A novel ligation mediated-PCR based strategy for construction of subtraction libraries from limiting amounts of mRNA

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Construction of subtraction libraries has become a popular method for isolating differentially activated genes. Several methods have already been published, but all of them require either ≥10 µg of polyA+ RNA (1), a large amount of total RNA (2) or a pre-made cDNA library (3–7). This could be a problem when dealing with expensive or scarce cell lines or tissues. The recent development of the ‘differential display’ technique (8) represents one means of overcoming the requirement for a significant amount of RNA, but some differentially expressed transcripts may not be isolated by this procedure because of peculiarities in their sequences. Here I present a ligation mediated-polymerase chain reaction based subtraction strategy which is quick, efficient, moderately expensive, and requires ≤0.5 µg polyA+ RNA.

Generally, subtraction libraries are made through a few common steps. Single-strand nucleic acids (either sense or antisense) are generated from normal cells. These nucleic acids are usually termed ‘driver’ sequences. Nucleic acids which are complementary to driver sequences are collected from activated cells and are called ‘target’ sequences. Target sequences are then removed. The remaining sequences represent a population of messages either exclusive to target cells or expressed in huge excess in target cells over driver cells.

For my particular purposes, I wished to isolate genes that are differentially expressed during macronuclear development in the ciliated protozoan *Euplotes crassus*. During the 100 h period that follows mating, this organism transforms a copy of its micronucleus into a transcriptionally active macronucleus via a process involving extensive genome rearrangement (reviewed in 9,10). Little is known concerning the molecular mechanisms mediating these processes, and isolating genes differentially expressed during macronuclear development represents one potential means of initiating studies in this area.

The strategy employed for isolating such genes is outlined in Figure 1. An *Ecrassus* cDNA library was made from vegetative cell mRNA (LEVR library; 11) in the λ UniZAP XR vector (Stratagene). This vector allows the conversion of a page library to a plasmid library (12), which after linearization with *XhoI*, can be *in vitro* transcribed with T3 RNA polymerase to generate sense (polyA+) RNA. Using this system driver RNA sequences were generated which were biotinylated to aid in their removal during the subsequent subtraction step.

A mixture of mRNA (0.5 µg) isolated from cells between 25 and 44 h of macronuclear development were used to generate target nucleic acids. During this process, these molecules were tagged at both ends with specific oligonucleotides, so that those molecules remaining after subtraction could be amplified via PCR. The 3′ tag is the oligo-dT-*XhoI* adapter–primer (Stratagene cDNA Synthesis Kit) added during cDNA synthesis. The 5′-tag (the ‘anchor adapter’) from an AmpliFINDER RACE kit (Clontech) was then ligated to the cDNAs using single-strand RNA ligase and other reagents. A quantity of tagged cDNA (0.2 µg) was recovered.

For the subtraction procedure, the adapter-tagged target cDNA (0.2 µg) was hybridized to the biotinylated driver RNA (6.5 µg) as described in Duguid et al. (7). Target–driver hybrids and unhybridized driver DNA were then removed by passage over a streptavidin agarose column (see Fig 1 legend for details). Subtracted RNAs were next amplified by PCR. The primers used were the ‘anchor primer’ from the AmpliFINDER RACE kit (Clontech), which is complementary to the anchor adapter and contains an EcoRI restriction enzyme recognition site, and the oligo-dT *XhoI*-adapter primer (Stratagene). The PCR products were cloned into λ UniZAP XR after purification, restriction enzyme digestion and size selection (following standard methods). Cloning and packaging steps were done according to the protocols recommended by Stratagene.

Four hundred clones containing inserts were generated and 27 of them were chosen from this library (LESR library) for further analyses. Northern hybridizations to total RNA isolated from both vegetatively growing cells and developing cells were carried out using the inserts as probes (Fig. 2). Twenty-three of the 27 clones detected transcripts that were significantly increased during macronuclear development (Fig. 2).

These results indicate that the subtraction strategy was quick and effective in isolating differentially expressed transcripts. Using cDNAs made directly from mRNAs instead of a pre-made cDNA library as a source of target sequences has several advantages. First, it obviates the need to make a cDNA library. Second, construction of a good cDNA library usually requires ≥5 µg polyA+ RNA, which may be too difficult to obtain from rare and/or expensive tissues. Third, libraries, especially λ-libraries are usually amplified to ensure their long term stability, but this amplification process is highly sequence dependent. Thus, there is the possibility that some sequences are lost during amplification. (Using a library as a source of driver sequences in
the current protocol does not pose this problem because the pseudo-positives screened will be eliminated in subsequent screening steps). Fourth, such libraries cannot be used directly as a source of target sequences for subtraction, but must be converted to yield single-stranded forms (3–7). Subsequently, the subtracted nucleic acid must be modified again for cloning. In these respects, the current method is much simpler, because single-stranded target sequences are generated by a single step (reverse transcription) and they become easily clonable in the subsequent PCR step.

In summary, the procedure described represents an efficient means of constructing subtraction libraries in cases where the target mRNA is scarce or limiting, and it may be adapted to many other systems.

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