# PROCESSING OF mRNA FROM HUMAN LEUKOCYTES BY BIOMAGNETICAL SEPARATION: COMPARISON WITH CURRENT METHODS OF RNA ISOLATION

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The article refers to the technique of biomagnetical separation (BS) of mRNA using "Dynabeads$^R$ mRNA DIRECT". After a brief survey of RNA isolation methods authors describe a general procedure of BS as well as its concrete application for mRNA isolation from bronchoalveolar cells. Authors report results of specific experiments to assess the efficiency and accuracy of mRNA isolation by BS. The applicability of BS technique for the gene expression studies is discussed in the end of the article.

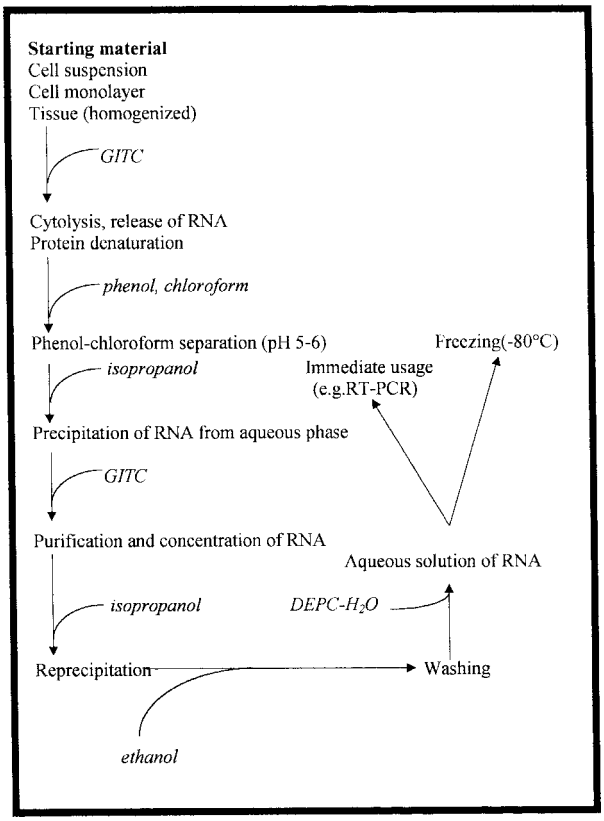
## INTRODUCTION

Today's state of development in molecular biology research is difficult to imagine without methods based on analysis of RNA. Applications of these procedures range from gene expression studies and detection of viral RNA to construction of cDNA libraries for the genetic transfection or determination of interspecies relationship. Each of these applications requires RNA in sufficient amount and of adequate quality.

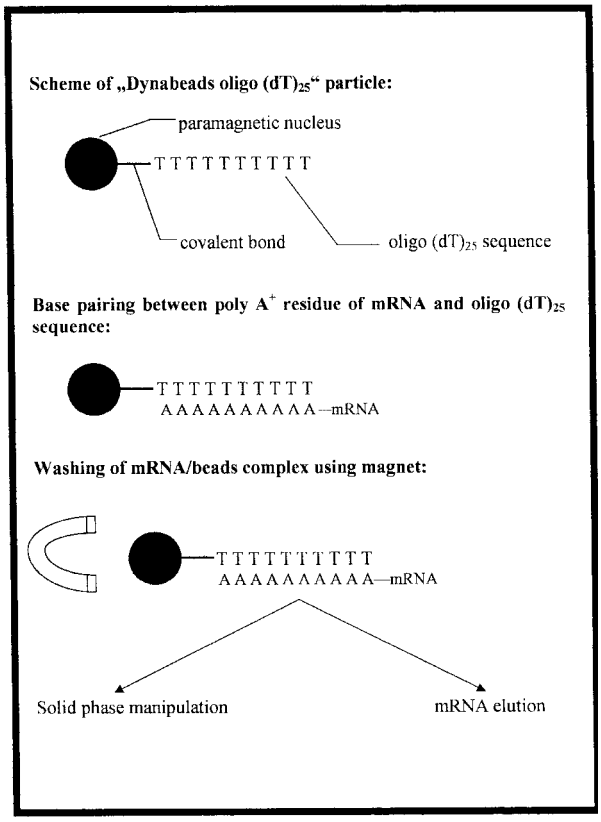
When isolating RNA its biochemical and biological specifics have to be taken into account. In contrast to DNA, elementary biological role of RNA does not consist in long-term conservation of genetic information and, therefore, RNA may be relative instable due to degradation by the enzymatic activity of common ribonucleases (RNAses). For successful and reliable RNA separation RNAse activity must be strictly avoided. Precautions aiming at RNAse elimination vary from technical arrangements (chemicals, glassware and plasticware reserved only for RNA processing) or procedural specifics (low temperature manipulation, fast deproteination of isolation mixture) to reagent additives (non-specific or specific RNAse inhibitors)[1]. Diethyl pyrocarbonate (DEPC), a non-specific RNAse inhibitor, is often used for treatment of reagent solutions, glassware or plasticware. As specific RNAse inhibitors may serve human placental RNAse inhibitor "RNAsin" or vanadyl-ribonucleoside complexes (VCR).

A number of methods has been developed for RNA isolation. The first group, simple cellular extractions, is represented by extraction by boiling[2], cytolysis by detergent Nonidet P40[3] or extraction using cationic surfactant Catrimox-14. These techniques suffice for any unpretentious applications, however, further purification steps are usually necessary for satisfactory results. In contrast, RNA separation using Catrimox 14 combined with phenol-chloroform extraction is very efficient and accurate[4]. Methods of the another autonomous group are based on ultracentrifugation of cellular deproteinated homogenate through a cushion of cesium chloride solution[5]. This technique can be also used for purifying of contaminated RNA from polysaccharides. As standard in RNA isolation one can consider methods based on phenol-chloroform extraction in a number of modifications. In our laboratory, RNA has been isolated by acid quanidinium thiocyanate-phenol-chloroform extraction (**Scheme 1**) either according original protocol[6] or in commercial variant "RNA Blue" (Exbio, Prague). Micro-adaptation of original procedure was developed by Rappolee[7]. Another micromethod is "mini-prep" separation[8] of cytoplasmatic RNA which combines Nonidet P40 cytolysis and phenol-chloroform extraction. The principle of the technique consists in gentle lysis of cytoplasmatic membrane and elimination of intact nuclei.

All above mentioned methods isolate total (cytoplasmatic, resp.) RNA on the basis of its biochemical properties. In contrast, biomagnetical separation of mRNA (BS) is based on selective separation of eucaryotic mRNA or polyadenylated viral RNA taking advantage of presence of polyadenyl (poly A) sequence on 3 end of these macromolecules[9]. As shown in **Scheme 2**, the method works on the basis of specific complementary hybridisation between poly A sequence of isolated mRNA and oligo $(dT)_{25}$ sequence covalently bound to the surface of paramagnetic particles, which will further be referred to as "beads". Beads/mRNA complex originating in the above described interaction can be simply manipulated using magnet and utilized according to the demanded purpose.




**Scheme 1.** Procedure of single step acid-GITC method simplified from Chomczynski & Sacchi[6]
Starting material is initially homogenized (if necessary) and lysed by guanidiniumisothiocyanate (GITC) solution. Total RNA is released into the reaction solution and proteins are denatured. After addition of phenol and chloroform the mixture is thoroughly vortexed. Subsequent centrifugation divides the mixture into aqueous phase, organic phase and interphase. Total RNA contained in the aqueous phase is precipitated by isopropanol. After purification and concentration using GITC, the gained RNA is reprecipitated by isopropanol, washed several times with ethanol and finally dissolved in corresponding volume of DEPC-treated sterile deionized water. RNA solution can be used immediately or stored at –80°C.




**Scheme 2.** Principle of biomagnetical separation of mRNA. Adapted from ref. 9; for description see introduction.

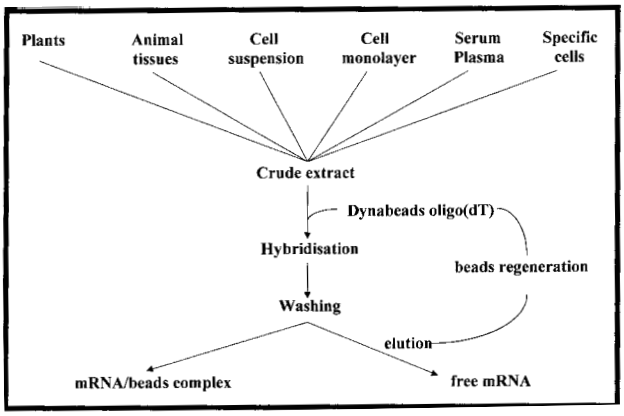
We have tested commercially available version of BS technique named "Dynabeads$^{R}$ mRNA DIRECT" (Dynal, Norway). According to the manufacturer, the kit should be capable to isolate highly purified, intact mRNA directly from crude extracts of plants, animal tissues, cell suspensions or monolayers as well as from the cells specificated by their surface markers (**Scheme 3**). Starting material is lysed by specific lysis solution that gives rise to the crude extract. After addition of oligo $(dT)_{25}$–coated beads to the extract, free mRNA is caught from the reaction mixture to the beads surface and beads/mRNA complex is washed using magnet. Except direct application of mRNA in complex with beads, mRNA may be also eluted which enables to fully regenerate beads up to four times and to reuse them for isolation of new mRNA. The obtained mRNA can be used in many downstream applications which are not inhibited by the presence of paramagnetic beads. Except reverse transcription – polymerase chain reaction (RT–PCR), other possible applications e.g. in vitro translation, Northern blotting, sequencing, subtractive hybridisation have been reported (reviewed in ref. 9). Special possibility of using of mRNA/beads complex is construction of solid phase cDNA libraries[10,11].

The aim of this work was to compare BS technique with standard acid-GITC-phenol-chloroform extraction with regard to its efficiency and accuracy, and further to evaluate its suitability for mRNA isolation from human mononuclear cells recovered from the lung and airways.

## MATERIALS AND METHODS

### Collection and processing of the clinical material for RNA isolation

The bronchoalveolar lavage (BAL) performed according to the standard protocol[12] was used to obtain bronchoalveolar cells. The cells were recovered from the BAL fluid by centrifugation at 200 g, 4 °C for 7 minutes. Peripheral venous blood, collected from cubital vein of healthy subjects, was anticoagulated by EDTA. Peripheral blood mononuclear cells (PBMC) were then separated by density gradient centrifugation using standard protocol[13]. 1% diethyl pyrocarbonate in phosphate buffered saline (PBS-DEPC) was used for final washing of either bronchoalveolar or peripheral blood cells. The number of cells in suspension was de-




**Scheme 3.** General procedure of biomagnetical separation of mRNA. Adapted from ref. 9; for details see introduction.

termined for each sample and cells were pelleted for subsequent RNA isolation.

**Biomagnetical separation (BS) of mRNA: evaluation of efficiency and accuracy**

Efficiency of mRNA isolation by "Dynabeads$^R$ mRNA DIRECT" was tested by experiments designed to compare BS with the standard single step acid-GITC method. Efficiency of isolation by the two compared methods was assessed indirectly based on the results of subsequent analysis of isolated RNA. At first, general methodical procedures are described, description of special experiments follows.

**A. General procedures**

*1. mRNA extraction by biomagnetical separation*

Procedure was performed according manufacturer's instructions[9]. Briefly, lysis/binding buffer (LBB) was added to a cell pellet and complete lysis was achieved by repeated pipetting. The lysate was pressed through a 21-gauge needle to reduce viscosity. In parallel, oligo $(dT)_{25}$-coated beads were prewashed by resuspending in LBB and supernatant was removed using a magnetic particle concentrator (MPC, Dynal). After the cell lysate was added to the beads, hybridisation phase followed (10 minutes) and afterwards, the supernatant was removed using MPC. Beads/mRNA complex was washed repeatedly with two different specific solutions and then it was resuspended in the elution buffer and heated (65 °C) for 2 minutes. The eluate, containing mRNA, was transferred to a new tube and mRNA solution was immediately used as a template for reverse transcription. Beads were regenerated for repeated separation according to manufacturer's instructions. RNAse-free plasticware was used throughout the whole procedure.

*2. Extraction of total RNA by single step acid-GITC method*

The pellet, containing defined number of cells, was lysed in adequate volume of GITC buffer and the lysate was stored in –80 °C until subsequent extraction which was performed according to Chomczynski and Sacchi[6], see Scheme 1. Extracted, total RNA was stored in 50 µl of sterile deionized water with 40 U of human placental RNAse inhibitor (RNasin, Promega) at –80 °C until further use.

*3. Reverse transcription(RT) and polymerase chain reaction (PCR)*

RNA samples obtained by both compared methods were reverse transcribed to cDNA. The reaction was primed by oligo(dT) primer. For information about a composition of reaction mix and reaction conditions see ref. 14. Obtained cDNA was stored at 4 °C.

To determine presence or absence of sufficient amount of cDNA suitable for amplification in the postRT samples, PCR reactions with sequence-specific oligonucleotide primers for β-actin[15] gene were performed. Except β-actin, the primers for chemokine RANTES and SCM-1α gene were also used in addition for amplification of selected cDNA samples (data not shown). For the sequences of used primers, composition of PCR mixes as well as reaction conditions see ref. 14. Negative controls were included within each PCR experiment: cDNA was replaced by sterile water.

The PCR products were visualised by ethidium bromide fluorescence after 2.0% agarose gel electrophoresis (AGE) at 5 V/cm for 20 minutes; the agarose was "Molecular Biology Grade" (Promega). The sizes of the products were distinguished by comparison with PCR marker (Promega). The UV transilluminated gels were photographed on Polaroid 665 negative film.

**B. Specific experiments: efficiency and accuracy of mRNA isolation by BS; applications of BS**

*1. Comparison of parallel size-scaled RNA isolation from mononuclear cells by BS and by acid-GITC method*

PBMC, isolated by density gradient procedure, were used to prepare two identical sets scaled by amount of cells ($0.3 \times 10^6$, $0.9 \times 10^6$, $2.7 \times 10^6$). Total RNA was isolated from the samples of the first set by acid-GITC method and mRNA was separated from the second one by BS; amounts of used reagents were adapted to the size of the samples (data not shown). Gained RNA was reverse transcribed to cDNA and adequate volumes of cDNA solutions (corresponding to the equal amount of starting material for both isolated cell sets) were amplified in β-actin specific PCR. The results were evaluated by comparing the intensity of the bands obtained after electrophoretic separation of the PCR products.

*2. Freezing of the cell lysate after the first step of BS procedure: effects on the mRNA separation*

Two identical samples of PBMC ($0.8x10^6$) were lysed in the first step of BS by LBB. While processing of the first sample continued until reverse transcription of eluted mRNA, processing of the second sample was (immediately after the lysis) interrupted by freezing at –80 °C; the biomagnetical separation followed by RT was completed after 24 hours. The cDNA obtained from these two differentially handled samples was amplified with β-actin specific primers and the amplicons were compared after AGE.

*3. Detection of contamination of cDNA by genomic DNA*

Several randomly selected cDNA samples originating in mRNA gained by BS from bronchoalveolar cells were tested for possible contamination by genomic DNA using PCR amplification of a segment of the human histidyl-tRNA synthetase gene[16]. Genomic contamination in the cDNA sample is revealed by presence of a 400 bp amplification product in addition to usual 128 bp product.

*4. Application of BS to gene expression studies: mRNA separation from bronchoalveolar cells*

mRNA was isolated by BS from 29 samples of freshly isolated bronchoalveolar cells. BS was also exploited to isolate mRNA from 50 samples of bronchoalveolar cells cultivated *in vitro*. The usual size of particular isolation was scaled in a number of cells from $4 \times 10^5$ (cultured cells) to $8 \times 10^5$ (freshly lavaged cells). A solution of free mRNA was obtained by the elution from the beads for each sample and the yield of isolation was checked by β-actin specific RT-PCR and AGE. The cDNA samples were stored at 4 °C and used for chemokine gene expression studies.

## RESULTS

**1. Comparison of parallel size-scaled RNA isolation from mononuclear cells by BS and by acid-GITC method**

As shown in Fig. 1, the PCR reactions with the β-actin specific primers resulted in the products of predicted size of 315 bp, no products were detected in the negative controls. Positive results (bands of the specific size) were obtained for all of the three samples of the size scaled set originating in isolation by BS ($0.3 \times 10^6$, $0.9 \times 10^6$, $2.7 \times 10^6$ of cells), but only for two larger samples of set isolated by standard acid-GITC procedure ($0.9 \times 10^6$, $2.7 \times 10^6$ ). Negative result (no band) was for the remaining smallest acid-GITC sample ($0.3 \times 10^6$). Intensity of the obtained specific bands grew with the amount of starting material.

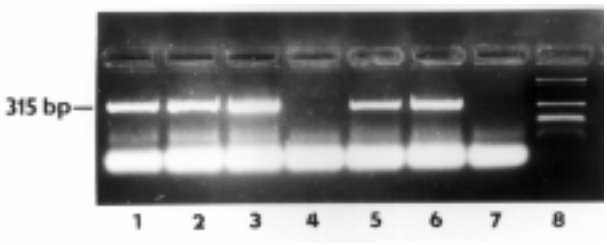



**Fig. 1.** Comparison of efficiency of RNA isolation methods- β-actin specific RT-PCR
Lanes 1–3: Amplicons corresponding to mRNA gained by BS from size scaled mononuclear cell samples ($0.3 \times 10^6$, $0.9 \times 10^6$, $2.7 \times 10^6$ of cells), lanes 4–6: amplicons corresponding to total RNA isolated by standard acid-GITC method from the analogous samples, lane 7: negative control with β-actin-specific primers, lane 8: PCR marker (Promega): the bands are 1000 bp, 750bp, 500 bp, 300 bp, 150 bp and 50 bp from the top. The size of the β-actin amplification product was 315 bp.

**2. Freezing of the cell lysate after the first step of BS procedure: effects on the mRNA separation**

Specific bands corresponding to the β-actin amplification products were obtained for the both tested samples, i.e. frozen and unfrozen in the course of separation. No influence of cell lysate freezing during BS procedure to separation quality was detected: both RT-PCR products were of equivalent intensity (data not shown).

**3. Detection of contamination of cDNA by genomic DNA**

As shown in **Tab 1.**, after amplification of a segment of the human histidyl–tRNA synthetase gene, a single specific product of 128 bp was obtained in 100% of selected cDNA samples. The absence of additional 400 bp amplicon confirms that the selected cDNA samples, originating in mRNA obtained by BS, were free of any genomic contamination.

**Table 1.** Detection of contamination of cDNA by genomic DNA according to Corrochano[16] (for details see Methods)

| Sample | Product size | | Result | |
|---|---|---|---|---|
| cDNA No. | 128 bp[a] | 400 bp[b] | Contaminated | Pure |
| 1 | + | - | - | + |
| 2 | + | - | - | + |
| 3 | + | - | - | + |
| 4 | + | - | - | + |
| 5 | + | - | - | + |

[a] The size of the cDNA-specific PCR product
[b] The size of the genomic DNA-specific PCR product

**4. Application of BS to gene expression studies: mRNA separation from bronchoalveolar cells**

Representative results of the β-actin specific RT PCR of mRNA samples isolated from fresh bronchoalveolar cells are shown in Fig. 2. Presence of the specific amplification products indicates that mRNA was yielded by BS and successfully transcribed to the cDNA. Negative results showing failure of mRNA separation (reverse transcription, resp.) were obtained only for 2 of 29 samples (7%). In case of BS of mRNA from cultured bronchoalveolar cells, the failure rate reached 16%. In contrast, up to 20% of RNA isolations from fresh cells by acid-GITC method used to be unsuccessful in our laboratory, and extraction of RNA using GITC from the small number of cultured cells was impossible.

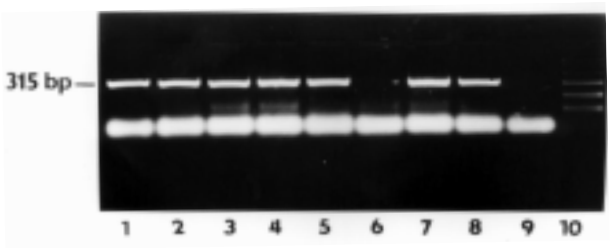



**Fig. 2.** Examination of mRNA samples gained by BS from bronchoalveolar cells by β-actin specific RT-PCR (representative results) Lanes 1–8: Amplicons corresponding to the examined samples, lane 9: negative control, lane 10: PCR marker (Promega). For the details about the amplicon and PCR marker see legend to the Fig 1.

## DISCUSSION

Current possibilities of choice of suitable method for isolation of RNA are extensive. A selection of a technique depends on intentions and laboratory facilities of concrete investigator[1]. Assessing a particular RNA isolation method one must take into account many diverse criteria: amount, integrity and purity of isolated RNA; time, labour and equipment requirements; toxicity of used reagents and also the costs. Techniques based on phenol-chloroform extraction and a number of their commercial variants (RNAzol, RNABlue), have been successfully exploited for RNA separation, and are considered as standard procedures. Recently, biomagnetical separation (BS) of mRNA has been introduced into a palette of RNA isolation techniques, which enables to proceed further and isolate mRNA[9,17]. We have tested commercial version of BS ("Dynabeads$^{R}$ mRNA DIRECT") to separate mRNA from human mononuclear cells and we have compared its results with those of standard acid-GITC phenol-chloroform extraction[6] of total RNA.

There are several ways to compare efficiency and accuracy of isolation of total RNA. At first, it may be directly checked by spectrophotometric determination or by denaturing agarose gel electrophoresis[18]. The presence and purity of isolated total RNA may be appreciated from the electrophoretic results, concentration of total RNA can be also assessed by comparison with the sample of known RNA concentration. However, regarding directly isolated mRNA (e.g. by BS), checking of isolation efficiency by agarose gel electrophoresis is complicated because a large amount of mRNA is required for detection, which is also aggravated by the absence of main ribosomal RNA (rRNA) bands on electrophoretic image[1,18]. Further, to our knowledge no possibility is available for direct determination of concentration of mRNA/paramagnetic beads complex.

We have, therefore, controlled efficiency of our mRNA isolation indirectly by assessment of the results of subsequent application (RT-PCR). Comparison of RT-PCR results based on parallel isolation of mRNA by BS and of total RNA by acid-GITC phenol-chloroform showed that BS is much more efficient for RNA isolation; this was apparent especially in case of the smallest amount of starting material. This feature can be exploited in any procedure requiring division of obtained material into many minute parts such as when examining pharmacological regulation of cytokine expression in cultures *in vitro*. Troubles of reliable mRNA separation in this and similar applications can be successfully avoided using BS.

Simultaneous isolation of RNA from a greater number of clinical samples is often accompanied by technical problems. Possibility of interruption of BS procedure after the first step and freezing of the cell lysate for storage enables to postpone a completing of RNA separation according to investigator's requirements.

On the basis of acquired experience we can report further advantages of the BS technique, first of all speed, and also low labour and equipment requirements. Additional benefit of BS in contrast to phenol-chloroform extraction is the absence of toxicity of the used reagents which would be appreciated by laboratories without capacity for disposing of toxic chemicals. The BS technique is reliable, and once set-up, it can be easily mastered by an inexperienced worker. This cannot be said for tedious and cumbersome acid-GITC method, which fails much more frequently and sometimes yields total RNA contaminated by genomic DNA.

High yield of mRNA obtained by BS is accompanied by its sufficient purity (without contamination by genomic DNA). Technique can be therefore used in applications requiring high purity of isolated mRNA. In addition, mRNA/beads complex can be used for a special application – construction of cDNA solid phase libraries[10,11].

Relative drawback of the BS, due to its specific principle, is its restriction to separation of mRNA; the technique thus cannot be applicated for isolation of further RNA types (rRNA, tRNA), but is quite sufficient for studies of the structural gene expression. Costs of the mRNA separation by BS are higher than those of phenol-chloroform extraction, but this difference is signifi-

cantly reduced by time and labour saving, and also by increased yield of the isolated mRNA.

In conclusion, comparison of BS (version "Dynabeads$^{R}$ mRNA DIRECT") with the standard phenol-chloroform extraction procedure, together with our experience from using of BS for mRNA separation from lung cells, showed high efficiency, accuracy and reliability of the technique. In our opinion, BS is especially suitable for mRNA separation from the small amount of valuable biological material, for example for investigation of the clinical samples gained by invasive procedures.

*ACKNOWLEDGEMENTS*

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