imm434-P1:Cre*$^+$)] (4, 5). After phage absorption at 37°C for 15 min without shaking, 0.8 ml of prewarmed Luria broth was added and incubation was continued at 37°C for 35 min. Cells were centrifuged for 30 sec and the resuspended pellet was spread on a Luria/kanamycin (25 μg/ml) agar plate supplemented with 5% sucrose. The plates were incubated overnight at 37°C to allow colonies to form.

Arraying and Pooling the Library. The library was arrayed using a Beckman Biomek 1000 robot running Biotest Software (Beckman) with a sterile, Biomek pipette tip used as the picking tool for each colony (Fig. 1). The transfer was performed by first imaging colonies on the agar surface using a video-based charged-coupled device camera. The colony coordinates were then identified in reference to four fixed points and used to position the arm and tablet of the Beckman Biomek robot. Each colony was picked into a unique well of a microtiter dish (Corning no. 25855) containing 210 μl of sterile Luria broth with 25 μg of kanamycin per ml and 10% glycerol. Sterile lids were added and the dishes were incubated at 37°C without shaking. On the next day, the culture in each well was mixed with the Biomek eight-channel pipettor and processed as described in *Results*.

Screening the Library using PCR. For each gene isolated, a single pair of PCR primers was prepared using DNA sequence information from GenBank. The cycling and buffer conditions for the PCRs were dependent upon the specific primer set and are available upon request. In general, reaction mixtures were 25 μl final volume with AmpliTaq DNA polymerase (Perkin–Elmer/Cetus) and reagents (with or without gelatin). Samples were placed in a boiling water bath for 1 min prior to addition of enzyme and mineral oil. The template was usually 1 μl of resuspended cells. However, for the most complex pools of the library (the 24 pools labeled A–X), DNA was prepared (5, 6) and 1 μl of that DNA was used as template.

P1 Clone Analysis. When a positive well in the library was identified, an aliquot was removed and streaked out on Luria/kanamycin (25 μg/ml) agar plates that were incubated overnight at 37°C. The next day several isolated colonies were screened for the desired recombinant by PCR analysis to ensure that the identified well does not contain more than one recombinant clone. A positive colony was used to inoculate a 10-ml culture that was subsequently induced to replicate at high copy number with isopropyl β-D-thiogalactoside (IPTG) and P1 recombinant plasmid DNA was isolated using a modified alkaline lysis procedure (5, 13, 15). The DNA was digested with either *Not* I or *Sal* I and analyzed by either field-inversion gel electrophoresis (4, 6) or CHEF electrophoresis using conditions described in the legend to Fig. 2. The insert size for individual clones was determined by summing the size of the resulting *Not* I or *Sal* I fragments and subtracting the 17-kb portion of the pAd10SacBII vector that is present in all recombinant clones (4). Note, for the initial analysis of insert DNA, sufficient quantity of P1 clone DNA can also be isolated from a 1.5-ml overnight culture that has not been induced with IPTG, as described for clones generated by the bacterial artificial chromosome system (8).

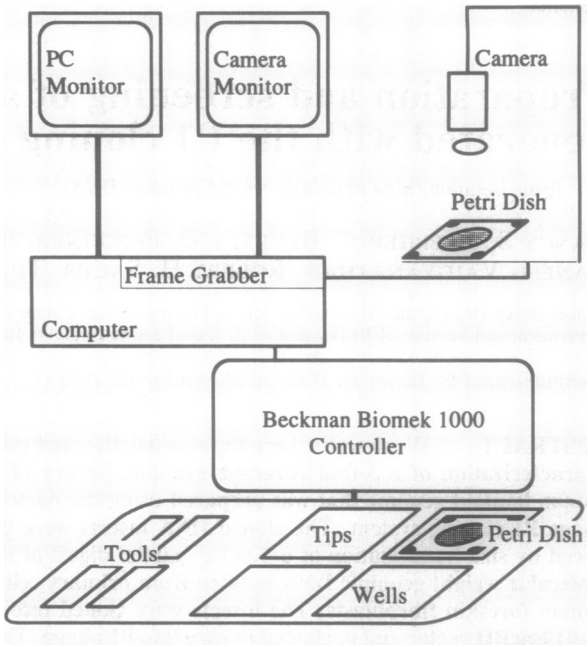

FIG. 1. Schematic diagram of the colony-picking process. The computer system includes a Dell 325D personal computer (Dell, Austin, TX) with 4-MB RAM running MSDOS4.01 (Microsoft) and a high-resolution frame grabber (DT2853; Data Translation, Marlboro, MA). An IBM 8515 monitor was used for display of text and commands and a Sony Trinitron color video monitor (PVM-1342Q) was used for visualization of colonies. Images were captured using Image-Pro Plus v.1.0 software (Media Cybernetics, Silver Spring, MD) and a charged-coupled device video camera module (Sony model XC-77) with a Fujinon CF-25B 25-mm fl.4 lens.

RESULTS

P1 Clone Preparation. For any library, the source and quality of DNA are a primary concern. We chose to prepare DNA from low-passage, primary diploid cells of HFFs from a single individual to avoid clone discrepancies that might arise from genomic rearrangements of cells in long-term culture. Another concern was the preparation of genomic insert DNA that would be largely in the 70- to 100-kb size range with as few fragments as possible below 60 kb. Although the P1 vector arm system is designed to eliminate the cloning of DNA fragments smaller than 70 kb (10), these fragments can be recovered with reduced efficiency (4, 12) and may also contribute to clone scrambling due to coligation events. The DNA shown in Fig. 2, lane 4, was largely between 65 and 110 kb and was used for the construction of ≈75% of the arrayed library, with a cloning efficiency similar to that reported previously (5, 6, 10). Another 20% of the clones were prepared from a fraction with a greater average insert size (data not shown). Such a fraction contains DNA that is on average slightly too large for the P1 phage head and, therefore, results in a drop in packaging efficiency. Never-

theless, it was used because that size of the cloned inserts was near the maximal that could be recovered with P1 (>90 kb). Care was taken to avoid fractions such as that shown in Fig. 2, lanes 5 and 6. Clones generated with these fractions contain DNA inserts that are <60 kb in size.

Insert DNA was ligated to dephosphorylated vector arms prepared from the pAd10SacBII vector, packaged *in vitro*, and used to infect the *E. coli* host strain NS3529 to generate a single-copy, recombinant plasmid (4, 5, 13). The isolated human insert DNA and vector arm preparation were found to be quite stable at 4°C, with the major variability in cloning efficiency attributable to variation in the *in vitro* packaging extracts and the particular insert DNA fraction used.

Arraying and Pooling the Library. As described in *Materials and Methods* and the legend to Fig. 1, recombinant P1 clones were displayed as colonies on agar plates and each was then transferred robotically into a well of a microtiter dish containing growth medium, and the contents of the wells were grown overnight. We found that the separate growth of each P1 clone was desirable because the size of colonies on the original agar plates was quite variable and significant biases would be produced if the colonies on a plate were pooled. Consistent with this view we routinely observed that the smallest colonies from the agar plates invariably produced the poorest growth after overnight incubation in wells. The arraying process continued in a serial fashion to produce the entire library (designated DMPC-HFF#1, series A), which is contained in 1500 microtiter dishes and is stored frozen at −70°C. A record of the multiple independent ligation and packaging reactions for clone preparation and picking was maintained in a data base. In general, clones separated by more than four dish numbers were from separate phage infections and, therefore, must represent independent cloning events.

To reduce the library to a more manageable size for library distribution and for screening purposes, a simple rows and columns method of pooling was chosen (Fig. 3 *Left*, top to bottom). First, 15 μl was taken from each of the 12 wells of a row of series A dishes and transferred into a single well of series B dishes (Fig. 3). This results in the representation of the cultures of a single series A dish as an eight-member column of a series B dish and has the effect of reducing the total number of dishes in the library from 1500 (series A) to 125 (series B). A second 15 μl was taken from each well of a series A dish and was combined in a modular reservoir unit (Beckman no. 372788) to form a pooled sample representing all 96 wells of that dish. This volume of 1.44 ml was manually divided into two sterile freezer vials (one for immediate use and one for long-term storage). The remaining sets of pools were established manually as outlined in Fig. 3 and all were stored at −70°C.

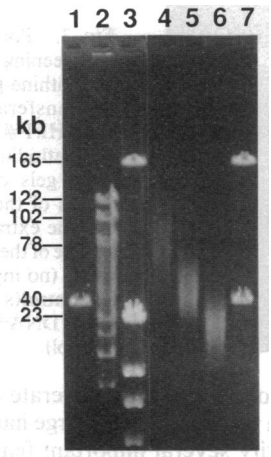

FIG. 2. Analysis of size-fractionated, *Sau*3AI-digested human DNA by pulsed-field gel electrophoresis. Three consecutive fractions from a 10–40% sucrose gradient separation of human HFF DNA partially digested with *Sau*3AI (lanes 4–6) was subjected to electrophoresis in an agarose gel [1% SeaKem GTG-grade agarose (FMC) and 0.5× TBE buffer (16)] at 14°C using Bio-Rad CHEF mapper with autoalgorithm set for 10–165 kb (120° angle, 6 V/cm, 20.2 hr, linear ramp with switch times from 0.47 to 14.22 sec). DNA size standards are 40 kb of bacteriophage T7 DNA (lanes 1 and 7; Sigma), 165 kb of bacteriophage T4 DNA (lanes 3 and 7; Sigma), DNA standards of 122, 102, 78, and 25 kb and smaller from restriction digestion of the bacteriophage T5 genome (lane 2; DuPont/NEN), and λ DNA *Hin*dIII digest of 23, 9.4, 6.6, and 4.4 kb (lane 3; BRL).

Library Screening. Once pooled, the library was screened for clones containing a particular DNA sequence using a PCR primer set that robustly amplified the expected size fragment with total human genomic DNA as template. For example, the isolation of a positive clone using primers prepared from the HPRT gene is illustrated in Fig. 3 (*Center* and *Right*, bottom to top). The primers were initially tested with DNA from each of the 24 most complex pools (A–X) as template. A positive signal in 1 or more of these 24 pools was then followed by a series of PCRs using cells from the simpler pools that were used to generate the complex positive pool. This process was continued until a single well of a microtiter dish from series A of the library was shown to contain the positive clone (e.g., well D11 of dish 0171 for HPRT). The strength of the PCR signal from a positive pool varies during the screening process. This occurs not only because the concentration of the positive clone is not constant in the various pools but also because the 24 most complex pools (pools A–X) were screened by PCRs with DNA while all subsequent pools were screened by PCR with cells. It is our impression that if the PCR signal is weak for a given primer set, it may be necessary to use DNA rather than cells from even the pools of lesser complexity (e.g., those containing 768 clones per well).

In general, we have found the choice of PCR primer set is critical for the screening process, with difficulty arising if the primer set amplifies several bands with human genomic DNA as template or if the amplification conditions are unusually sensitive to variations such as magnesium concentration or thermal cycling conditions. When such problems occur, we generally develop new primer sets.

Results to date indicate that 94% of the PCR primer sets used (132 of 140), which are derived from unique sequence loci and produce a single PCR band with genomic DNA, yield at least one positive clone from the library (Table 1; D. Smoller and P. Gold, personal communication; J. Gingrich, personal communication). Moreover, on average, each positive primer set produces two or three different positive clones.

Clone Analysis. An example of 17 clones that we have identified by the PCR screening method and that were isolated from single wells of the series A library is shown in Table 1. The results indicate that 15 of the 17 clones varied in size from 70 to 95 kb, with two smaller clones containing inserts that were 49 and 62 kb in size. This is consistent with an estimate of an overall average insert size of 80 kb, which agrees with our original determination that was made during library preparation by testing various randomly chosen clones. An insert size of ≈95 kb is at the limit of the P1 headful packaging system with the pAd10sacBII vector, and several isolated clones were at that limit. In the case of HSA, we originally identified three complex library pools that were positive with the HSA primer set used, but we followed through and isolated only one of those clones. This clone (DMPC-HFF#1-029E03) is of particular interest because it was identified using a PCR primer set derived from the 5′ portion of the HSA gene but was also positive when a primer set specific for the 3′ end of the nearby human α-fetoprotein

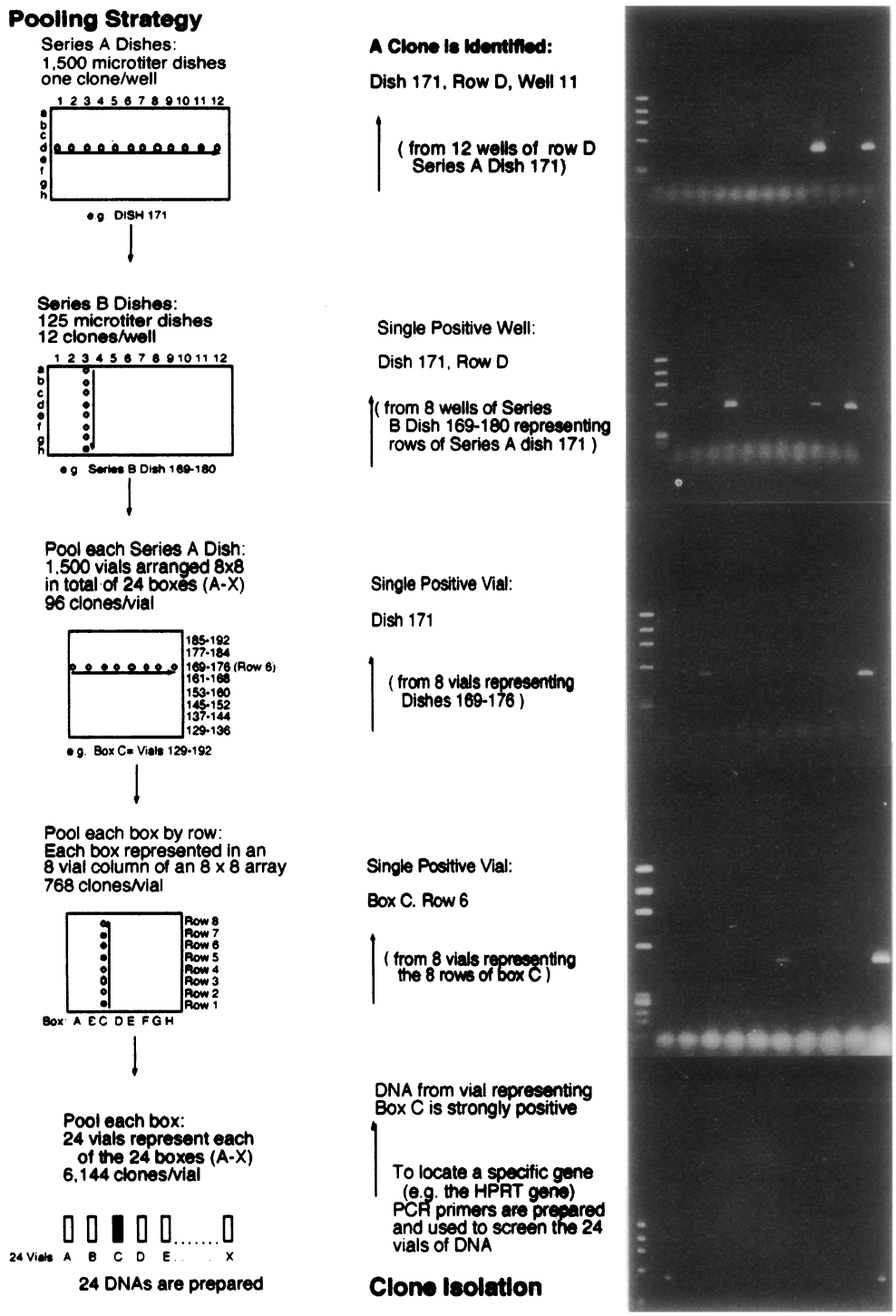

FIG. 3. Pooling and subsequent PCR screening of the library for the hypoxanthine-guanine phosphoribosyltransferase (HPRT) clone DMPC-HFF#1-0171D11. Photos are of ethidium bromide-stained agarose gels containing the PCR products of the indicated wells or vials. The extra lanes on the right-hand side of the gel contain negative controls (no input DNA) and positive control(s) (human genomic DNA or DNA from a previous positive pool).

(AFP) gene [40 kb away (18)] was used. This suggests that the clone (HSA-AFP) contains both of these genes as well as the intervening region between them.

DISCUSSION

Since its development in 1990 (7), and following several vector improvements (4, 13), the P1 cloning system has come to be used for the production of genomic libraries from various cells including a human pre-B-leukemia cell line (11), adult fly tissue of *Drosophila melanogaster* (9), a mouse C127 fibroblast cell line (6), a suspension culture of cells of *Pinus radiata* (19), *Schizosaccharomyces pombe* (17), Sprague–Dawley rat spleen tissue (12), and, most recently, an E14 mouse embryonic stem cell line (N.S., unpublished data). In this report we show that we can generate an arrayed library from an organism with a relatively large haploid genome (3×10^6 kb) and identify several important features that make it superior to the human leukemia cell P1 library previously reported (10). First, we document that this library represents ≈3-fold coverage of the human genome based on the recovery of 2 or 3 clones with average insert size of 80 kb at several different unique loci. The leukemia cell P1 human library was at best 1-fold coverage. Moreover, the choice of the new pAd10sacBII P1 vector (1) and the use of genomic DNA derived from primary human cells for the preparation of inserts should facilitate the analysis of cloned inserts and should maximize the fidelity of cloning (4–6). Neither of these two features was incorporated in the original leukemia cell library (10).

Table 1. Examples of clones identified in DMPC-HFF#1 library

Gene	Clone name (DMPC-HFF#1-)	Insert size, kb	PCR primer pair sequence
AP endonuclease	0319H10	91	5′-GTGCAGATACGGCGTTGCTC-3′
	1324G04	94	5′-CATTCCCGTTACGAACGCCC-3′
β-Polymerase	0232G03	62	5′-GAGCTGGGTTGCTCCTGCTC-3′
	0570B01	89	5′-GAGCATGTCGGTGATTCCCC-3′
	1150E07	95	
Cyclooxygenase I	0577G05	85	5′-CAGCCATCTCCTTCTCTCCT-3′
	1027E09	78	5′-ATGTGGCTGTGGATGTCATC-3′
	1077A10	79	
HPRT	0171D11	81	5′-GGAATTCCTGAACGTCTTGCTCGAGATG-3′
	0501G10	ND	5′-ACATCGAACCGATGTAGCTCAAGAGAGG-3′
HSA	0295E03	84	5′-ATGAAGTGGGTAACCTTTATTTCCC-3′
			5′-AACCGATGAGCAACCTCACTCTTGT-3′
p53	0642E05	80	5′-AATGGATGATTTGATGCTGTCCC-3′
	0754B10	ND	5′-CGTGCAAGTCACAGACTTGGC-3′
	1357E09	49	
Ligase	0577A02	73	5′-GGATAGTGACAAGGGCATCT-3′
	0699H06	77	5′-GTAGGTATCTTCAGGGTCAG-3′
	1235B10	67	

HSA, human serum albumin; AP, apurinic; ND, not determined.

Perhaps most important for future screening, the members of this human library are individually arrayed in microtiter dish format. Although costly in up-front preparation time, money, and storage, this arrayed library has several key advantages over a library existing only as pools of clones (6, 10, 12). (*i*) By being arrayed it is a renewable resource that has a defined clone representation and position. Thus, copies of the series A library (one clone per well) may be faithfully generated without allowing the variable growth rates of individual recombinant clones to modify clone representation. Lack of growth competition is also advantageous in the screening process, where the relative representation of a clone producing a positive signal will only vary in a defined manner that is determined by the pooling strategy. Moreover, every clone has a unique identifier of library dish and well number that permits the easy recognition of independent cloning events and facilitates data handling of multiple, independent screens. (*ii*) An arrayed library is amenable to the application and development of various automated screening manipulations that are more difficult to implement if the library consists of multiclone pools. For example, using high-density colony hybridization filters, an arrayed library may be used to simultaneously characterize the relative copy number and extent of homology of clones to various probes. In addition, chromosome-specific hybridization probes from various sources may be used to identify and regroup the genomic library into chromosome specific P1 libraries. This has definite advantages over other methods such as cloning from flow-sorted chromosomes or hybrid cell lines (14). (*iii*) Information retained during computer-aided arraying and screening processes should be extremely helpful in tracking data relating to each clone and in resolving any inconsistencies that may be found in the library at a future date.

Although it may to a bit be premature to evaluate the fidelity of cloning with the human P1 library, three results suggest it is likely to be quite good. (*i*) When a variety of clones were grown for 40–80 generations in *E. coli* we were unable to detect any evidence of rearrangement in isolated P1 plasmid DNA digested with the restriction enzyme *Bgl* II. (*ii*) Reports from many laboratories that have obtained clones from the library (D. Smoller, personal communication) indicate that those clones contain segments of genomic DNA that could not be isolated from either cosmid or YAC libraries. (*iii*) Three hundred-500-kb P1 contigs have been generated with P1 mouse and rat libraries (11, 12) that could not be generated using multiple cosmid libraries. Based on these results we believe the P1 library described here may be especially useful for cloning and mapping efforts.

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