

# High Quality RNA Retrieved from Samples Obtained by Using LMPC (Laser Microdissection and Pressure Catapulting) Technology

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## Summary

Isolation of intact RNA in high quality is the first and often the most critical step in performing many fundamental molecular biology experiments, and is essential for many techniques used in gene expression analysis. As many factors influence nucleic acid preservation, RNA isolation should include some important steps before and after the actual RNA extraction. We tested the influence of fixation and staining protocols regarding RNA integrity and concentration.

A factor that is often underestimated is the absolute necessity for homogenous starting materials. Application of the LMPC technology allows for a rapid and highly precise procurement of purified cell populations suitable for a variety of downstream analyses.

**Key words:** LMPC – Laser microdissection – Gene expression – RNA integrity – Homogeneous material

## Introduction

For RNA experiments expected to generate good results, homogeneous starting material and extraction of high quality RNA from this material are needed. This is of particular importance for e.g. gene expression analysis methods.

The steps involved in acquiring cells or groups of cells from tissues are as follows: tissue harvest, tissue fixation, sectioning, fixation after sectioning, staining,

and finally targeting and capture. Molecular investigations will be carried out by subsequent DNA or RNA extraction and analysis. The analysis of gene expression is based on the investigation of ribonucleic acids and proteins, which, in turn, requires the accurate and reliable isolation of these molecules as undamaged as possible. Archival tissues are most often formalin-fixed and paraffin-embedded. However, RNA extraction from these tissues is less than ideal because of the crosslinking properties of aldehydes. Therefore, this article focusses on the isolation of high quality RNA from cryosections.

Regarding the evaluation of physiologic and pathologic processes, morphologic studies and identification of cell differentiation within tissues have been the basic focus of attention for a long time. It is now generally accepted that whole tissue specimens have important disadvantages when they are used e.g. for gene expression analyses. The application of tissue homogenates and bulk material bears the risk of obscuring genetic deviations, and, as a consequence, leads to expression changes of an individual cell type by the mass of surrounding cells. For example, a section of liver contains hepatocytes, stellate cells, Kupffer's cells, vascular cells, bile duct epithelium, etc. Neoplasms are generally composed of tumor cells, as well as stromal components, inflammatory cells, vessels, and others. Thus, to overcome the problem of tissue heterogeneity, cells

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have to be collected selectively for further analyses [4, 9].

Laser microdissection and pressure catapulting (LMPC™ with PALM's MicroBeam) is the state-of-the-art technology to generate homogeneous material, e.g. for RNA extraction (for information regarding this technology see ref. 6, 11, 12 and Stich's contribution in this issue).

## Materials and Methods

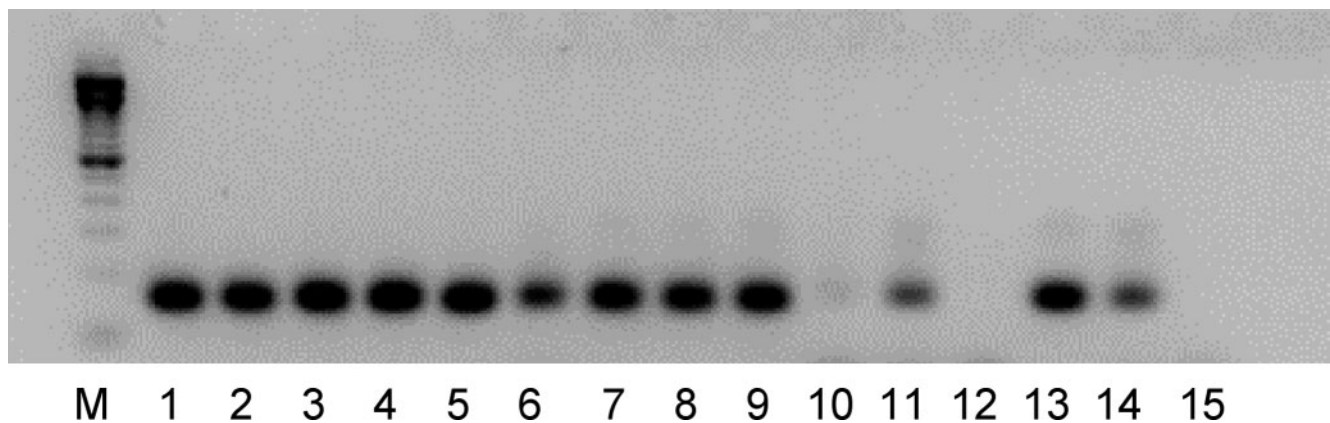
### Tissue treatment

Materials used for microdissection can be of different biologic origin. The way these samples are acquired and processed influences the quality of subsequently extracted RNA. The type of tissue preparation, storage, and fixation are major factors for analytical success [7]. The time between tissue extraction and start of fixation may vary. During this time, at room temperature, enzymes such as endogenous RNases are able to degrade their targets. Therefore, RNA is often of poor quality independent of any microdissection procedures.

Specimens intended for LMPC can be prepared by any of the techniques commonly used in molecular genetics. They can be frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ ; they can be fixed in formalin and embedded in paraffin, and stained with standard chromogenic-histologic, fluorescent or immunohistologic procedures.

If full length cDNA is required for subsequent analyses, tissue specimens should be snap frozen at  $-80^{\circ}\text{C}$  or in liquid nitrogen. If fixation is desired because of easier storage and handling, specimens may be processed in a number of different precipitating fixatives, such as ethanol, methanol/acetic acid, and acetone [2], followed by paraffin wax embedding. It is recommendable to use a non-cross-linking fixative combined with low-temperature embedding. A mild neutral, buffered-formalin fixation can be considered when accepting a higher degradation rate of nucleic acids.

Cross-linking fixatives, such as routine formalin fixation used in surgical pathology departments for clinical specimens, yield highly fragmented nucleic acid of very poor quality. RNA derived from these specimens is significantly degraded compared to snap frozen material. However, this material is still suitable for reverse transcriptase polymerase chain reaction (RT-PCR)-based assays [5] using primer pairs, resulting in small-sized PCR products (best smaller than 200 bp). Fig. 1 is an example of RT-PCR. As the histologic morphology of sections processed by these methods is better, this may be a reasonable compromise for some investigators. Given the amount of archival material embedded in paraffin collected in the laboratories of pathologists, it is hoped that conditions are ultimately developed that permit the extraction of good quality RNA even from formalin-fixed, paraffin embedded samples [8].



**Fig. 1.** RNA was isolated from approx. 2000 murine liver cells. RT-PCR of PBGD gene was performed resulting in a cDNA product of 154 bp. Investigations were made in triplets.

- |            |                                 |
|------------|---------------------------------|
| 1, 2, 3    | HE-stained tissue               |
| 4, 5, 6    | Methylgreen-stained tissue      |
| 7, 8, 9    | Nuclear Fast Red-stained tissue |
| 10, 11, 12 | Methylene Blue-stained tissue   |
| 13         | control 1ng/μl                  |
| 14         | control 0,1 ng/μl               |
| 15         | mixcontrol                      |

### Sectioning of frozen material

Tissue blocks frozen in liquid nitrogen and e.g. embedded in OCT compound in cryomolds, stored at  $-80^{\circ}\text{C}$  until use, are dissected into sections using a cryotome. Four to 10 microns thick cryostat sections were cut. The sections are mounted onto slides. To achieve the best results regarding RNA retrieval, we use membrane-mounted slides (PALM<sup>®</sup>MembraneSlides). Before use, they were made RNase-free by high temperature sterilization for 4 hours at  $180^{\circ}\text{C}$  (PALM<sup>®</sup>MembraneSlides can be also purchased already RNase-free). The sections are air dried on ice for 20 seconds.

### Fixation after sectioning

After mounting the sections onto slides, there are many possibilities of fixing the material [2]. If RNA is intended to be prepared with these frozen sections, we recommend ethanol fixation. This is done (after 20 seconds of air drying on ice, see above) by dipping the mounted sections for 1 to 5 minutes into ice cold ( $-20^{\circ}\text{C}$ ) 70% ethanol.

If OCT or another tissue freezing medium is used, it is important to remove the media on the slide before Laser Microdissection, because these media will interfere with laser efficiency. The removal of the medium is very easily done by gently washing the slide for about 1 minute in RNase-free water. If the sections are stained, the supporting substance is removed “automatically” in the aqueous staining solutions or the diluted ethanols.

### Staining

After tissue processing and subsequent sectioning, tissue sections can be stained with a number of different histologic dyes. For routine histologic staining, the intention is to visualize cell nuclei and cytoplasmic features with sufficient detail to allow for the selection of the target cell population.

Using frozen tissue, one has to be aware of endogenous RNases that may still be active after short fixation steps. Therefore, it is recommended to keep all incubation steps of histochemistry as short as possible. RNase-free water, solutions, and ethanol series should be used. To get solutions RNase-free, they might be treated with DEPC (diethylpyrocarbonate). DEPC destroys enzymatic activity by modifying -NH, -SH and -OH groups in RNases. Hands and dust particles may carry RNases, which are the most common sources of RNase contamination. It is recommended to wear latex or vinyl gloves while handling reagents and RNA samples to prevent RNase contamination from the surface of the skin or from dusty laboratory equipment.

We performed Hematoxylin/Eosin (H&E), Methylene Blue, Methylgreen and Nuclear Fast Red (NFR)

staining applying shortened routine protocols, using RNase-free solutions only. After the last step of staining, for example H&E, we used ethanol series of 70%, 96% and 100% ethanol. Subsequently, the samples were air dried for 5 to 15 minutes. The slides are then ready to use for LMPC (even if they are somewhat wet) or they can be deep frozen at  $-80^{\circ}\text{C}$ .

### Capture of selected material (LMPC<sup>™</sup>)

Sample preparation of highest purity without contamination from unwanted cells is a prerequisite for meaningful expression analyses that could potentially interfere with detection of important transcripts. Laser microdissection and pressure catapulting (LMPC) with the PALM MicroBeam allows for non-contact isolation of individual cells or cell populations in a simple and fast way without any surrounding or contaminating cells [11, 12, and Stich's contribution in this issue].

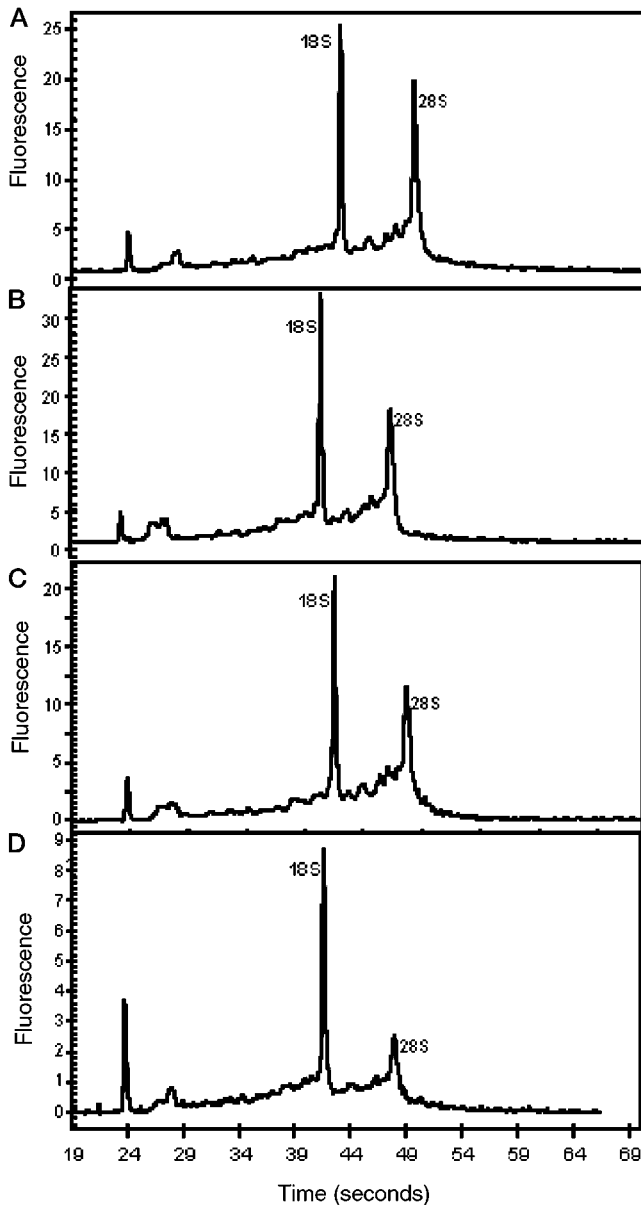
### Isolation of RNA

Strategies for the isolation of nucleic acids involve a number of steps, which can lead to fragmentation and degradation of RNA, resulting in a low recovery of an already limited amount of input material. Different technologies and methods are available for isolating RNA. In general, the methods involve lysis of the starting material, followed by removal of proteins, DNA, and other contaminants. Some points have to be considered for RNA preparation:

1. The value of gene expression studies is principally dependent on the quality of the starting RNA. The samples must be pure and of high quality. All chemicals and solutions used must be free of RNases.
2. To protect RNA from degradation, it might be advisable to catapult the selected material directly into RNA-protective solutions.
3. After catapulting, the samples are mixed with lysis buffer (provided in the respective RNA extraction kit), which is located in the tip of the reaction vessel. Mixing is performed by inverting as soon as possible after catapulting to protect RNA best.
4. Most suitable are freshly cut or deep frozen specimens. Freezing should be performed after ethanol fixation or after staining and drying. Frozen sections should be stored  $-80^{\circ}\text{C}$  for only a few days.

### Checking RNA integrity

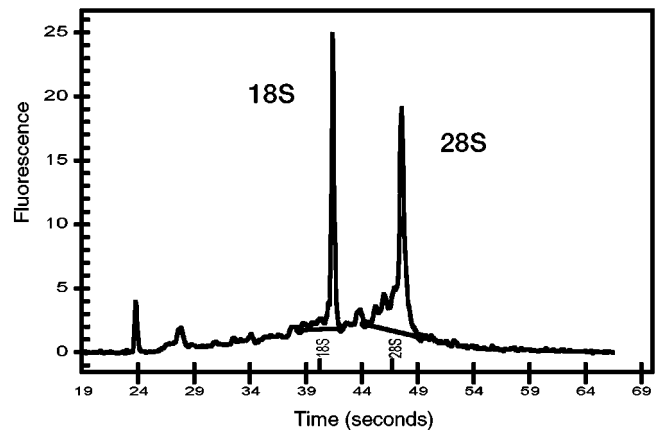
The main parameter influencing the quality of experiments is specimen fixation, which critically determines the conservation and integrity of the nucleic acids. For instance, fixation leads to some degree of fragmentation of RNA and induces several chemical modifications, including cross-linking between amino groups, resulting



**Fig. 2.** RNA was isolated from mouse liver tissue sections. Each microdissected area was chosen to represent approx. 2000 cells. **A:** Methylgreen-stained tissue, **B:** Hematoxylin/Eosin-stained tissue, **C:** Nuclear Fast Red-stained tissue, **D:** Methylene Blue-stained tissue (RNA degraded and in low yield).

in potentially poor PCR amplification. Staining may influence quality and yield of RNA [3]. As many of the separation procedures are time- and labor-intensive, one should take appropriate steps at the end of the isolation procedure to check the purity and quality of the isolated material before starting an experimental design.

The most common method used for assessing the integrity of total RNA is to run an aliquot of the RNA sam-



**Fig. 3.** Frozen mouse liver section was prepared on a PALM®MembraneSlide and stained with Methylgreen. 2000 cells were catapulted, RNA was extracted, and RNA integrity and concentration were analyzed.

ple on an agarose gel. A drawback of this method is the amount of RNA required for visualization. In general, at least 200 ng of RNA must be loaded onto the gel. RNA preparations using microdissected specific cell preparations naturally result in low yields. In these cases, it may be impossible to spare 200 ng of RNA for the assessment of integrity before proceeding with the expression profiling application.

Currently, there exists an alternative to traditional gel-based analysis that integrates the quantitation of RNA samples with quality assessment in one quick and simple assay. The Agilent 2100 Bioanalyzer (Agilent Technologies) is a microfluidics instrument that provides detailed information about the condition of RNA samples, i.e., RNA concentration and purity. The microchannels of the Bioanalyzer are filled with a sieving polymer and fluorescence dye. Samples are detected by their fluorescence and translated into electropherograms or into gel-like images.

We tested the integrity and concentration of RNA obtained from microdissected samples (LMPC) in a single step using Agilent RNA 6000 Pico LabChip kit.

## Results

Snap frozen material, stored at  $-80^{\circ}\text{C}$ , was the best starting material for obtaining high quality RNA. We achieved highest RNA quality by using frozen sections mounted on PALM®MembraneSlides and catapulting the cells directly into RNase-free water containing 0.5% Igepal CA-630 or into PALM's Catapult Buffer.

The four staining procedures showed differences in RNA yield and quality. Best results were obtained using

the Methylgreen and Nuclear Fast Red staining. H&E and NFR gave very similar results in terms of RNA yield and quality (Fig. 2A, B, C). In the case of Methylene Blue staining, we saw partially degraded RNA. The 28S band is almost entirely missing (Fig. 2D).

RNA isolation from catapulted cells requires the same procedure as a standard RNA isolation, but on a small scale. Many manufacturers are now producing kits for RNA isolation from small numbers of cells and, in our hand, all the kits tested gave reasonable yields for the number of cells processed, although there are differences in quality [3]. PALM has recently released its own version of RNA isolation kit optimized for the use with laser microdissected samples.

RNA integrity and concentration was analyzed in one step. Figure 3 shows an electropherogram presenting total RNA after extraction from 2000 catapulted murine liver cells. Clearly visible 18S and 28S peaks indicate excellent RNA quality.

## Discussion

Many factors influence nucleic acid preservation, extraction and further manipulation. Changes in RNA can occur during the handling of the sample and isolation of the RNA. These changes, including down-regulation of genes and enzymatic degradation of RNA, can occur very rapidly. Immediate stabilization before RNA isolation is therefore a prerequisite for accurate and meaningful gene expression analysis.

Unlike DNases, RNases are very stable and represent active enzymes that generally do not require functioning metal ion cofactors. Since RNases are ubiquitous and difficult to inactivate (they can maintain activity even after prolonged boiling or autoclaving), and even minute amounts are sufficient to destroy RNA, great care should be taken to avoid inadvertently introducing RNases during or after the isolation procedure.

Traditional methods for stabilizing nucleic acids rely on rapid freezing of the sample in liquid nitrogen or on dry ice. However, it is important to remember that freezing does not permanently stabilize the nucleic acids in the sample. Freezing generates ice crystals, which can disrupt cellular compartments where endogenous RNases are sequestered, giving them access to the RNA. Subsequent thawing during sample processing can allow these endogenous RNases to act. Multiple freeze/thaw cycles can induce further degradation.

A factor that is often underestimated, particularly in gene expression studies, is the absolute necessity for clean and contamination-free starting materials. The studied material will be decisive for the results obtained. Investigation of functional aspects of gene expression essentially requires the use of sophisticated methods allowing for the isolation of specific and narrowly cir-

cumdissected cell populations. However, primary tissues are a complex mixture of various cell types, and tumors contain an abundance of stromal and inflammatory cells, which frequently exceed the neoplastic population. This complexity can critically influence the results of molecular genetic analyses. Using standard detection methods, many alterations of genes or their expression products can remain undiscovered if the rate of contaminating cells reaches a certain threshold. Moreover, early pathologic lesions, such as dysplasia or carcinoma *in situ*, are frequently not detectable by commonly used molecular analyses.

Applying the method of LMPC™, rapid and highly precise procurement of purified cell populations can be achieved, suitable for a variety of downstream analyses. Indispensable for expression analysis, particularly for microarray analyses, is the use of RNA of good integrity and quality. We could demonstrate that RNA extracted from samples obtained by LMPC shows excellent quality. This technology has been applied even for microarray analyses [1, 10].

With the possibility of getting easily access to specific single cells by LMPC, a desired experimental sequence – isolation of a specific cell, amplification of its genome, characterization of its mRNA expression profile, identification of characteristics leading to its specialized role – is no longer problematic.

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