specific PCR primers for very similar sequences. It is particularly useful in situations in which limited sequence information is available for primer design for at least one of the fragments that is to be amplified. Selective suppression can also be used to specifically amplify individual members of a variety of highly homologous gene families using DNA obtained in the course of population studies of genetic polymorphisms—in essence, using similarity of a sequence as an advantage rather than as an obstacle. Finally, the selective suppression technique will potentially be even easier to apply when thermostable restriction endonucleases become widely available (10).

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Simplified Protocol of Solid-Phase cDNA Libraries for Multiple PCR Amplification


Reverse transcription (RT) followed by polymerase chain reaction (PCR) amplification is a widely used, highly sensitive method to analyze the expression of low abundance messenger RNAs extracted from very small samples (down to one or a few cells). Nucleic acids can also be extracted from limited tissue fragments on slides using microdissection technology (5). In these cases, the number of mRNAs amplified with PCR technology is reduced, even when using multiplex PCR amplification, and then the detection of multiple mRNAs requires multiple sampling. This limiting factor becomes a very important problem; for instance, when the phenotype of a well-delimited cellular group must be precisely determined, or when the sample contains various intermingled cell populations of interest to be characterized.

The use of magnetic beads technology is well adapted to the extraction of small quantities of mRNA and facilitates the production of solid-phase cDNA libraries (2,6). Protocols described for subsequent PCR recommend removal of the beads after the second-strand cDNA synthesis (6). Here we report the use of immobilized cDNA libraries for multiple amplifications with beads present in the PCR tube. The main advantages of this method are (i) simplicity, (ii) economic and time savings, one extraction allowing the amplification of several genes, and (iii) convenient cDNAs storage.

We have developed this method in order to study genes expressed in a very limited region of the rat lateral hypothalamus. Adult male Sprague-Dawley rats (IFFA, Credo, France) were used for this study and killed by decapitation. Brains were immediately dissected and frozen in liquid nitrogen without a fixation step. They were cryostat-sectioned into 20-μm-thick sections thaw-mounted on glass slides. Slides were kept at -20°C until use. Tissue samples...
were taken on individual sections brought to room temperature, using a micromanipulator under a stereomicroscope. Samples were about 500 × 500 × 20 μm (ca. 5 μg). For mRNA extraction, we tested guanidinium thiocyanate buffer and lithium dodecyl sulfate (LiDS)/LiCl lysis buffer (Dynabeads® mRNA DIRECT kit; Dynal, Oslo, Norway) methods. Tissue fragments were collected in RNase-free microtubes containing 10 μl guanidinium thiocyanate buffer or LiDS buffer. Microtubes were kept on ice for 30 min and vortex mixed twice in this time. Lysates were then spun at 12 000 × g for 1 min in a centrifuge, and supernatants were transferred to new vials. Poly(A)+ mRNAs were then extracted from the crude suspension using 5 μl Dynabeads oligo(dT)25 (Dynal), following the manufacturer’s recommendations (2). After three washes with cold RT buffer (Promega, Madison, WI, USA), cDNA synthesis was performed with Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega) as recommended by the manufacturer (60 min at 42°C). At the end of the RT, the samples were denatured 5 min at 95°C. The beads were collected with a magnet, and the RT mixture containing the mRNA was discarded. The beads were resuspended in PCR buffer (75 mM Tris-Cl, pH 9.0, 20 mM (NH₄)₂SO₄, 0.01% Tween® 20, 2.5 mM MgCl₂, 0.2 mM each dNTP, 50 pmol primers and 0.5 μl Taq DNA Polymerase (Goldstar; Eurogentec, Liege, Belgium). The reactions were covered with 50 μL mineral oil, denatured for 5 min at 94°C and subjected to thirty cycles of 94°C for 1 min, 40°C to 55°C (depending on the primers used) for 1 min and 72°C for 2 min in a thermal cycler (Minicycler™; MJ Research, Watertown, MA, USA) with beads present in the reaction. After completion of cycling, the reactions were incubated at 72°C for 10 min and held at 4°C. Three microliters of the first reaction were used for a second round of amplification, with the same primers or nested primers when available. Fifteen microliters of the second PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide (Figure 1).

After the first amplification, beads can be stored at 4°C in the amplification medium for weeks or months. Before performing another amplification, beads were carefully resuspended in the reaction mixture, and hybridized nucleic acid was denatured for 5 min at 95°C. This step proved to be important to prevent adhesion of the beads to the tube and their loss. The beads were then collected with a magnet, and the supernatant was removed. Beads were washed twice in 20 μl PCR buffer and finally resuspended in a PCR mixture containing a new set of primers.

This method enabled us to amplify the DNAs complementary to mRNAs in the perifornical region of the rat lateral hypothalamus. Primers were used to specifically amplify several dihydodeoxyribonucleotides of interest in our laboratory: dynorphin (DYN) (primers: 5′-TGCC-GGGCGTTTGCTCT-3′ sense, 5′-CTGGGACGAGTCCACCAC-3′ antisense, 522 bp; nested primers: 5′-CTCATCAGCCAGGTTGGC-3′ sense, 5′-CCACTTAAAGCTTGGGCG-3′ antisense, 278 bp, annealing temperature of 55°C); secretogranin (SgII) (primers: 5′-AGAGTTGTCCAGACATG-3′ sense, 5′-GGGAGGACCGAGTCACCAC-3′ antisense, 684 bp; nested primers: 5′-CTCAGCAGTTGTTTTATGGAC-3′ sense, 5′-GGGAGGACCGAGTCACCAC-3′ antisense, 324 bp, annealing temperature of 50°C); melanin concentration hormone (MCH) (primers: 5′-CCGCCCACCTCATCAAGG-3′ sense, 5′-AGGTATCGAAGCTTGGCC-3′ antisense, 433 bp, annealing temperature of 58°C); GAPDH, 207 bp (7th amplification); lane 4, PRL, 402 bp (8th amplification); lane 6, CYC, 509 bp (11th amplification) and genomic DNA was not involved in these reactions. We tested the tissue specificity of PCR by using MCH primers on cDNAs obtained from rat pituitary sections; no signal was obtained from immobilized cDNA library, with beads present in the first amplification reaction. mRNA extractions were performed from rat pituitary sections; no signal was obtained from immobilized cDNA library, with beads present in the first amplification reaction.

![Figure 1. 1.5% agarose gel stained with ethidium bromide, showing amplification products obtained from immobilized cDNA library, with beads present in the first amplification reaction.](image-url)
was detected after electrophoresis. This result is correlated with the fact that this tissue does not express this gene (data not shown).

Semi-solid-phase cDNA libraries were shown to be representative of rare tissue mRNAs, as the number of neurons expressing MCH, DYN or SgII is small in our samples: about 30 cells expressing the MCH gene could be counted in representative samples. Furthermore, there is no loss of the amplification signal. When the presence of a transcript has been detected once, it is possible to find it again after several other amplifications. We tested this technique using 5-µm-thick paraffin-embedded sections, as recently shown for DNA extraction (1), and from paraformaldehyde-fixed brain sections. The results were similar to those obtained with unfixed tissue sections. Immobilized cDNA libraries also appeared to be very stable when stored at 4°C, because beads could be reused after a four-months storage, and a sequence that had never been amplified before could be demonstrated.

In conclusion, cDNA libraries, immobilized on magnetic beads, allow multiple gene amplifications from very small tissue samples (100 ng–10 µg range). Then the phenotypic expression of cells present in the sample can be analyzed. This protocol should be of great interest in laboratories studying gene expression in very precious and restricted material.

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Benchmarks

Removal of RT-PCR Inhibitors from RNA Extracts of Tissues


Detection of cytokine mRNA from tissues such as the lung is an important approach in defining the local immune response to disease. Procedures for extracting total RNA such as the use of acid guanidinium thiocyanate-phenol (3) usually provide RNA suitable for use in reverse transcription-polymerase chain reaction (RT-PCR).

By using the acid guanidinium thiocyanate-phenol method and other standard procedures to prepare total RNA, we had difficulty in consistently detecting cytokine mRNA expression from bovine or ovine lung tissue by RT-PCR. The inability to detect these mRNA species may be attributed to either the rapid degradation of cytokine mRNA or the presence of inhibitory substances. The degradation of cytokine mRNA is a finely controlled process due in part to the presence of AUUUA motifs in the 3’ untranslated regions of cytokine mRNA (2). Inhibition of RT-PCR has been identified by Hänni et al. (6) and by Lévesque et al. (7). Lévesque et al. identified heme from blood as one inhibitory substance.

In our attempts to increase the purity of template for RT-PCR, RNA was extracted from 1 g of lung tissue (frozen in liquid N₂, then stored at -70°C until required) using one of the three methods outlined below.

Method A: RNA was extracted as outlined in Chomczynski and Sacchi (3) using guanidinium thiocyanate with further precipitations using lithium chloride (LiCl) to obtain total RNA. Briefly, 10 µL of 8 M LiCl (Merck, Kilsyth, Victoria, Australia) were added to 100 µL RNA resuspended in diethyl pyrocarbonated water (DEPC-H₂O). The solution was mixed and allowed to precipitate on ice for 2 h. The RNA was centrifuged at 15 000 × g for 20 min at 4°C and redissolved in DEPC-H₂O. Precipitation with LiCl was repeated, and the pellet was then washed twice in 70% ethanol and finally resuspended in 100 µL DEPC-H₂O.