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.

REFERENCES

Address correspondence to Florence Fellmann, Laboratoire d'Histologie Embryologie Cytogénétique, Faculté de Médecine, Place Saint-Jacques, 25030 Besançon Cedex, France.

Received 11 March 1996; accepted 18 April 1996.

Florence Fellmann, Jean-Luc Pretet and Dominique Fellmann
CNRS URA5G1 and Institut d’Etude et de Transfert de Gènes Besançon, France

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.

Method B: RNA was extracted as outlined in Chomczynski and Sacchi (3), with the following modifications. In replacement of the second precipitation with isopropanol (3), a precipitation with LiCl was used. The crude RNA was redissolved in 600 µL of guanidium thiocyanate (Fluka Chemicals, Castle Hill, NSW, Australia) (3), and then 60 µL 8 M LiCl were added. The RNA was placed on ice for 2 h, centrifuged at 15,000 × g for 20 min at 4°C and redissolved in DEPC-H2O, followed by a repeat of the LiCl precipitation step. The RNA was then washed twice in 70% ethanol, resuspended in DEPC-H2O and stored at -70°C.

Method C: RNA was extracted in urea and LiCl, instead of the guanidium thiocyanate, followed by further precipitations with LiCl. Briefly, 1 g of tissue was placed in 5 mL of 6 M urea (BDH Chemicals, Kilsyth, Victoria, Australia) and homogenized. To this, 500 µL of 2 M sodium acetate (pH 4.0) were added, followed by 5 mL acid phenol and 1 mL chloroform:isoamyl-alcohol (49:1 vol/vol) (BDH Chemicals). After mixing, the solution was incubated on ice for 15 min and then centrifuged at 10,000 × g for 20 min at 4°C. Five milliliters of the aqueous phase were transferred to a new tube containing 500 µL of 8 M LiCl. The RNA was allowed to precipitate for 2 h on ice, then centrifuged at 15,000 × g for 20 min at 4°C, resuspended in 600 µL 6 M urea and 3 M LiCl and transferred to a 1.5-mL tube. Precipitation with LiCl was repeated, using 60 µL 8 M LiCl, and the pellet was washed twice in 70% ethanol and finally resuspended in DEPC-H2O.

RT reactions were carried out in a 20-µL final volume containing 5 µg total RNA, 2 µL 0.1 M dithiothreitol (DTT) (Life Technologies, Mulgrave, Victoria, Australia), 4 µL 5× RT-reaction buffer (Life Technologies), 20 U RNase inhibitor (Life Technologies), 300 ng random decamers (Ransom Hill Bioscience, Ramona, CA, USA), 4 µL 5 mM dNTPs (Promega, Sydney, Australia) and 20 U reverse transcriptase (Life Technologies). The reaction was incubated at 37°C for 2 h, and then the enzyme reaction was terminated by incubation at 95°C for 2 min.

PCR amplifications were performed containing 2 µL of the RT sample in a 40-µL final volume using a DNA Thermal Cycler (Hybaid, Teddington, England, UK). One unit of Taq DNA Polymerase (Boehringer Mannheim, Mannheim, Germany), 2 µL 5 mM dNTPs and 4 µL PCR buffer (Boehringer Mannheim) were also added. Forty PCR cycles were carried out as follows: 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. PCR primers for cytokines and the class II region of the major histocompatibility complex (MHCII), specific for the DR-β allele, were designed from published bovine or ovine sequences. MHCII primers are used as a positive control, since MHCII is expressed on alveolar macrophages and B cells, as well as activated T cells (5), and these cell types are present in significant numbers in lung tissue. PCR products were visually analyzed after electrophoresis in agarose gels.

RNA was extracted, using the standard acid guanidium thiocyanate-phenol (3) method, from bovine and ovine lung tissue samples taken 30 min after death of an experimental animal.
RT-PCR with the positive control MHCI primers showed no PCR product. However, a product was detectable when further precipitations of the RNA with LiCl (method A) were included in the extraction procedure (Figure 1, lanes 1 and 2). A comparison of RNA obtained by the Chomczynski method and by LiCl precipitation showed no obvious differences (data not shown). Cytokine mRNA, including IL-1α and IFNγ (Figure 1, lanes 3 and 4) were detected, both of which contain 3’ AUU-UA sequences (4,8). These results strongly suggest that some form of inhibitory factor is preventing either the RT or PCR process and that there is no detectable mRNA degradation in tissues taken from animals up to 30 min after death.

Variations of method A were then used to identify the optimal procedure of extracting RNA from lung tissue to give a consistent RT-PCR product. Both methods B and C were not reliable for generating RNA ideal for use in RT-PCR. Upon repeated extractions of various lung tissues, only some extractions provided RNA suitable for RT-PCR. The variable RT-PCR results are most likely due to varying amounts of the inhibitory substance(s) present in the tissues, suggesting that these extraction methods are insufficient and inadequate in removing all the inhibitory substances. RNA from over 30 lung specimens has been obtained that is consistently suitable for cytokine RT-PCR. Generation of RNA from lung parenchyma suitable for RT-PCR thus requires an additional two precipitations of the RNA with LiCl following RNA extraction using guanidinium thiocyanate.

Method A was used to quantitate differences in the efficiency of the RT and PCR reactions of RNA samples obtained with and without LiCl precipitation by inclusion of radiolabeled [33P]dATP in the dNTP solutions. This showed that the RT and PCR process were 80% and 310% more efficient, respectively, following LiCl precipitations. Thus, the total accumulated increase in efficiency was approximately 400% more than that obtained when using RNA not precipitated with LiCl.

We believe that this method provides a simple but effective modification to a widely used method for the extraction of RNA for use in RT-PCR. This method has been developed for the isolation of RNA from lung tissue but is an approach that should also be applicable to other tissues where RT-PCR inhibitors may be present.

REFERENCES


Address correspondence to Nathalie L. Mathy, Centre for Animal Biotechnology, Cnr. Park Dve. & Flemington Rd., Parkville, Victoria 3052, Australia. Internet: natlm@rubens.its.unimelb.edu.au

Received 11 December 1995; accepted 18 March 1996.

Nathalie L. Mathy, Rogan P. Lee and John Walker
University of Melbourne
Parkville, Victoria, Australia

Oven Cooking Bags Allow an Inexpensive Alternative in Oil-Free Competitive RT-PCR


Plastic oven baking bags (Pingvin; Hoechst, Wiesbaden, Germany) are made of a tough heat-resistant plastic that is readily adapted to any application involving moderate heat and pressure. We have discovered that this plastic readily forms a vapor-proof seal with the polycarbonate plastic of the vacuum-formed plastic plates that are commonly used in 96-well polymerase chain reaction (PCR) thermal cyclers. They therefore provide a simple, rapid and inexpensive means for carrying out high-volume semiquantitative competitive reverse transcription (RT)-PCR without the need for expensive accessories using any 96-well format thermal cycler equipped with an after-market heated lid such as the HL-1 (Techne, Princeton, NJ, USA). Our method involves using this heated lid with only minor modifications in combination with sheets of cut oven baking bag plastic to enable oil-free thermal cycling on standard thermal cyclers.

Heated lids are designed to enable oil-free thermal cycling by maintaining the upper part of the reaction vessels at a constant temperature. They are necessary to prevent any evaporation, which may alter the relative concentration of the reaction components in the PCR and therefore significantly affect the efficiency of the cycling protocol. Even though any heating element that can simply maintain a constant temperature above the highest cycling temperature of the PCR over an area the size of the upper portion of the reaction vessels can be classified as a heated lid, this valuable accessory is missing from many older thermal cyclers. Many thermal cyclers purchased in the early 1990’s were not outfitted with heated lids as original equipment, leaving operators faced with the dismal choice of either continuing to use oil overlays, which are messy and extend sample handling time, or having to purchase new thermal cyclers (3). Recently, a