Agar Plug/Serial Dilution Approach for Rapid PCR Screening of Phage Libraries

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The “superpool”/pool format has widely been used for PCR screening of BAC, PAC, or YAC genomic libraries. While these vectors have become the tools of choice for genome analysis, such libraries are not available for all species. Furthermore, for specific applications such as the construction of knockout vectors, small insert libraries are useful, as they enable one to obtain clones containing the gene of interest and its closest sequence directly, with no need for tedious subcloning steps. With genomic libraries built in λ bacteriophages, the number of clones necessary to cover the genome (i.e., three or four genome equivalents) is generally too high for isolating single clones in microplates. Therefore, the screening is often performed by classic colony hybridization and requires the plating of a large number of phage plaques (at least 4–6 × 10⁵ for a phage library with 15 kb average insert size). When homologous primers can be designed, PCR of “superpools” and pools has been proposed as a useful alternative to hybridization (1), but these protocols involve a liquid growth step for the phages, and this procedure sometimes results in bias and under-representation of some phages. The possibility of serial dilutions from plaques was mentioned with the use of nitrocellulose membranes that are first lifted from the agar plates and then split into a series of smaller sections (4). Here a significant improvement on these techniques that does not require radioactivity or membranes is proposed. This technique makes use of a series of dilutions and exploits the possibility of performing PCR directly on agarase slices derived from an LB-agar plate.

Embryonic stem cells derived from 129 SV mice are the principal targets for knockout experiments. Therefore, obtaining DNA from a mouse 129 genomic library for the genes under scrutiny is an essential step toward their targeted inactivation. The current technique has been successfully applied to identify mouse phages encompassing two genes involved in sex determination, PISRT1 and FOXL2 (2,3). The phage library was constructed in λGEM-12 (Promega, Madison, WI, USA), with MboI-digested 129sv genomic fragments. The phage particles were used to infect E. coli strain KH802. One million bacteriophages plaques from the initial phage library were plated on 50 plates at about 20 000 plaques/8.5-cm Ø plate (Figure 1). To each plate 2 mL 10 mM MgSO₄ were added, and the plates were shaken gently for 10 min. Each supernatant was poured in 50 × 2 mL Eppendorf tubes. One hundred microliters of each solution were transferred to one well of a 96-well microplate, to which 20 µL Proteinase K (ICN Biomedicals, Costa Mesa, CA, USA) were added to a final concentration of 1 mg/mL. The plate was placed in a MJ thermal cycler (MJ Research, Waltham, MA, USA) and incubated for 1 h at 65°C and 10 min at 100°C. Five microliters were used in 20-µL reactions with the specific primers in standard conditions. Positive agar plates were identified, and the agar was divided into 50 blocks with a sterile scalpel. Each block, representing approximately 400 different phage plaques, was put in a 2-mL Eppendorf tube containing 10 mM MgSO₄. After a 30-min incubation at 37°C with gentle shaking, 100 µL were placed in PCR tubes, and the Proteinase K/PCR procedure was resumed. Ten microliters from the positive plug supernatants were serially diluted in 100 µL MgSO₄ in six successive 10-fold dilutions. Each of these dilutions was mixed with an equal volume of KH802 bacteria and incubated for 20 min at 37°C, mixed with 5 mL 0.5% LB-agarose, plated on an LB-agar plate, and incubated overnight at 37°C. The next morning, 2 mL 10 mM MgSO₄ were added, and the supernatants were collected from the plates and tested by PCR. The agar from the positive plate with the lower phage concentration out of the six tested dilutions was cut again in 50 plugs representing 10–20 different plaques, and the procedure was resumed. After three successive steps, for the two PCR systems tested, the lower positive dilution contained less than 100 clones. Each clone was picked with a sterile toothpick into 10 µL water and incubated 10 min at 100°C. The PCR mixture was then added to a final volume of 20 µL, and the samples were subjected to cycling according to the standard protocol, allowing the identification of the positive clones. An example of this last screening test is given in Figure 1 with primers designed for the mouse homologue of PISRT1. Because of the weak level of interspecific sequence conservation, a specific amplification product of only 81 bp could be defined. In such a case, PCR screening is the alternative of choice compared with classical hybridization. At the last step, 28 individual phage clones were picked, of which

Figure 1. PCR for PISRT1. Twenty-eight phage plaques from the last screening step were tested by PCR for the presence of mouse homolog of PISRT1. PCR products were loaded on an ethidium bromide-stained 4.5% agarose gel. The strong band corresponds to the expected amplified product at 81 bp (PISRT1m) for 17 of 28 clones (lanes 1–28). The smaller bands that are sometimes visible are primer dimers. The size marker (1 kb; Invitrogen, Carlsbad, CA, USA) is positioned on both size of each row of the gel and duplicated on the left side with enhanced contrast to render two of the smaller bands visible (154 and 134 bp).
17 were positive in PCR.

One apparent pitfall of the technique was the fact that reactions were carried out first on the plate supernatants and then on individual plugs from the plates, suggesting the possibility of a general contamination of the plugs. We could see that it was not the case and that the contamination level was very low compared with the signal obtained with positive plugs. Therefore, this technique makes it possible to screen a phage library in about a week, without the expense of membranes, and at a high stringency using PCR.

REFERENCES


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Pseudogene-Free Amplification of Human GAPDH cDNA

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RT-PCR has become a common method in molecular biology laboratories involved in research, diagnostics (9), and forensic medicine (4,7,10). Much effort has been spent to improve this powerful method (12). However, there is still an ongoing discussion about the reliability of the method for quantitative mRNA analysis. One reason is the possibility of amplifying contaminating DNA, which is normally easily distinguished from the cDNA PCR product by its greater size because of the presence of introns. However, if a pseudogene exists that arose because of retrotransposition of mRNA (6), then amplification of cDNA and contaminating DNA results in PCR products of the same sizes. This would lead to false-positive results or at least to an unknown error in the quantitative data. A complex method that avoids pseudogene amplification was introduced (11), but specific primers that fail to amplify related pseudogenes are rare. Housekeeping genes are used to normalize quantitative RT-PCR data. Here accurate determination of cDNA concentration is essential. Primer pairs for human and rat β-actin, which do not amplify their corresponding pseudogenes, were designed (5,8). We demonstrate the problem and then solve it for the human housekeeping gene GAPDH (1) since a corresponding pseudogene (2) exists and the GAPDH mRNA (13) is frequently used to normalize RT-PCR.

First, genomic DNA was isolated from human whole blood with the QIAamp® DNA Blood Mini Kit (Qiagen, Hilden, Germany). No RNase treatment was performed. The human monocytic cell line U-937 (ACC 5) obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) was used as RNA source. Total RNA was isolated with the RNeasy® Mini Kit (Qiagen). No DNase treatment was performed. Subsequently, cDNA was synthesized from total RNA using the Reverse Transcription System (Promega, Mannheim, Germany). The reaction volume of total 20 µL contained 1 µg RNA, 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton® X-100, 1 mM each of four dNTPs, 5 mM MgCl2, 20 U recombinant RNasin® ribonuclease inhibitor, 0.5 µg Oligo(dT)15 Primer, and 15 U avian myeloblastosis virus reverse transcriptase. The mixture was incubated at 42°C for 60 min and then heated at 95°C for 5 min to inactivate the reverse transcriptase.

Second, 1 µL cDNA and 100 ng genomic DNA were amplified simultaneously under the same conditions. Initially, nucleic acids were denatured at 95°C for 3 min, amplified with 29 cycles of 95°C for 30 s, 60°C for 45 s, 72°C for 45 s, and finally extended at 72°C for 5 min in a Mastercycler® gradient (Eppendorf, Hamburg, Germany). The reaction volume of total 25 µL contained 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100, 200 µM each of four dNTPs, 1.5 mM MgCl2, and 0.2 U Dynazyme™ II (Biometra, Göttingen, Germany). The human GAPDH sense primer 5′-TGAAGTCCGAGTCAA-CGGATTGCT-3′ and antisense primer 5′-CATGTTGGGCGATGAGTTCC-AACC-3′ (BD Biosciences Clontech, Heidelberg, Germany) were used at a final concentration of 200 nM. The amplification of cDNA and genomic DNA resulted in a PCR product of the same size (Figure 1). The PCR product size

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![Figure 1. Amplification of cDNA and genomic DNA. PCRs were performed from cDNA (lane 1) and genomic DNA (lane 2) as described using a commercially available human GAPDH primer pair for RT-PCR. Negative control (lane 3) without nucleic acid. PCR products were separated on a 1.5% agarose gel and visualized by ethidium bromide staining. The gel was photographed by a Polaroid® camera. Phage X174 DNA-HaeIII digest and λ DNA-HindIII digest (Biometra) served as a molecular weight marker (M).}