0023-6837/03/8304-599$03.00/0
LABORATORY INVESTIGATION
Vol. 83, No. 4, p. 599, 2003
Printed in U.S.A.

BRIEF METHOD

Assessment of RNA Quality Extracted from Laser-Captured Tissues Using Miniaturized Capillary Electrophoresis

Virginie Copois, Caroline Bret, Frédéric Bibeau, Jean-Paul Brouillet, Maguy del Rio, Marie-Laurence Berthe, Thierry Maudelonde, and Nathalie Boulle

Unité INSERM 540 and Service de Biologie Cellulaire (CB, JPB, MLB, TM, NB), Centre Hospitalier Universitaire de Montpellier, Hôpital Arnaud de Villeneuve, and Laboratoire d'Anatomie Pathologique, and CNRS-UMR 5094 (VC, FB, MDR), CRLCC Val d'Aurelle, Montpellier, France

Analysis of gene expression in specific tumor cells is a valuable approach to understanding the molecular events that lead to tumor development. This approach has usually been performed on whole tumor extracts, therefore not allowing the molecular analysis of specific cell populations. Laser-capture microdissection (LCM) was developed recently to collect pure cell populations from a tissue section, overcoming the problem of tissue heterogeneity (Emmert-Buck et al, 1996), but this new technology has been challenged by the small amounts of RNA that can be extracted from the limited number of microdissected cells. As real-time RT-PCR is a sensitive method of quantitative mRNA measurement, it has been associated with LCM to improve the sensitivity of gene expression analysis from laser-captured cells (Bustin, 2002; Fink et al, 1998). However, mRNA integrity plays a major role in the determination of gene expression, and it is essential to extract a high-quality RNA from laser-captured cells to ensure reliability of mRNA quantitative analysis. One major problem encountered with this approach is assessing the quality of RNA extracted from microdissected tissues because of the small amounts of RNA available.

The aim of this study was to find a valuable protocol to assess the quality of RNA extracted from laser-captured cells and to evaluate its sensitivity. This has been achieved by taking advantage of the sensitive "Lab-on-a-chip" technology (Caliper Technologies Corporation, Agilent Technologies) based on miniaturization of RNA capillary electrophoresis, which allows the analysis of minute amounts of total RNA. This procedure was further validated by comparing its results with those obtained by real-time RT-PCR quantification of two housekeeping genes, using the same RNA samples extracted from laser-captured cells.

A tumor sample from a metastatic melanoma was snap-frozen in liquid nitrogen immediately after surgery and stored at −80° C. The frozen sample was sectioned at 7 μm using a cryostat, and tissue sections were stained with RNAse-free reagents. After staining, tissue sections were stored in a desiccated container for at least 15 minutes before LCM using the PixCell II LCM System (Arcturus Engineering, Inc., Mountain View, California). Total RNA was extracted from laser-captured cells according to DiLella (2001) procedure using the RNeasy Mini kit, with an additional DNAse I digestion step (Qiagen, Valencia, California). The total RNA was not concentrated by ethanol precipitation but eluted in a small volume of RNAse-free water (20 μl, loaded twice on the column). The extracted total RNA was divided in two aliquots and kept at −80° C. One aliquot was used to assess RNA integrity using the RNA 6000 Pico Assay kit with Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, California), which is adapted to small RNA concentrations (qualitative range from 200 pg to 5 ng/μl total RNA). For this purpose, 1 μl of the aliquot was loaded onto the chip according to the manufacturer's instructions and analyzed with Bio-sizing software. The other aliquot was subjected to a reverse-transcription step using the Sensiscript Reverse Tran-

DOI: 10.1097/01.LAB.0000062853.92828.20

Received January 13, 2003.
VC and CB contributed equally to this work.
This study was supported by the Centre Hospitalier Universitaire de Montpellier, the Institut National de la Santé et de la Recherche Médicale, and the Ligue Contre le Cancer. NB is a recipient of a grant from the Association pour la Recherche sur le Cancer (ARC).
Address reprint requests to: Dr. N. Boulle, Laboratoire de Biologie Cellulaire et Hormonale, Hôpital Arnaud de Villeneuve, 34295 Montpellier, France. E-mail: n-boulle@chu-montpellier.fr

scriptase kit (Qiagen) with 12 μl of total RNA extracted from LCM-captured cells and oligo dT primers (Boylan et al, 2001). The cDNA product was then analyzed by real-time PCR using a SYBR Green approach (Light Cycler; Roche, Mannheim, Germany). This method was used to quantify mRNA levels of two housekeeping genes, β2 microglobulin and HPRT, chosen for their different expression levels: a high expression level for the β2 microglobulin gene and a low expression level for the HPRT gene (Vandesompele et al, 2002).

In a first step to examine the quality of samples preparation, total RNA was extracted from a whole tissue section, stained and stored in the same conditions as the tissue sections used for LCM. As illustrated in Figure 1A, we obtained electropherogram profiles showing total RNA of high quality, indicating that RNA integrity is maintained during all of the steps before LCM (cryosection, staining, and storage of tissue sections). The integrity of RNA extracted from laser-captured melanoma cells (from 130 to 1200 cells) was also tested. In total, 51 RNA samples extracted from whole tissue sections and laser-captured melanoma cells were analyzed. Among them, six RNA samples (from 200, 600, and 1200 laser-captured cells) were found to be degraded. Analysis of the 45 remaining electropherograms showed reproducible profiles indicating the integrity and quality of the extracted RNA. Typical profiles are shown in Figure 1B. With the use of the LCM and RNA extraction protocols described here, as few as 130 melanoma cells resulted in a reliable electropherogram profile (Fig. 1B). RNA extracted from fewer microdissected cells (80 to 100 cells) gave background signals and did not allow accurate RNA analysis (data not shown). Evaluation of RNA concentrations from microdissected samples using the Biosizing software indicated a total amount of RNA per cell of 22.1 $\pm$ 2.5 pg (mean $\pm$ SD, n = 51), which is in accordance with the typical amount of total RNA in mammalian cells (10 to 30 pg RNA per cell). Similar electropherogram profiles could be obtained with RNA extracted from laser-captured breast tumor cells (data not shown).

Those electropherogram profiles were compared with the results obtained using real-time RT-PCR by measuring, on the same LCM samples, the expression levels of two housekeeping genes. As predicted, real-

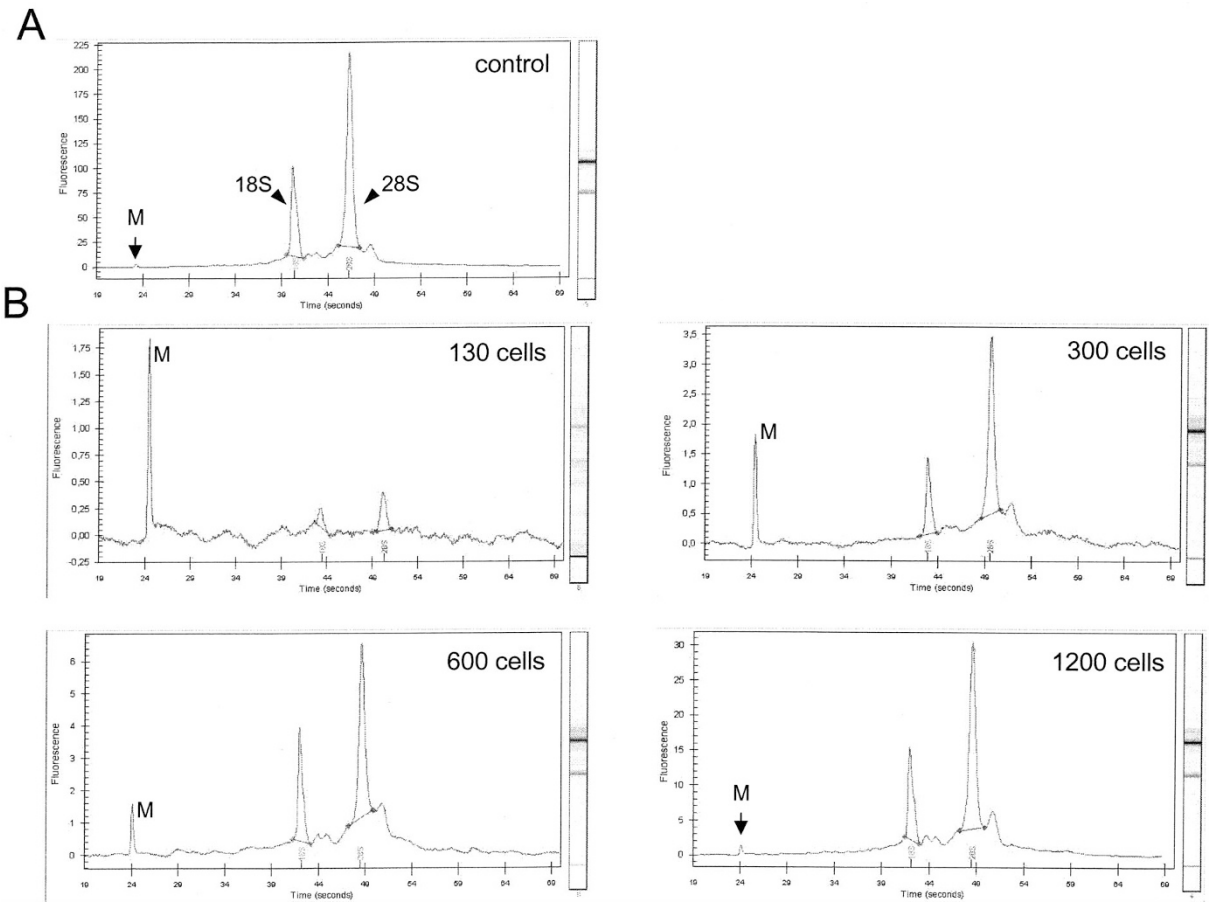

Figure 1.

Electropherogram profiles of total RNA extracted from laser-captured melanoma cells. One microliter of total RNA extracted from whole tissue section (control; A) or various numbers of laser-captured melanoma cells (B) was analyzed by capillary electrophoresis (Agilent 2100 Bioanalyser, RNA 6000 Pico Assay kit). Representative electropherograms are shown. Peaks corresponding to the 18S and 28S rRNA are indicated. *M*, the marker peak that allows to compare sample intensity. The number of laser-captured cells was calculated by taking into account the total volume of laser-captured tissue, the tissue section thickness (7 μm), and the average size of a melanoma cell (20 μm). For each LCM experiment, cell transfer efficiency was >90%.

time RT-PCR quantification indicated higher expression levels of the β2 microglobulin gene compared with the HPRT gene in melanoma cells, with a ratio (cycle threshold [Ct] β2 microglobulin)/(Ct HPRT) of 0.78 ± 0.03 (mean ± SD; $n = 45$). For all samples, we found a high correlation between HPRT and β2 microglobulin mRNA levels ($p < 0.001$; Fig. 2). The results obtained for the HPRT gene are detailed in Table 1. As expected, the Ct for HPRT were inversely correlated with the number of laser-captured melanoma cells ($R^2 = 0.91$, $p = 0.01$), the smallest cell number tested (130 cells) still being in the range of quantitative results (Ct < 35). Moreover, reproducible HPRT mRNA levels (in terms of Ct) were obtained between assays (coefficient of variation ≤6.5%). Altogether, these results confirm the integrity of the RNA extracted from laser-captured melanoma cells and demonstrate that the protocol reported here allows the obtaining, in a reproducible way, of a high-quality RNA that can be used for real-time PCR quantification.

Comparison of electropherogram profiles and real-time RT-PCR results showed a good parallelism between the two approaches, as all RNA samples showing a high-quality total RNA on electropherogram profiles displayed expected values of HPRT and β2 microglobulin Ct after real-time RT-PCR. In the same way, degradation of RNA led to a typical degraded electropherogram profile together with high HPRT and β2 microglobulin Ct values (>35 to 36). However, 11 of the 51 RNA samples tested showed degraded-like electropherogram profiles, contrasting with HPRT and β2 microglobulin mRNA levels similar to those obtained with the high quality RNA. This phenomenon was also observed when other commercial RNA extraction kits were used, which gave satisfactory results after real-time RT-PCR but unreliable profiles using the Agilent 2100 Bioanalyser (data not shown).

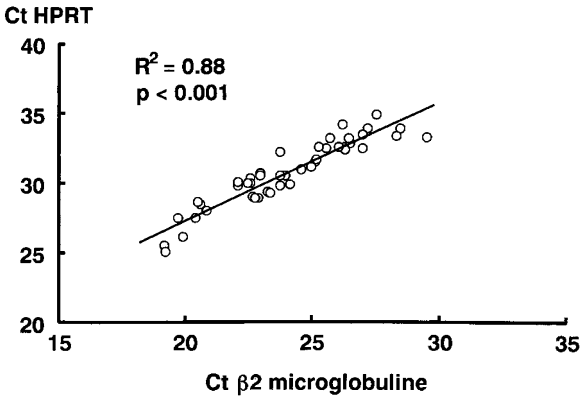

Figure 2.
Correlation between β2 microglobulin and HPRT mRNA levels in laser-captured melanoma cells. β2 microglobulin and HPRT mRNA levels from laser-captured melanoma cells or whole tissue section (control) were quantified by real-time RT-PCR using the SYBR approach. The Ct of HPRT and β2 microglobulin from 45 different samples are presented. Ct obtained for the six samples with RNA degradation have been eliminated. Primers for HPRT were as follows: forward 5'-CTGACCTGCTGGATTACA-3', reverse 5'-GCGACCTTGACCATCTTT- 3'; the primers for β2 microglobulin have been published elsewhere (Rey et al, 2000). PCR efficiency for both genes were >90%.

Table 1. Real-Time RT-PCR Quantification of HPRT mRNA from LCM-Captured Melanoma Cells

No. of LCM-captured melanoma cells	No. of microdissected samples[a]	Mean Ct HPRT ± SD	CV (%)
1200	8	28.6 ± 1.53	5.3
600	11	30.1 ± 1.95	6.5
300	10	31 ± 1.26	4.1
200	8	31.9 ± 1.10	3.5
130	6	32.7 ± 1.52	4.6
Control[b] (whole tissue section)	2	25.5; 25	

[a] Microdissected samples from four independent experiments except for the "130 cells" group (three independent experiments).
[b] Individual values from two independent experiments.

These discrepancies may be due to buffer interferences with the RNA 6000 Pico LabChip system, preventing accurate analysis of electropherograms. The DNAse I digestion step during RNA extraction was also found to be critical to avoid genomic DNA contamination that precludes electropherogram analysis by clogging micro channels of the RNA chip system. Finally, we observed, for a small number of RNA samples, that a unique 18S peak occurred on the electropherogram profile with recovery of the two ribosomal RNA peaks 18S and 28S after dilution of the sample. The obtaining of this particular profile is under investigation.

In conclusion, the association of LCM with real-time RT-PCR allows accurate quantification of gene expression in a small number of selected cells. However, this combined approach requires optimization and standardization of the multiple steps from the laser capture procedure to the extraction of RNA and RT-PCR quantification. One critical point is the ability to analyze the quality of the small amounts of total RNA extracted from laser-captured cells. We achieved this aim using the RNA 6000 Pico Assay kit combined with the Agilent 2100 Bioanalyser. This technology was found to be an easy-to-use, RNA nonconsuming and reliable tool to assess the quality of RNA sample extracted from few microdissected cells, before its use for various applications such as real-time RT-PCR quantification.

Acknowledgements

We thank Marie-Christine Lecq for careful technical assistance and Drs. Piona Dariavach and Pascal Pujol for critical reading of the manuscript. The RNA 6000 Pico Assay kits were kindly provided by Agilent Technologies.

References

Boylan S, Honda S, Hjelmeland LM, and Handa JT (2001). An optimized protocol for first strand cDNA synthesis from laser capture microdissected tissue. Lab Invest 81:1167–1169.

Bustin SA (2002). Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): Trends and problems. J Mol Endocrinol 29:23–29.

DiLella AG (2001). Successful isolation and RT-PCR of RNA from small numbers of laser capture microdissected cells. Qiagen News 4:3–5.

Emmert-Buck MR, Bonner RF, Smith PD, Chuaqui RF, Zhuang Z, Goldstein SR, Weiss RA, and Liotta LA (1996). Laser capture microdissection. Science 274:998–1001.

Fink L, Seeger W, Ermert L, Hänze J, Stahl U, Grimminger F, Kummer W, and Bohle RM (1998). Real-time quantitative RT-PCR after laser-assisted cell picking. Nat Med 4:1329–1333.

Rey JM, Pujol P, Callier C, Cavailles V, Freiss G, Maudelonde T, and Brouillet JP (2000). Semiquantitative reverse transcription-polymerase chain reaction to evaluate the expression patterns of genes involved in the oestrogen pathway. J Mol Endocrinol 24:433–440.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, and Speleman F (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3:1–12.