

Quality assurance of RNA derived from laser microdissected tissue samples obtained by the PALM[®] MicroBeam System using the RNA 6000 Pico LabChip[®] kit.

Application

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Abstract

Gene expression profiling of material isolated by microdissection has become a very popular method for analyzing cellular behavior in a micro scale and is used in a wide range of research and clinical applications. Laser-assisted microdissection allows isolation of RNA from specific cell populations out of their surrounding tissue environment. Usually, the sample yield that can be obtained by this method is too low for quality control of RNA using a standard technique. On the other hand, extraction of high quality RNA is crucial for all subsequent steps and the success of the overall experiment. Since the introduction of the Agilent RNA 6000 Pico LabChip[®] kit, it is possible for the first time to analyze total RNA samples in concentrations down to 200 pg/μl, which is in the range of the sample concentration one can expect from microdissected tissue or cells. In this study we analyzed RNA samples that were obtained by laser microdissection and pressure catapulting (LMPC; PALM[®] MicroBeam System) from tissue sections. The influence of different staining procedures and RNA extraction kits on RNA quality was investigated. RNA was tested for quality and approximate yield, as well as the reproducibility within homologous sample replicates. The data revealed clear differences between the different isolation kits and also between the staining procedures that were used.



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Introduction

The analysis of gene expression has become one of the major scientific tools for understanding the behavior of cells in vivo and in vitro and is used in many biological and medical applications. Using microarray hybridization, the abundance of a large number of transcripts can be analyzed in a single experiment. Comparison of different expression patterns enables scientists to correlate the changes in the transcriptome to various external factors influencing the cellular behavior. Especially in clinical research there is the need for methods that allow expression analysis from very specific cells. Prerequisite for meaningful results are samples of high purity without contamination from unwanted cells that could potentially interfere with detection of important transcripts. Laser-assisted microdissection with the PALM[®] MicroBeam system allows isolation of individual cells in a simple and fast way. Using this technology, researchers are able to procure pure cell populations from specific areas of tissue sections for isolation of DNA, RNA, and proteins with visual control using a microscope. An UV-A-laser-mediated process dissects selected specimen from various sources and transfers them contact free directly into collection vessels for subsequent extraction (see figure 1). Together with novel methods for linear RNA amplification it is possible to perform gene expression profiling analyses even from very limited amount of cell material. The most

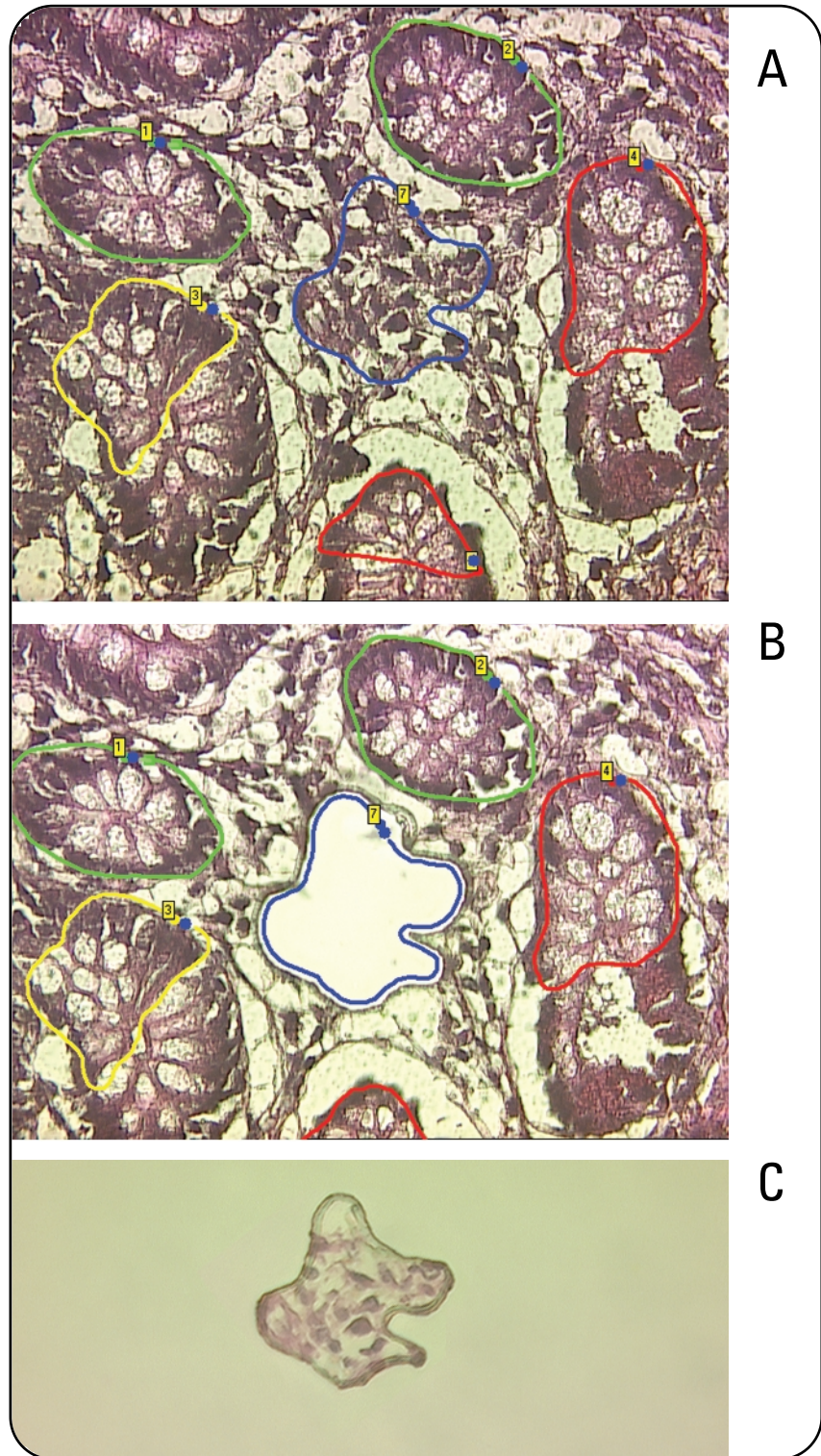


Figure 1
LMPC procedure (example)
A Laser microdissection
B Laser pressure catapulting: Section after catapulting of selected area
C Catapulted area in the collection device

critical parameter for the success of such an experiment is the integrity and purity of the RNA.

Experimental case study

Tissue preparation and microdissection

Snap-frozen mouse liver tissue stored at -80°C was cut in $7\ \mu\text{m}$ serial sections on a cryotome at -25°C . The sections were transferred to PALM[®] MembraneSlides (1mm glass slides covered with a $1.35\ \mu\text{m}$ thin Polyethylene-naphthalate-membrane to facilitate the laser pressure catapulting procedure) and air dried for 10 seconds. After a fixation step of 5 minutes in 70% ethanol at -20°C and a short wash in RNase free water (10 seconds) the sections were further processed according to standard histochemical procedures. Depending on the subsequent tests either Hematoxylin/Eosin (HE), Nuclear Fast Red (NFR), Methylgreen (MG) or Methylene Blue (MB) staining was applied. After staining and a short increasing ethanol series the sections were air-dried and either used immediately or refrozen at -20°C for up to 2 days. The PALM[®] Micro-Beam System was used to precisely excise the selected tissue areas with the UV-A-laser and then to catapult the areas of interest contact free against gravity into collecting caps filled with $12\ \mu\text{l}$ of RNase-free water. For lysis the isolated material was then immediately mixed by inversion with the respective lysis buffers for RNA extraction (see below).

RNA Purification

For this study RNA isolation kits from 3 different manufacturers were used. Two are based on a column-purification, while the third one uses an extraction-/precipitation-based approach. RNA cleanup was carried out according to the manufacturers' protocols with the only variation that uniformly a volume of $30\ \mu\text{l}$ of elution buffer or water was used in the final step to collect the purified RNA. For the column-procedures the elution process was repeated once with the primary eluate of $30\ \mu\text{l}$ to improve the yield. The scope of this work was the demonstration of feasibility to perform RNA sample quality control from microdissected material and not the evaluation of commercially available RNA extraction kits. Therefore, the manufacturers are not explicitly named here, but instead referred to as manufacturer A, B, and C.

RT-PCR

Selected RNA samples were reversely transcribed with the First Strand cDNA Synthesis Kit for RT PCR (AMV) cDNA Synthesis Kit for RT-PCR (Roche) according to the manufacturers' protocol. Briefly, $5\text{-}8\ \mu\text{l}$ of each RNA solution were transcribed by AMV-RTase with random primers in a total volume of $20\ \mu\text{l}$ for one hour at 42°C . For the subsequent quantitative RT-PCR analyses $1\text{-}2\ \mu\text{l}$ of each cDNA-solution were used as templates. The PCR amplification of the cDNA was performed in a LightCycler[®]

instrument (Roche) in $20\ \mu\text{l}$ reactions using protocols and components of the QuantiTect SYBR Green PCR-Kit (Qiagen). cDNA-specific primers for murine PBGD (porphobilinogen deaminase) were used as model system producing a PCR-fragment of $154\ \text{bp}$. Resulting crossing-point values were used to compare extraction efficiencies of different kits or influences of different staining procedures.

RNA 6000 Pico Assay Protocol

All chips were prepared according to the instructions provided with the RNA 6000 Pico LabChip Kit. In brief, $550\ \mu\text{l}$ of the RNA 6000 Pico gel matrix were placed on a spin filter, centrifuged at $1500\ \text{g}$ and divided in $65\ \mu\text{l}$ aliquots. After addition of $1\ \mu\text{l}$ RNA 6000 Pico dye concentrate to one gel aliquot, the mixture was vortexed and centrifuged at $13000\ \text{g}$ for 10 min. A RNA 6000 Pico chip was filled with gel-dye mix using the chip priming station, followed by addition of Conditioning Solution and Marker. $1\ \mu\text{l}$ of RNA 6000 ladder (Ambion) and RNA samples were added in the designated wells, the chip was vortexed for 1 minute and run on an Agilent 2100 bio-analyzer.

Results and Discussion

RNA quality and yield obtained by different isolation kits

All 3 kits tested in this study appeared to work well together with the RNA 6000 Pico assay (figure 2). For the comparison of the different kits we used a standard HE tissue staining and dissected areas corresponding to 1000, 2000 and 3000 cells. Quality and yield of the isolated RNA showed a high variability with respect to the different kits and also within the replicates of one specific method. This may be caused either by differences in the tissue samples (different areas were isolated from the same section) or by some variances in sample handling. On average the extraction-/precipitation-based method (manufacturer C) showed a higher RNA recovery than the two column-based methods, which is most probably caused by irreversible RNA-binding at the column material. The average yield (manufacturer C) obtained from a 1000 cell area was approximately 700 pg/μl when resolving the pellet in 30 μl of water. This would result roughly in a theoretical average RNA recovery rate of approx 21 pg per cell. In contrast to this the yield obtained by the two column-based kits varied between approx. 130 and 380 pg/μl (1000 cells; 30 μl elution volume). Note that the quantitative results from an analysis with the RNA 6000 Pico kit are somewhat dependent on the salt content of a sample. Only a rough estimation of the RNA concentration of a sample can be obtained.

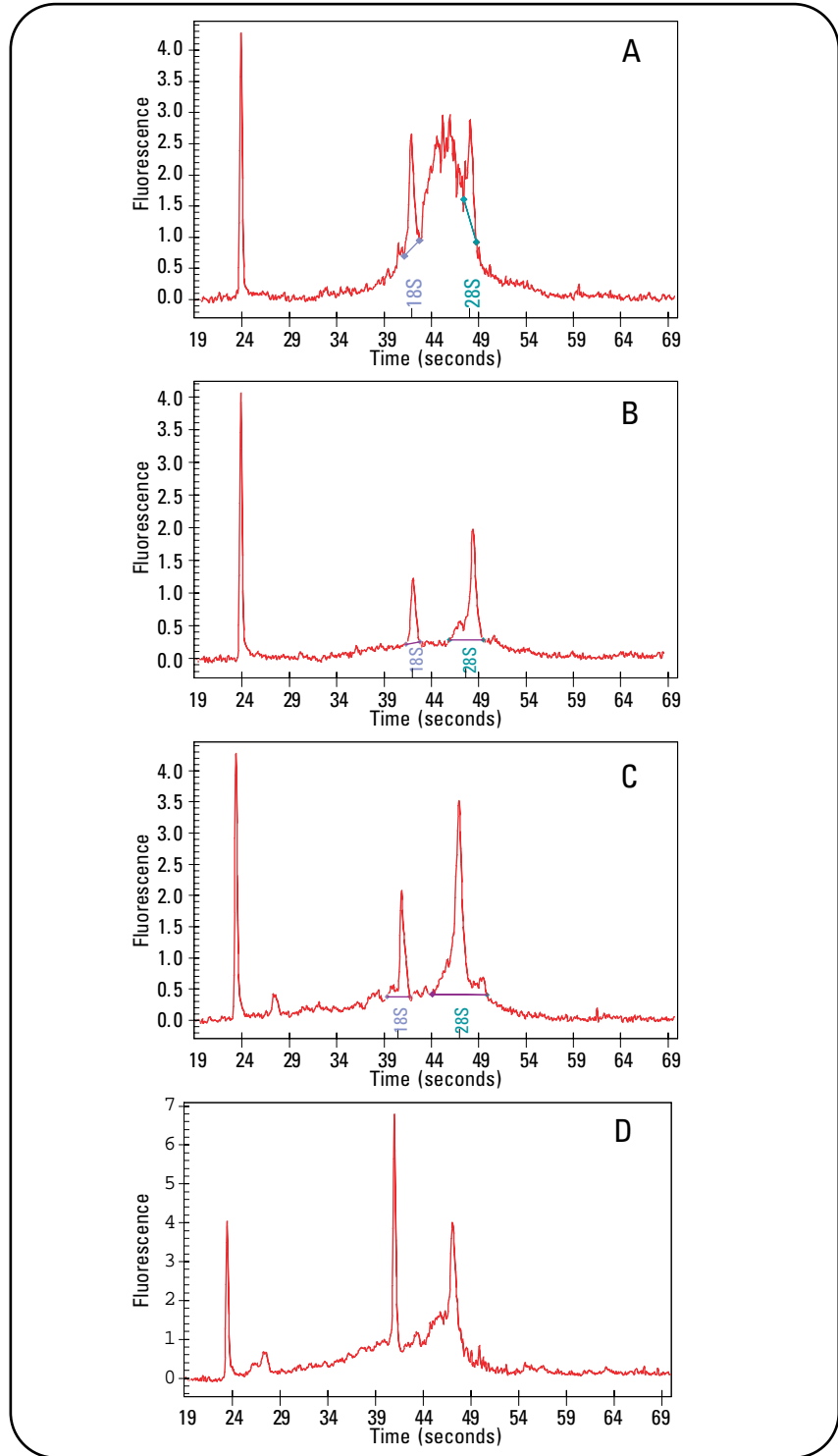


Figure 2

RNA was isolated from mouse liver tissue sections using different RNA isolation kits. Histological staining was carried out using a standard HE procedure. Microdissected areas represent approx. 1000 cells each. A: Manufacturer A (column-based; no DNase digest) B: Manufacturer A (column-based; including DNase digest on the column during cleanup as recommended by the supplier). C: Manufacturer B (column-based; no DNase digest) D: Manufacturer C (extraction-/precipitation-based, no DNase digest)

RNA quality and yield obtained from tissue stained with different methods

In this test, we compared RNA quality and yield when using different standard histochemical staining methods. Therefore, we dissected areas corresponding to 2000 cells and purified the RNA using the kit of manufacturer C (3 replicates for each sample). Serial tissue sections were stained using either Hematoxylin/Eosin (HE), Nuclear Fast Red (NFR), Methylgreen (MG) or Methylene Blue (MB). In general, all four staining procedures were compatible with the RNA Pico Assay, but significant differences in RNA yield and quality were observed. Best results were obtained using the MG stain (average yield: approx. 3100 pg; highly intact RNA; see fig. 3A). HE (figure 3B) and NFR staining (figure 3C) gave very similar results in terms of RNA yield and quality (average yield: approx. 1600 pg; slightly degraded RNA). In the case of MB staining, ambiguous results were observed (average yield: approx. 730 pg; partially degraded RNA; figure 3D). The 28 S band is almost entirely missing, which could be due to RNA degradation or due to a salt content that exceeds the salt specifications of the Pico kit. RNA-extraction with the kit of manufacturer B from the same sections with all four staining methods yielded slightly different relations (see in RT-PCR results below), and overall lower concentration levels as expected from the comparison of the kits from above. It should be noted that this short investigation does not put a value on the use of certain cell stains for work with microdissected material but rather points out the value of the RNA 6000 Pico kit for optimization of experimental protocols.

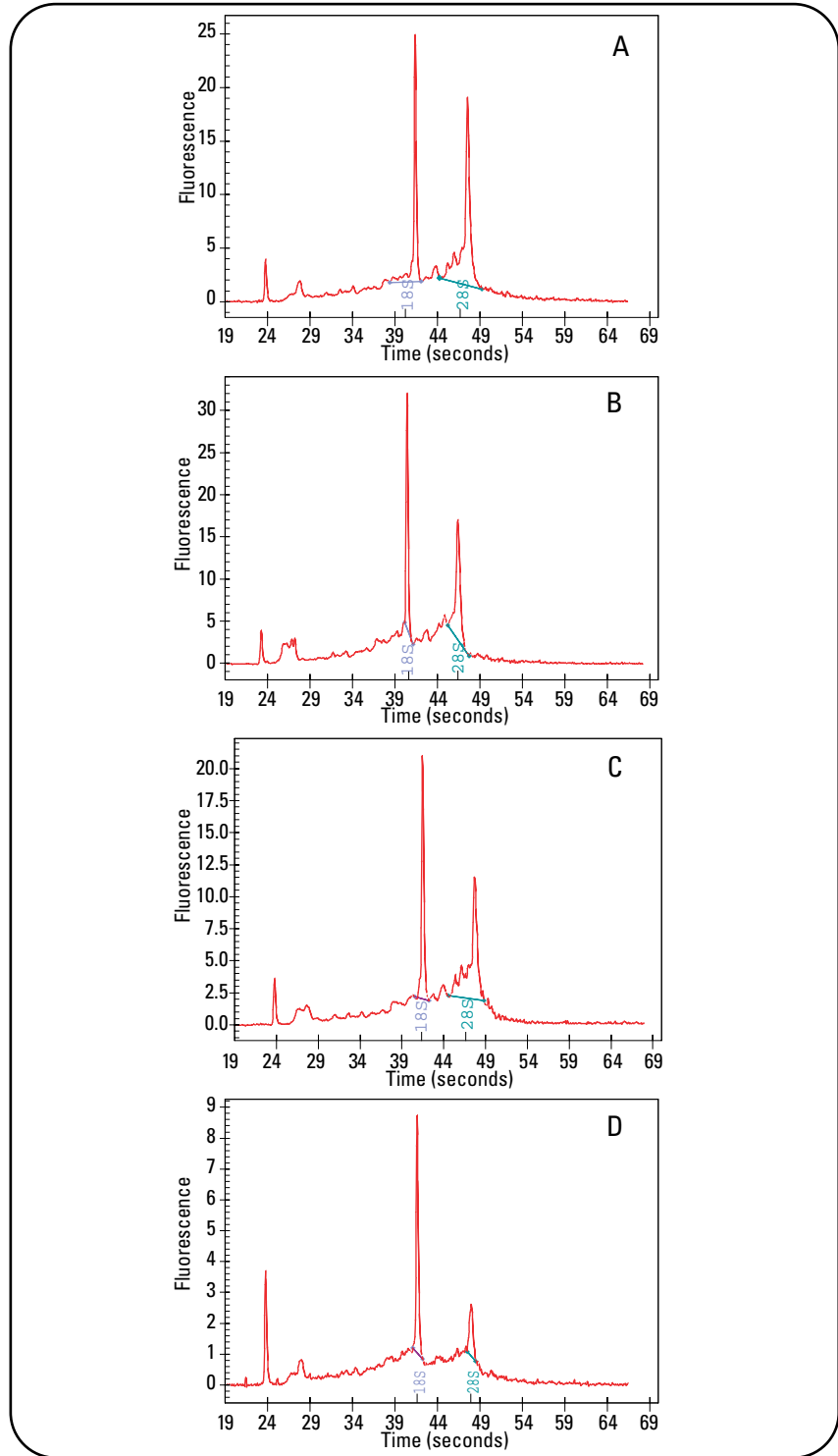


Figure 3
RNA was isolated from mouse liver tissue sections using Manufacturer C (extraction-/precipitation-based). Each microdissected area was chosen to represent approx. 2000 cells.

- A: Methylgreen stained tissue**
- B: Hematoxylin/Eosin stained tissue**
- C: Nuclear Fast Red stained tissue**
- D: Methylene Blue stained tissue**

Correlation of the results obtained by the RNA Pico Assay to real-time RT-PCR

Comparison of RNA extraction kits:

All 3 sample extraction kits that were tested yielded good quality RNA that could be used for RT-PCR (figure 4). For the comparison we used HE-stained sections to microdissect and catapult areas of 1000 cells each (3 replicates per sample). After RNA isolation with the three different kits real-time RT-PCR with the LightCycler, was performed. The amount of cDNA used as template was half of the RNA used for the 2100 bioanalyzer analysis (fig. 4A). The specificity of the PCR-fragments was proven by the melting curve (figure 4B). The crossing points (Cp)* of the growth curves (figure 4C) being a scale for the initial specific RNA-amount were close together (about one cycle distance each) correlating to the Bioanalyzer values although Manufacturer A was overestimated there probably due to the DNA-contamination (figure.1).

*(CpA: 31.9; CpB: 33.1; CpC: 30.7)

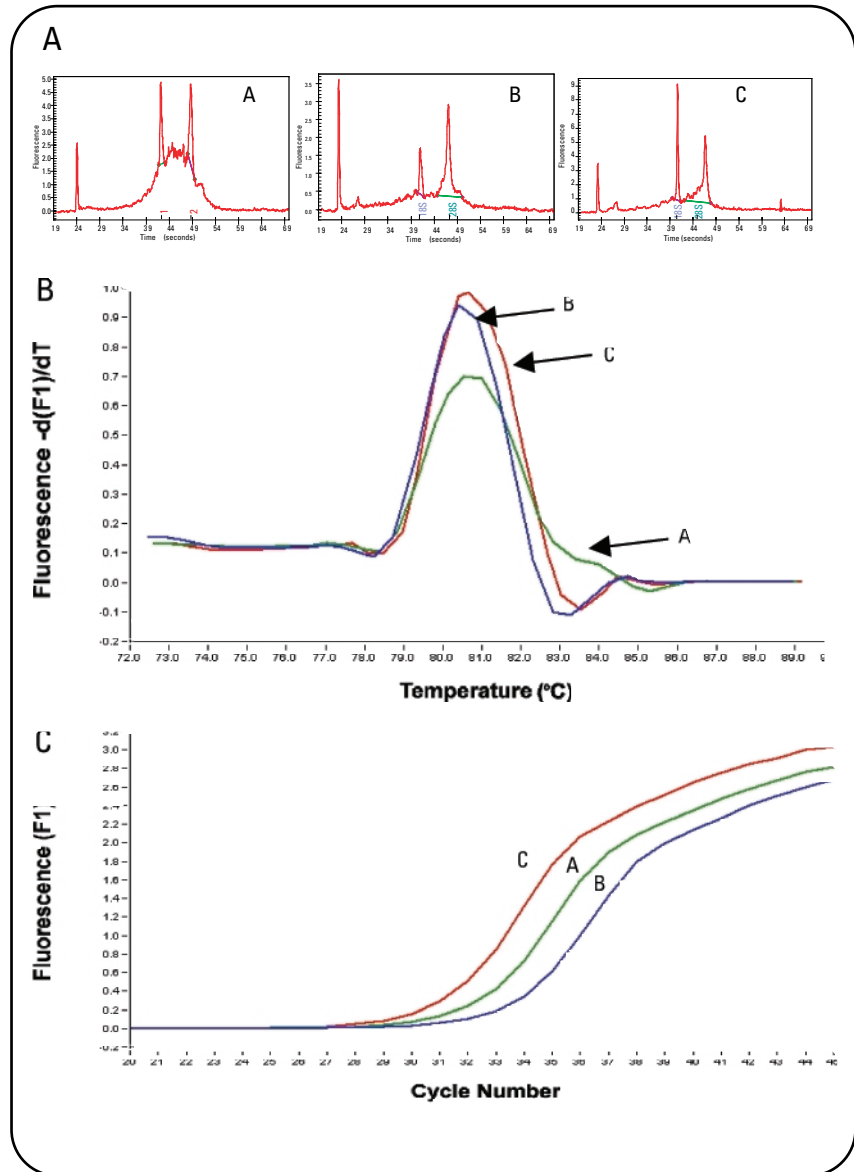


Figure 4
 RNA was isolated from microdissected mouse liver tissue sections using different procedures (Manufacturer A, B, C) and used for real-time RT-PCR with the LightCycler®. Histological staining was carried out using a standard HE procedure. Microdissected areas represented approx. 1000 cells each.

- A: Bioanalyzer results (RNA from A, B, C)
- B: specific melting curves (PCR products from A, B, C)
- C: LightCycler growth curves (PCR products from A, B, C)

Comparison of staining procedures

In this test serial tissue sections were stained using either Hematoxylin/Eosin (HE), Nuclear Fast Red (NFR), Methylgreen (MG) or Methylene Blue (MB). Areas corresponding to 2000 cells were dissected, the RNA purified using Manufacturer B (3 replicates each) and analyzed with the 2100 bioanalyzer (figure 5A) and the Light-Cycler® as above. The cDNA input for the PCR corresponded to a fourth of the RNA amount used for the 2100 bioanalyzer run. The melting curve proves the specificity of the products (figure 5B), while the growth curve reflects the starting amount of RNA (figure 5C). In contrast to the test with Manufacturer C (see above) NFR was the optimal stain, though the Cp-values lay very close together (only half a cycle difference) except for MB which showed worse results. (CpNFR: 34,7; CpHE: 35,2; CpMG: 35,8; CpMB: 37,6). This real-time RT-PCR results again correlated well with the 2100 bioanalyzer values.

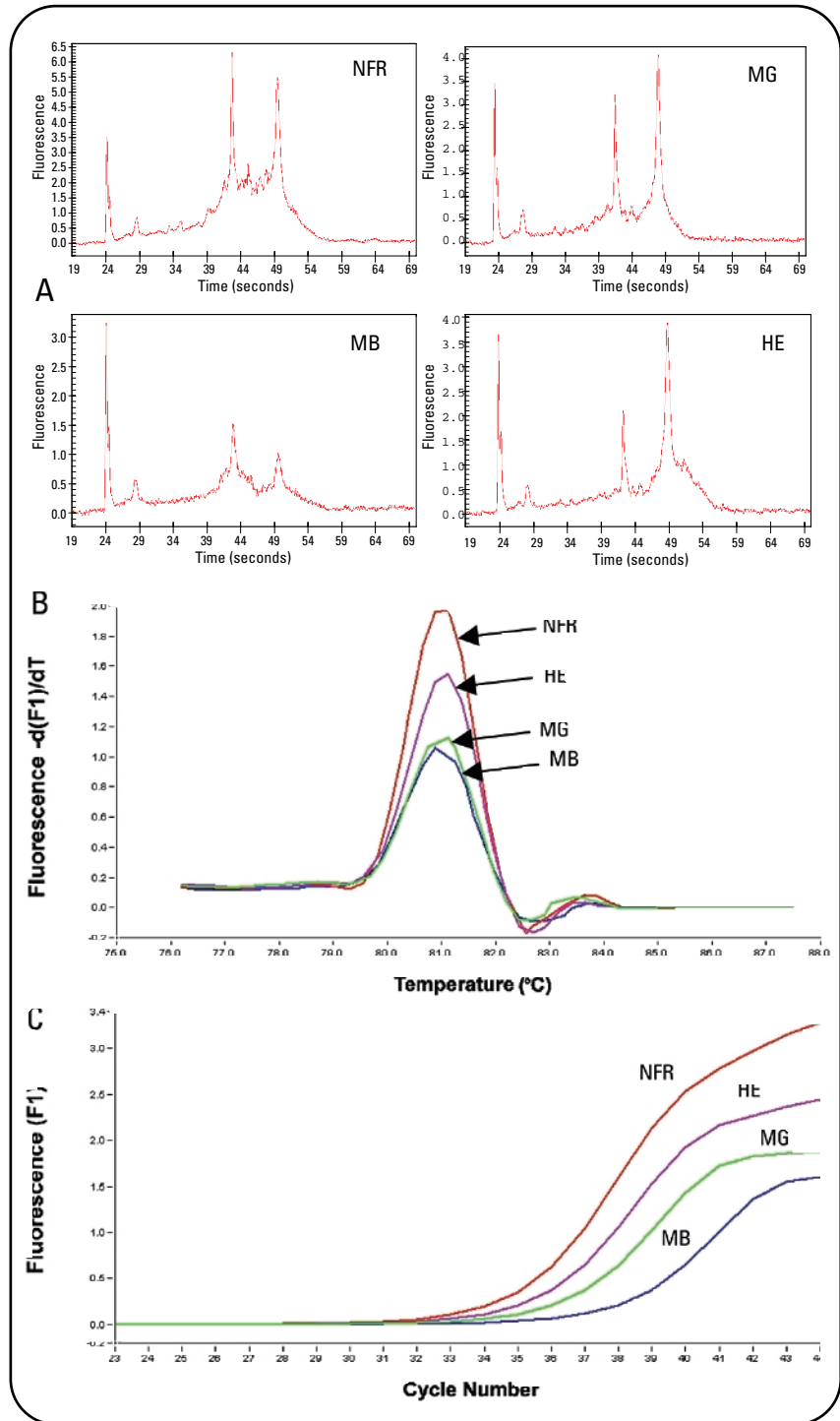


Figure 5
RNA was isolated from mouse liver tissue sections using Manufacturer B and used for real-time PCR with the LightCycler®. Four different stainings (HE, NFR, MG, MB) were compared. Each microdissected area was chosen to represent approx. 2000 cells.

- A: 2100 bioanalyzer results
- B: specific melting curves
- C: LightCycler growth curves

Conclusion

RNA derived from laser-microdissected tissue isolated by the PALM[®]MicroBeam system is of high quality and can be analyzed efficiently using the RNA 6000 Pico assay with the Agilent 2100 bioanalyzer. RNA-purification kits of different manufacturers and various common staining procedures have been tested and were compatible with the Pico Assay. Nevertheless, it seems to be advisable that any extraction protocol and staining method should be tested in combination to find the optimal procedure for tissue microdissection experiments. The RNA 6000 Pico kit is well suited to show differences in RNA quality and, therefore, is an ideal tool to optimize experimental conditions. Its unprecedented sensitivity allows for the first time quality control in the context of microdissection experiments ensuring successful gene expression profiling experiments.

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