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Systematic screening of an arrayed cDNA library by PCR

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ABSTRACT **We have developed a PCR-based method for rapid and effective screening of arrayed cDNA libraries. This strategy directly addresses the limitations of conventional hybridization-based schemes and provides a more rapid, cost-effective, and sensitive method compatible with large-scale and routine cDNA clone recovery. To prepare arrayed libraries, 1–2 × 10^6 cDNA clones were propagated as individual plaques on solid medium in 24-well culture dishes at ≈250 plaque-forming units per well. Phage suspensions were prepared from each well and transferred to a 96-well format. To screen the library, pools were generated that correspond to each individual 96-well plate and to each row and column within "blocks" of six plates each. Library screening for specific cDNA clones was conducted in a systematic and hierarchical fashion beginning with the plate pools. Next, the row/column pools corresponding to each positive plate pool were screened. Finally, isolated clones from within each positive well were identified by hybridization. We have applied this approach to the screening of an arrayed human brain cDNA library resulting in the recovery of cDNAs corresponding to >25 genes and expressed sequence tags.**

The efficient identification and recovery of expressed sequences from large segments of complex genomes are central to the development and integration of long-range physical and transcription maps. Construction of such maps facilitates the ongoing efforts of the genome initiative and heightens understanding of genome structure and organization as well as the molecular basis of development and disease.

Three basic strategies have emerged that are amenable to large-scale identification of expressed sequences. (*i*) Exon amplification (1–4) employs *in vivo* splicing systems to identify and select for functional splice sites in genomic DNAs, resulting in the generation of cloned exon libraries. (*ii*) Hybrid selection (5, 6) relies on hybridization of cDNA fragments to immobilized genomic DNAs to enrich for cDNAs encoded by specific genomic clones. (*iii*) Automated partial DNA sequencing of random cDNA clones produces catalogs of expressed sequence tags (ESTs) (7–10). All three strategies result in the identification of short (100–400 bp) cDNA fragments that, when localized relative to markers of known map position, pinpoint the location of an expressed gene (8, 9, 11–17). Subsequent closure of regionalized transcription maps, and/or in-depth analysis of genes identified as exons and/or ESTs, requires the recovery of a more full-length clone or cDNA.

Standard methods for the isolation and recovery of cDNA clones have changed little since they were first introduced over a decade ago (18, 19). These methods, plaque/colony hybridization, are limited by several inherent drawbacks that are contrary to larger-scale screening efforts. (*i*) Filter hybridization screening is labor intensive and time consuming, typically requiring three or four rounds of plating to purify clones. Practical limits to the number of filters that can be screened can lead to insufficient library complexity, resulting in the omission of less abundant clones, and may preclude the parallel screening of multiple probes. (*ii*) Filter hybridization often requires optimization of "signal-to-noise" ratios on a probe-to-probe basis. Variations in probe length, (G+C) content, and the presence of repetitive elements or homologous sequence motifs can contribute to background hybridization and lead to the pursuit of undesired cDNAs. (*iii*) Filter replicas of cDNA libraries have finite life-spans and must be periodically regenerated, resulting in the rapid consumption of unique and valuable resources.

Here, we report an effective and high through-put system for arraying and screening cDNA libraries. This system directly addresses the limitations of phage/colony hybridization schemes and provides a more efficient and sensitive screening method compatible with large-scale and routine cDNA clone recovery.¶

MATERIALS AND METHODS

Arraying and Pooling the Library. Approximately 250 phage were plated in 0.1 ml of NZYM low-melt top agar overlays in each well of 192 24-well culture dishes containing 1.0 ml of NZYM bottom agar. Following incubation at 4°C for 30 min and 37°C for 18–24 hr (until near-confluent lysis) phage suspensions were prepared from each individual well by the addition of 1 ml of SM buffer followed by incubation at 4°C for 1 hr. Each phage suspension was transferred to a unique well within a deep-well, polypropylene 96-well microtiter dish (see Fig. 1*A*). One hundred to 200 μl of $CHCl_3$ was added to each well to lyse host bacteria. Pools were manually collected corresponding to each 96-well dish (plate pool) and to each row or column (row and column pools) within every "block" of six dishes as diagramed in Fig. 1*B*. Media, host bacteria, and buffers for phage propagation and recovery were prepared as directed (20).

PCR Screening of Library Pools. Phage pools were prepared for PCR analysis by diluting 1:5 in 0.1% Nonidet P-40/0.25× TE (2.5 mM Tris, pH 8.0/0.25 mM EDTA). Screening was initiated with the complete collection of individual plate pools. The 20 row and column pools relevant to each positive plate pool were then screened to reveal the unique address of the positive well within each plate (see Fig. 2). PCRs were carried out in a total reaction volume of 25 μl consisting of 1 μM of each primer and 1.25 unit of *Thermus aquaticus* DNA polymerase in 50 mM KCl/10 mM Tris, pH 8.0/1.5 mM $MgCl_2$/0.01% gelatin/300 μM dATP, dCTP, dGTP, and dTTP with the following cycling parameters: 94°C for 3 min followed by

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Abbreviations: EST, expressed sequence tag; NF2, neurofibromatosis type 2.

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. L38282).

35–40 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min followed by 72°C for 10 min.

PCR Primers. Oligonucleotide primer sequences are as follows:

30a-1 5′-CTGTAGTCCCAGCCTGCTTA-3′

30a-2 5′-TGACTTAGAATGCCTGACCA-3′.

Hybridization Screening of Library Pools. Competent host bacteria were plated in NZYM low-melt top agar overlays on NZYM plates. Following incubation at 37°C for 1–2 hr, 2 μl of each phage pool to be screened was spotted onto the plate. Plates were incubated at 4°C for 3–4 hr and then at 37°C for 18–24 hr. Duplicate lifts were made for each set of "plaques."

cDNA Libraries. The human adult frontal cortex cDNA library was cloned into λZAP and purchased from Stratagene. The human fetal kidney cDNA library was cloned into λgt10 and purchased from Clontech. The human fetal brain cDNA library was cloned into λgt11 and purchased from Clontech.

Cell Lines. Human/hamster somatic cell hybrid J1-2 retains ≈12 Mbp of human chromosome 11p15 as its sole human component (ref. 21; D.J.M., unpublished data).

RESULTS

The cDNA library pooling strategy was designed with regard to the following considerations. (*i*) The library structure should support sufficient complexity to maintain representation of low-abundance clones ($>1 \times 10^6$ primary plaques). (*ii*) Primary clones should be propagated as individual plaques on solid medium to reduce representation bias due to differences in the growth characteristics of individual phage. (*iii*) The pooling of individual clones would clearly be impractical. Rather, a series of low-complexity clone pools should serve as

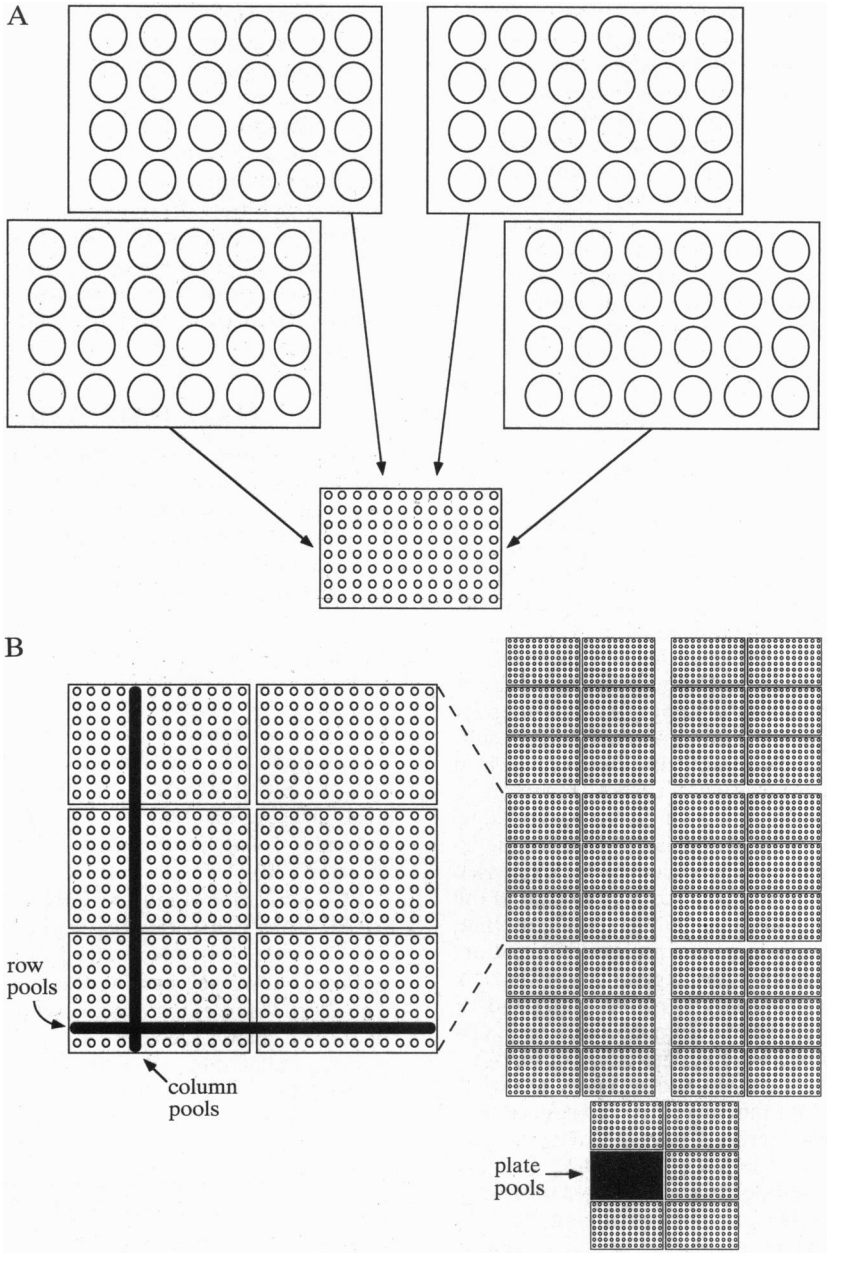

FIG. 1. Schematic representation of cDNA library configuration and pooling. (*A*) Primary clones were propagated as individual plaques on solid medium in 24-well culture dishes. Following near-confluent lysis, phage suspentions were prepared from each well by elution with SM buffer and transferred to 96-well microtiter dishes. (*B*) The 96-well microtiter dishes were arrayed into eight blocks of six plates each. Row, column, and plate pools were collected as described in the text.

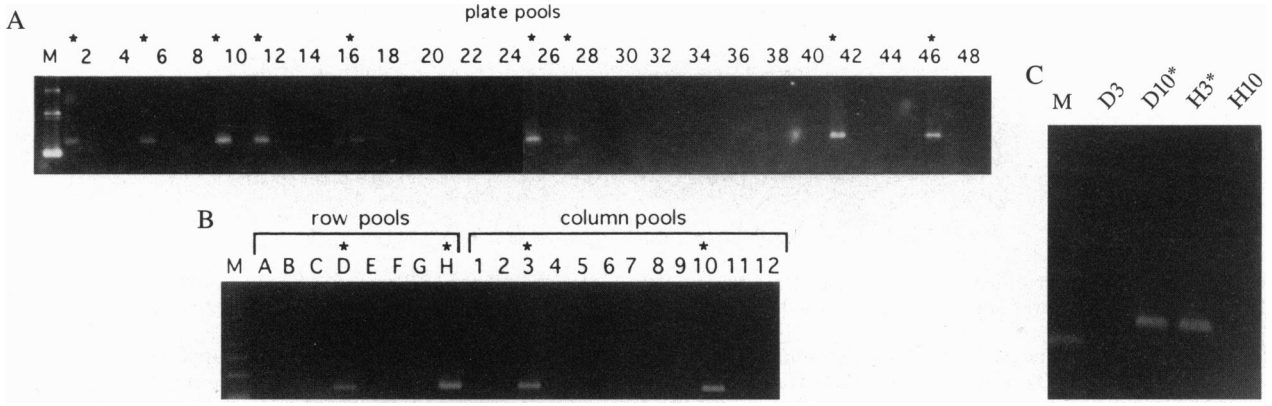

FIG. 2. PCR screening of arrayed brain cDNA library for cDNA clones containing exon 30a. (*A*) Library plate pools were screened by PCR with primers specific for exon 30a (see text). Positive signals (*) are found in plate pools 1, 5, 7, 11, 16, 25, 27, 41, and 46. (*B*) Row and column pools specific for plate 11 screened by PCR for exon 30a. Positive signals (*) are found in rows D and H and columns 3 and 10. (*C*) Individual wells from plate 11 screened by PCR for exon 30a. Positive signals (*) are found in wells D10 and H3. DNA size markers (M), 123-bp ladder.

the endpoint of PCR-based screening. These simple clone pools should maintain a complexity low enough to ensure subsequent single-step plaque purification (<500 plaques). (*iv*) Each of the simple pools should be uniquely addressed by the minimum number of complex pools that supports efficient PCR detection of single clones. Reports from the literature (22–24), as well as unpublished results from our laboratory, indicate that pool complexities in excess of 150 Mbp are consistent with reliable PCR detection of single loci.

Fig. 1 outlines the arrayed cDNA library pooling scheme. Competent host bacteria and bacteriophage stock were mixed and incubated at 37°C to allow adsorption of phage to host cells. The resulting phage/host conjugates were then suspended in molten top agarose and aliquoted [≈250 plaque-forming units (pfu) per well] into each well of 192 24-well culture dishes containing hardened bottom agar ($1–2 \times 10^6$ pfu total). Plates were incubated at 37°C until near-confluent lysis had been achieved. Phage stocks were prepared from each well by diffusion into SM buffer and transferred to a total of 48 96-well microtiter plates (Fig. 1*A*). Pooling was accomplished by arraying the 48 96-well microtiter plates into eight "blocks" of six plates each (Fig. 1*B*). Row and column pools were collected from each block, resulting in 24 row and 24 column pools per block (Fig. 1*B*). Each row or column pool contains phage from $\approx 6 \times 10^3$ primary plaques (24 wells × ≈250 phage per well). Similar pools are collected from the 96 wells corresponding to each of the 48 96-well microtiter plates ($\approx 2.4 \times 10^4$ primary plaques; 96 wells × ≈250 phage per well) (Fig. 1*B*). Library screening for specific cDNA clones was conducted in a systematic and hierarchical fashion beginning with the 48 plate pools. Next, the 20 row/column pools (8 row and 12 column) relevant to each positive plate pool were screened in a similar fashion. The exact well location of cDNA clone(s) positive for each PCR assay was determined by the intersection of positive row and column pools within the array (Fig. 1*B*). Finally, isolated clones from within each positive well were identified by plating at low density and screening by the method of Benton and Davis (19) using the radiolabeled PCR product as probe.

A human brain (adult frontal cortex) cDNA library has been arrayed and assayed by PCR as an initial test of the strategy outlined in Fig. 1. As a result, we have recovered cDNA clones corresponding to >25 known genes and anonymous ESTs. Experiments leading to the isolation of a cDNA clone that includes one such EST are shown in Fig. 2. This particular EST, exon 30a, was developed from a library of human chromosome 11p15 specific amplified exons (D.J.M., E.B., R.L., A.B., and D.E.H., unpublished data). A 154-bp PCR product representing exon 30a is clearly visible in 9 of the 48 plate pools (Fig. 2*A*). Subsequent screening of the row and column pools specific for one of these plate pools (plate 11) detects positive clones in rows D and H and columns 3 and 10 (Fig. 2*B*). These results indicate that a minimum of two wells on plate 11 contain cDNAs corresponding to exon 30a. Screening of individual wells D3, D10, H3, and H10 on plate 11 demonstrates that wells D10 and H3 were responsible for the positive row, column, and plate pool signals (Fig. 2*C*). Approximately 1×10^3 clones from well D10 were plated on a single 150-mm plate and screened by the Benton and Davis plaque lift method (19). Several isolated cDNA clones were identified based upon their hybridization to radiolabeled exon 30a PCR product. One such clone was sequenced with the same pair of 30a specific oligonucleotide primers that were used in the PCR screen of the library. A perfect match of the 30a exon sequence, located

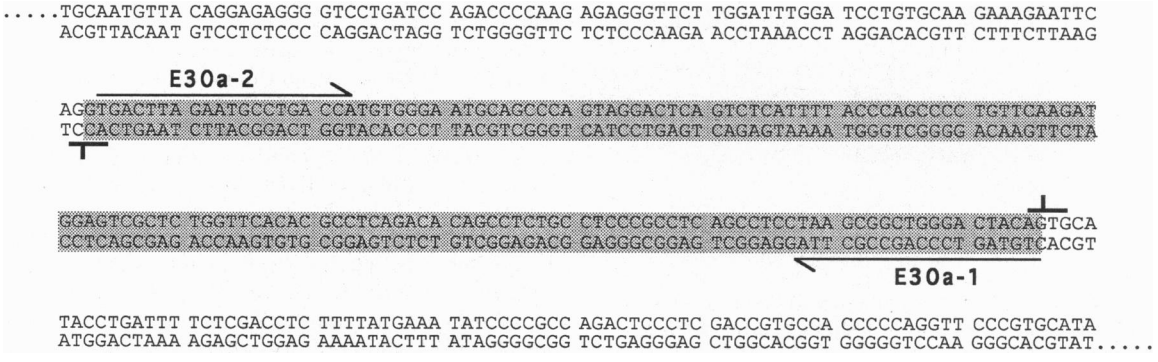

FIG. 3. Sequence analysis of cDNA 30a. cDNA 30a was sequenced by the dideoxynucleotide chain-termination method (25) using oligonucleotides E30a-1 and E30a-2 (arrows). The sequence of exon 30a is indicated by shading.

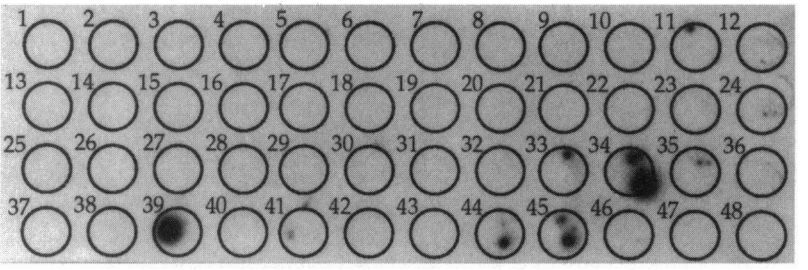

FIG. 4. Hybridization screening of arrayed brain cDNA library plate pools with radiolabeled exon 49c. The arrayed brain cDNA library plate pools were screened by hybridization with radiolabeled exon 49c. Strong positive signals are found in plate pools 34 and 39.

within the 30a cDNA clone, verifies that a *bona fide* 30a cDNA has been isolated (Fig. 3). Furthermore, consistent with the previously determined map position of the 30a exon, we have been able to localize the 30a cDNA to a specific *Not* I fragment on human chromosome 11p15.5 (26).

We have found that the arrayed cDNA library pools are also amenable to screening by hybridization. This was accomplished by spotting aliquots of each pool onto lawns of competent host cells immobilized in top agarose. Following incubations at 4°C, to allow efficient formation of phage/host conjugates, and 37°C, to allow lysis and phage propagation, the resulting pool-plaques were transferred to nylon membranes and hybridized with radiolabeled probe. Fig. 4 illustrates screening of the arrayed brain cDNA library plate pools by hybridization with one of our human 11p15 amplified exons. Exon 49c (134 bp) is clearly detected in plate pools 34 and 39 (Fig. 4). Subsequent screening led to the identification and isolation of a cDNA clone that included the exact nucleotide sequence of exon 49c (D.J.M., R.L., and D.E.H., unpublished results). Southern hybridization of cDNA 49c to blotted DNAs from a somatic cell hybrid that retains 11p15 as its sole human component clearly demonstrates that cDNA 49c maps to human chromosome 11p15 (Fig. 5). In other experiments we have further mapped this cDNA to a specific *Not* I restriction fragment within 11p15.5 (26).

These experiments demonstrate the effectiveness with which cDNA clones can be recovered from arrayed cDNA libraries. Typically, the time from initial PCRs to plaque-purified clone is 4 days. Furthermore, we have found that the manipulations required for library screening by PCR can easily be carried out for multiple ESTs in parallel and, with compatible PCR systems, in multiplex (D.J.M., E.B., R.L., D.P., and D.E.H., unpublished data).

DISCUSSION

In this report we describe an effective strategy for PCR screening of arrayed cDNA libraries that is amenable to high through-put. We have applied this approach to the screening of a human brain (adult frontal cortex) cDNA library that has been configured and pooled as outlined in Fig. 1. In initial tests we have been able to recover cDNA clones corresponding to >25 known genes and ESTs. The experiments leading to the recovery of these cDNAs clearly demonstrate that this system offers significant advantages over plaque/colony hybridization methods in terms of (*i*) sensitivity, (*ii*) specificity, and (*iii*) efficiency.

(*i*) *Sensitivity.* PCR-based approaches to library screening are considerably more sensitive than those that rely solely on hybridization (22). As a direct consequence it is practical to routinely screen libraries of sufficient complexity to ensure representation of lower-abundance clones ($>10^6$ clones). This enhanced sensitivity has enabled us on two separate occasions to identify and recover cDNA clones from the arrayed form of the brain library (Fig. 1) that were not detected when the same library was screened by plaque hybridization. The most notable of these cDNAs proved to be the neurofibromatosis type 2 (NF2) tumor suppressor gene (15) while the other represented an anonymous 11p15 amplified exon (D.J.M., R.L., E.B., and D.E.H., unpublished data).

(*ii*) *Specificity.* A second advantage over purely hybridization-driven approaches is the inherent specificity of PCR. Distinguishing between the desired cDNA and homologous clones that share related sequence motifs or membership in gene "families" can be difficult with conventional hybridization-based methods. However, as demonstrated in Fig. 2, screening by PCR provides confirmation of putative positive clone identity at every stage of screening via the characteristic size of each amplification product. This proved to be especially advantageous as we screened the brain library for "full-length" variants of the human MLH DNA mismatch repair gene with a set of oligonucleotide primers developed from sequence information available in the literature (27). Preliminary experiments established that while the MLH cDNA was well represented, the vast majority of clones contained out of frame deletions of up to 140 bp in length. Systematic PCR analysis of each successive pool of clones allowed us to identify wells containing the desired clone. As a result, a full-length cDNA was quickly recovered (D.J.M., D.E.H., and J. Pelletier, unpublished data).

(*iii*) *Efficiency.* PCR-based screening of arrayed libraries is considerably more efficient than plaque/colony hybridization with respect to time and effort. Conventional screening of cDNA libraries by plaque/colony hybridization typically requires three or more rounds of screening to obtain isolated clones. In contrast, PCR screening of arrayed libraries can be completed in 4 days or less. Furthermore, the manipulations required for PCR screening are such that several clones can readily be isolated in parallel and, with compatible PCR systems, in multiplex (D.J.M., E.B., R.L., D.P., and D.E.H., unpublished data). Another factor contributing significantly to the overall efficiency of this system is the library configuration in microtiter dishes. Although somewhat costly, in terms of initial preparation, arrayed libraries possess innate advantages

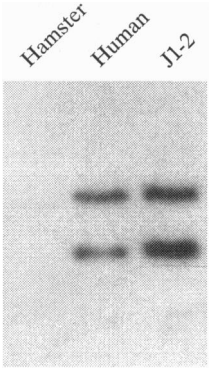

FIG. 5. Mapping of cDNA 49c by Southern analysis of somatic cell hybrids. Radiolabeled cDNA 49c was hybridized to a Southern blot containing genomic hamster, human, and somatic cell hybrid J1-2 DNAs that had been digested with *Hin*dIII. Fragments of 12 kbp and 5.3 kbp are detected in the human and hybrid J1-2 lanes.

over libraries that exist solely as pools of clones. With more conventional hybridization-based schemes, the filter replicas of cDNA libraries and the "master plates" from which the filters were derived have limited life-spans. These resources must periodically be regenerated, the net result being lost time, effort, and library stock. On the other hand, arrayed library pools and individual well stocks can be stored indefinitely at −80°C in 10% dimethyl sulfoxide (20) and represent enough material for >100,000 individual screens. In essence, once arrayed and pooled, these libraries represent an essentially permanent and maintenance-free resource. Another advantage, in terms of large-scale screening efforts, is that arrayed libraries are amenable to the development and application of automated screening at all stages with the possible exception of the final clone purification step. Finally, an added, although somewhat indirect, advantage of this system is that the same set of oligonucleotide primers developed for PCR-based clone identification/isolation can be used in sequence analysis to rapidly and unequivocally confirm that the desired clone has been isolated (Fig. 3).

The same principles that we have utilized here to screen a phage cDNA library can also be directly applied to the PCR screening of plasmid cDNA libraries. Likewise, with slight modification, a similar strategy can be adapted to the effective screening of expression libraries (28–30) by ELISA or RIA. Further development of this method should involve configuration of additional libraries representing a broad spectrum of tissues and developmental stages. Toward this end we have recently arrayed two human cDNA libraries derived from fetal tissue, one kidney and one brain (described in *Materials and Methods*). Of course, to fully exploit the potential of this system future targets will include normalized (31, 32) and primary (unamplified) libraries.

We believe that this method will contribute significantly to expanding the catalogue of cloned and sequenced cDNA clones. We have found it to be especially powerful when used in conjunction with techniques such as exon amplification (1, 2), which can be used to develop ESTs from defined physical locations without regard to the expression level of individual transcripts.

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