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A PCR-based method for high stringency screening of DNA libraries

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ABSTRACT

A rapid method for cloning genomic DNA utilizing a PCR-based screening protocol is described. A murine genomic library in lambda phage was subdivided into 64 wells, each containing 1000 clones, and propagated in bacteria. Amplified phage from each of 8 wells across columns, and each of 8 wells down rows, were pooled. The pooled phage were screened for the presence of murine M-CSF DNA by PCR using specific oligonucleotide primers. A single well that contained an M-CSF genomic clone was identified by the synthesis of a PCR product of the correct size that hybridized to an internal M-CSF oligonucleotide probe. This well was subdivided into 64 wells, each containing approximately 30 individual phage, reamplified, and rescreened utilizing the same protocol. A positive well was then subdivided and amplified a third time starting with an average of 2 phage per well, and rescreened for M-CSF DNA by PCR. Phage from a PCR-positive well, now highly enriched for M-CSF DNA, were grown as individual plaques. PCR-screening of randomly picked plaques demonstrated that the majority contained an M-CSF genomic insert. This method obviates the more labor and time intensive method of plaque hybridization screening of DNA libraries, and is more stringent since three oligonucleotides (the two PCR primers, and the hybridization probe) are required to give a true positive signal. Similar methodology has also been used to clone a cDNA gene contained within a plasmid library.

INTRODUCTION

Conventional cloning of genomic or cDNA fragments contained within phage vector libraries is performed by plaque hybridization (1). This method is laborious and time consuming, and often yields falsely positive recombinant clones. As an alternative to plaque hybridization, I describe a PCR-based protocol that streamlines the effort required to screen a library. This method requires that three oligonucleotides correctly anneal to the desired gene fragment, thereby increasing the stringency of the screening procedure and reducing the chance of obtaining false positive clones. To illustrate the utility of this screening method, I describe the cloning of the murine M-CSF gene (also known as CSF-1), a cytokine that stimulates proliferation and differentiation of cells of the monocytic lineage (2). The need for obtaining genomic clones has increased with the proliferation of transgenic animal technology, the initiation of the human genome project, and the ongoing structural and functional characterization of genes from a variety of organisms. The method described here may be used to screen a phage library for any genomic fragment, providing sufficient sequence information exists for designing the oligonucleotide primers and probe.

MATERIALS AND METHODS

Amplification of phage

A mouse genomic DNA library (Stratagene, mouse strain 129SV constructed in a Lambda FIX II vector) was used to infect an overnight culture of *E.coli* strain LE392 at the indicated titer of phage. Phage and bacteria were incubated together in 1 ml L broth + SM (1:1) at room temperature for 20 min. The culture was diluted with 20 ml L broth/10 mM $MgSO_4$, plated in an 8×8 matrix (64 wells total , 100 μl/well) in a 96-well U-bottom multiwell plate (Costar), sealed with acetate sealing tape (Dynatech Laboratories), and amplified at 37°C for 5–6 hr while shaking at 225 rpm. The phage titer was typically $1-2\times 10^9$/ml following amplification. For secondary and tertiary amplifications, phage from single positive wells (see below) were titered, used to infect LE392 at approximately 30 pfu/100 μl (secondary screen) or 2 pfu/100 μl (tertiary screen) and amplified as described above.

Pooling of phage

Amplified phage from 8 wells across a row or 8 wells down a column were pooled using a multiwell pipet (25 μl/well), and diluted 1:1 with glass distilled water. The matrix of 64 wells was therefore reduced to 16 pools which were used as templates for PCR analysis as described below. The pooled phage, and the 96 well plate (resealed with fresh sealing tape), were stored at 4°C. At all steps, extra care was taken to avoid cross contamination of samples.

PCR reactions

Oligonucleotide primers for PCR were synthesized from exon six of the murine M-CSF gene (2, 3). The 5′ primer (5′-CCAAGCCTGATTGCAACTGCC-3′) and 3′ primer (5′-GTGAGTCCTCAGTGAGTCTGTC-3′) were located at nucleotides 714–734 and 970–992 of the M-CSF cDNA sequence, respectively. An internal oligonucleotide (5′-GTGCCAAGCAGCGACC ACCCAGGAGTACCTG-3′), corresponding to

nucleotide 903–933 of the M-CSF sequence, and that did not overlap with either PCR primer, was synthesized for use as a hybridization probe to the PCR products. Each PCR reaction (25 μl final volume) contained 50 pmoles of each oligonucleotide primer, 0.25 μl Taq polymerase (Promega, 5000 units/ml), 200 μM each dATP, dCTP, dTTP and dGTP, 2.5 mM $MgCl_2$, 1× PCR buffer (Promega), and 0.5 μl template (phage stock or purified murine genomic DNA). For convenience, a PCR cocktail containing all the reagents except the template and Taq polymerase was made and stored at −20°C. Following addition of 1 μl Taq polymerase/100 μl PCR cocktail, the PCR cocktail was aliquoted (24.5 μl/tube) into GeneAmp reaction tubes (Perkin Elmer Cetus), and 0.5 μl template was added. Each round of screening contained a negative control, lacking template, and a positive control in which either 10 ng total mouse genomic DNA, or a phage population known to contain the M-CSF gene, was used for the template. Randomly picked individual plaques grown from a positive well from the tertiary screen were eluted in 0.5 ml SM and used as templates under the same reaction conditions. PCR was performed in a thermal cycler (Perkin Elmer Cetus) for 35 cycles, with each cycle consisting of 94°C for 1 min to denature the template, 55°C for 1 min for primer annealing, and 72°C for 2 min for polymerization. At the end of 35 cycles, samples were incubated for 7 min at 72°C, and held at 4°C prior to gel analysis. Preliminary experiments were performed using mouse genomic DNA as template, in which the number of PCR cycles and annealing temperature were varied. The PCR conditions which yielded a high amount of correct product with a minimum amount of side products were used for all subsequent reactions.

Analysis of PCR reaction products

PCR reaction products were electrophoresed through a 2% agarose/TAE gel and visualized with ethidium bromide. The gel was dried *in vacuo* at approximately 70°C and prepared for hybridization as previously described (4), except the gel was dried between Whatman 1chr paper and Saran Wrap prior to denaturation. The internal M-CSF oligonucleotide, end labelled with [^{32}P]-phosphate (5), was hybridized *directly* to the dried gel in 5× SSC, 5× Denhardt's reagent, 0.1% SDS, 100 μg/ml denatured salmon sperm DNA, at 55°C for 3hr-overnight. The hybridized gel was washed at 55°C in 5× SSC/0.1% SDS, and exposed to X-ray film for autoradiography.

Plaque hybridization

Plaque hybridization, using the same internal oligonucleotide probe used for analysis of the PCR reaction products, was performed as described previously (1). Filters were hybridized and washed as described above.

RESULTS AND DISCUSSION

The overall strategy for screening the library using PCR and oligonucleotide hybridization is diagrammed in Fig. 1. The library is subdivided and amplified in *E.coli* in an 8×8 grid in a 96-well multiwell plate. Amplified phage are pooled across rows and down columns, so that each pool contains phage from 8 individual wells. The pooled phage are then subjected to PCR analysis using oligonucleotide primers specific for the desired gene. PCR products are analyzed by agarose gel electrophoresis, and following hybridization to a specific oligonucleotide probe internal to the PCR primers (Fig. 1B), are visualized by

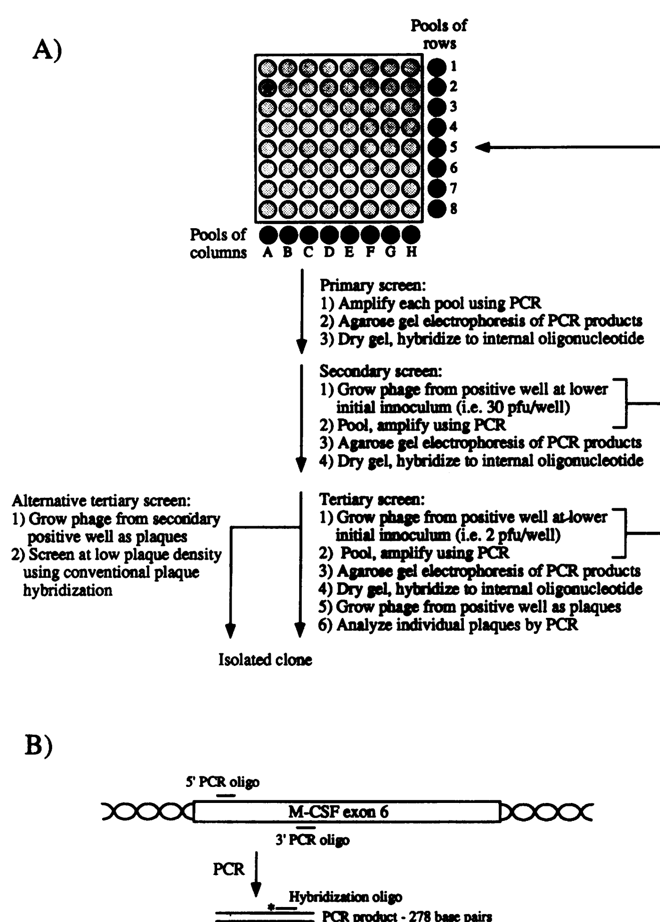

Figure 1. Panel A—Schematic diagram of the PCR screening procedure—The genomic phage library is amplified and analyzed by PCR as described in the text. At each stage of screening, the number of initial phage particles per well is decreased. By the tertiary level of screening, randomly picked phage can be grown as individual plaques and screened by PCR, yielding pure recombinant phage containing the desired insert. The library can also be highly enriched using the PCR screening protocol, allowing for conventional plaque hybridization screening of a small number of plates. Panel B—Diagrammatic representation of the PCR product detectable by hybridization—The locations of the PCR primers and the hybridization oligonucleotide within exon 6 of the M-CSF gene are shown. The correct PCR product of 278 base pairs hybridizes to the internal hybridization oligonucleotide.

Table I.

Sample	Frequency	Method of determination	Fold enrichment
Starting library	.003%	PCR titration	–
Primary screen	.12%	plaque hybridization	40
Secondary screen	5.4%	plaque hybridization	45
Tertiary screen	82%	plaque hybridization	15
Tertiary screen	63%	PCR amplification of individual plaques	–

The frequency of phage containing M-CSF inserts was estimated in the starting genomic library using PCR (Fig. 2). Wells containing the M-CSF gene were identified at the primary, secondary, and tertiary screens as described in the text, and screened further using plaque hybridization. At the tertiary level of screening, the frequency of M-CSF-positive phage was determined by both plaque hybridization and of PCR analysis of single plaques (Fig 4). The frequency is expressed as% of phage containing an M-CSF insert. The fold enrichment was calculated from the frequency of the M-CSF gene within the pool at each level of screening relative to its frequency at the previous level of screening.

autoradiography. The single well within a row that contains the desired gene is located at the column that is also PCR and hybridization positive. For example, pool 2 and pool A in Fig. 1A are PCR and hybridization positive due to phage contributed by the single well 2A. The phage from the positive well is titered by plaque formation, and used to infect bacteria at a lower number per well for secondary screening, and again for tertiary screening using the same protocol.

The appropriate number of phage particles per well used in the primary screen was determined by titering the starting library using PCR (Fig. 2). For the M-CSF gene, a minimum of 30,000 pfu was required to yield a PCR product that was detectable by hybridization to an M-CSF specific oligonucleotide (Fig. 2, lane 4). Assuming an average sized genomic insert of 20,000 bp, PCR amplification of 30,000 phage represents analysis of 6×10^8 bp

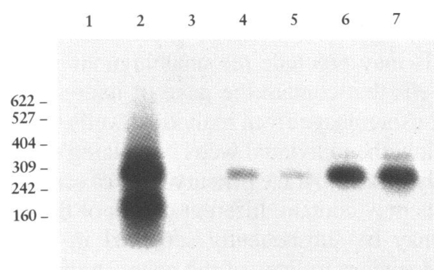

Figure 2. PCR titration of the library for the M-CSF gene—The limit of detection of the M-CSF gene in the starting library using the PCR-based assay was determined by using limiting amounts of phage for template. The lowest number of phage that yielded the correct PCR product defined the minimum frequency of the gene in the library, and was used to determine the number of initial phage for the multiwell screening protocol. The templates for each reaction were; lane 1, no template; lane 2, 10 ng mouse genomic DNA; lane 3, 10^4 phage; lane 4, 3×10^4 phage; lane 5, 10^5 phage; lane 6, 3×10^5 phage; lane 6, 10^6 phage. The migration of MspI-digested pBR322 is indicated on the left (base pairs).

of genomic DNA. The experimentally determined frequency of 1/30,000 for the M-CSF gene is within several fold of the hypothetical frequency of 1/100,000 in a library containing 20,000 bp inserts and a haploid genome size of 2×10^9 bp (6).

A genomic clone for M-CSF was isolated using the PCR screening protocol illustrated in Fig. 1. Based on the frequency of M-CSF clones detectable by PCR of approximately 1/30,000 within the genomic library (Fig. 2), 64 wells were inoculated in an 8×8 format with 1000 pfu/well. Following amplification of the phage, pools were made as diagrammed in Fig. 1 and analyzed by PCR. Ethidium bromide staining of the PCR products revealed that a complex pattern of bands were synthesized using the M-CSF-specific primers (Fig 3, panel A). A band of the expected size of 278 bp was observed using mouse genomic DNA as the template (Fig 3A, lane 10). However, due to the complexity of the PCR products from the pooled phage (Fig 3A, lanes 1–8 and A–H), the presence of a 278 bp band could not unambiguously be used to identify pools that contained an M-CSF gene.

Hybridization to an internal M-CSF oligonucleotide allowed confirmatory identification of the M-CSF PCR product. Pools 2 and A, as well as mouse genomic DNA (Fig 3B, lanes 2, A and 10, respectively) yielded a 278 bp band that hybridized to the M-CSF probe, while all other PCR products were negative. Thus, a single well (2A) containing an M-CSF genomic clone was identified, and screened further. Subsequent screens were performed exactly as diagrammed in Fig. 1, except the number of inoculating phage from well 2A was 30 per well for the secondary screen. Phage from a secondary-positive well were then used to infect bacteria at 2 pfu per well for the tertiary screen. Single wells containing the M-CSF gene at both the secondary and tertiary screens were identified by gel hybridization to PCR products of pooled phage exactly as described above (data not shown).

Individual phage were plaque isolated from a well that was positive in the tertiary screen. Randomly picked plaques were analyzed by PCR. The results demonstrate that 5 of 8 randomly

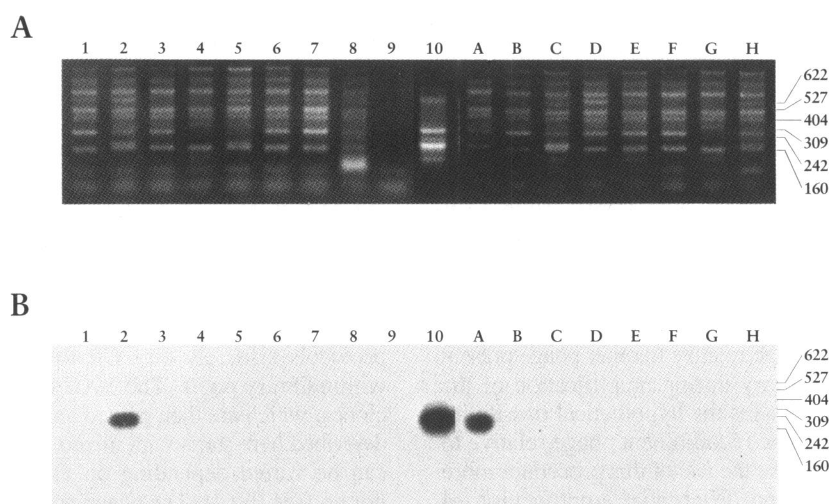

Figure 3. PCR screening of pooled phage for the M-CSF gene—64 wells were inoculated with 1000 phage/well, amplified and pooled in an 8×8 matrix. Pools from columns and rows were screened using PCR and hybridization. The agarose gel in panel A was vacuum dried and used for direct hybridization to the internal M-CSF oligonucleotide as described in Materials and Methods. Panel A, Ethidium bromide staining of PCR products; Panel B, hybridization to M-CSF specific oligonucleotide. The templates for each reaction were; lanes 1–8, pools of rows; lane 9, no template; lane 10, 10 ng mouse genomic DNA; lanes A–H, pools of columns. The migration of MspI-digested pBR322 is indicated on the right (base pairs).

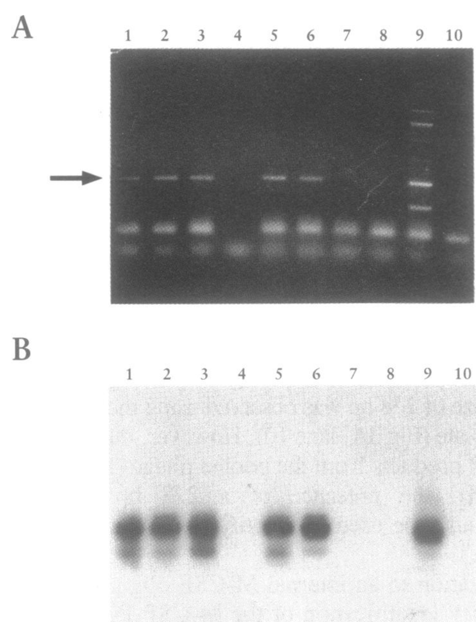

Figure 4. Screening of individual plaques for the M-CSF gene—Phage from a positive well following the tertiary screen were isolated from randomly picked plaques and used as templates for PCR reactions. The arrow indicates the 278 bp PCR product that hybridizes to the internal M-CSF oligonucleotide probe. Panel A, Ethidium bromide staining of PCR products; Panel B, hybridization to M-CSF specific oligonucleotide. Lanes 1–8, randomly picked plaques; lane 9, phage from the tertiary positive well; lane 10, no template.

chosen plaques yielded a 278 bp PCR product (Fig 4A, arrow). This band hybridized to the internal M-CSF screening oligonucleotide (Fig 4B), demonstrating that single plaques contained the M-CSF gene. Thus, the M-CSF gene was cloned from a library of high complexity entirely with the PCR-based screening procedure. Subsequent analysis of this phage demonstrated that it contains an approximately 15 kb insert spanning at least exons 4–8 of the murine M-CSF gene (data not shown).

The frequency of M-CSF genomic clones was determined at each stage of screening utilizing both PCR-based determination and plaque hybridization (Table I). The data demonstrate a high degree of enrichment of the M-CSF gene, ranging from 15 to 45-fold, at each level of screening. The cumulative 27,000-fold enrichment (Table I) for obtaining the M-CSF genomic clone is consistent with the gene frequency determined using PCR analysis of the starting library (Fig. 2).

The frequency of the gene following phage amplification is similar to the reciprocal of the initial pfu used to inoculate each well (Table I), suggesting that there is no significant enrichment or loss of M-CSF-containing phage relative to other phage present in the same subpool of the library during amplification of the phage. In other cases, there remains the hypothetical possibility of selective amplification of some recombinant phage relative to other phage (7), which could make the use of this procedure more difficult. However, there was no differential amplification of phage containing a fragment of the M-CSF gene (Table I) or the bone morphogenetic protein 1 (8) gene (data not shown) relative to other recombinant phage within the library, allowing for the cloning of these genes using this approach. The possibility of genetic recombination during repeated phage amplification also exists (7), and can be addressed using Southern blot analysis of the purified genomic clone versus total genomic DNA.

Several technical suggestions should be considered in order to successfully use this protocol for screening libraries: 1) optimize PCR conditions—both the number of cycles and annealing temperature for the primers may be varied, using total genomic DNA or the starting phage library as template, in order to increase the amount of correct PCR product relative to side products; 2) titer the library using PCR—the frequency of the gene in the library should be determined (see Fig. 2) to establish that the gene exists within the library and to determine a suitable number of phage for the primary screen; 3) don't rely solely on ethidium bromide staining—the correct PCR product can be easily and rapidly identified by drying the agarose gel and hybridizing to an internal probe as described in Materials and Methods. False PCR products may be readily be distinguished from correct products using hybridization (compare panel A to panel B in Fig. 3); 4) when multiple positive clones are present, perform a second round of PCR analysis using individual wells—Multiple positive wells may preclude the unambiguous identification of the single wells that contain the gene of interest. A second set of reactions, using phage from individual wells within a positive pool, will allow the individual wells containing the desired gene to be precisely located. At the primary level of screening, multiple positive wells may contain different clones of the desired gene. Each well may be subsequently screened in order to obtain multiple independent isolates of the gene. In the secondary and tertiary screens, multiple positive wells will contain the same clone (refer to Fig. 1). While a second set of reactions may be required to unambiguously identify a positive well, only a single well should be taken to the next level of screening in these cases, and; 5) properly store and handle the primary plate and pooled phage—The primary plate should be sealed to prevent evaporation and cross contamination. Short term storage of the primary plate and pooled columns and rows can be at 4°C. The phage can be frozen in 15% glycerol or 7% dimethyl sulfoxide for prolonged storage (9). In this way, the library can be repeatedly screened for different genes simply by performing PCR analysis of the already pooled columns and rows (providing that the appropriate number of phage for the gene of interest was used for inoculating the primary plate).

This procedure has also been successfully used to screen a cDNA plasmid library for a propeptide cleaving enzyme gene (Donna Michnick, personal communication). For screening the plasmid library, the procedure was slightly modified by allowing the host bacteria to grow in the 96 well plates for 16 hr. This assured a sufficient bacterial density so that all cDNA clones were represented in the bacterial pools.

Several protocols have been described previously for screening yeast artificial chromosome (YAC) libraries (10, 11). These protocols also rely on PCR to detect a given DNA sequence within library pools. The YAC libraries are grown as individual clones, which are then pooled and analyzed, whereas the protocol described here starts with mixed pools of phage, the size of which can be varied depending on the frequency of the gene in the library (see Fig 2). The phage pools are then successively reduced to smaller pools at each level of screening. The procedures for PCR screening of YAC libraries employ purified DNA, whereas PCR screening of phage and plasmid libraries utilizes whole bacterial culture medium, and doesn't require the purification of template DNA. These procedures share the fundamental

principal of PCR analysis of subpools of the library as a means to isolate a particular DNA fragment contained within the library.

The method described here provides an alternative to plaque hybridization for screening genomic libraries. The procedure should be applicable to phage and plasmid libraries of all types and for all genes in which sufficient sequence information exists for designing appropriate PCR primers and an internal hybridization probe. In many cases, particularly when conventional plaque hybridization yields a high proportion of false positive phage, this method may be preferable to plaque hybridization due to its ease, and to its more stringent requirement of the correct annealing of three oligonucleotides for obtaining a true positive signal.

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