NOTE *Pathology*

# One-Step RT-PCR without Initial RNA Isolation Step for Laser-Microdissected Tissue Sample

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(Received 21 November 2002/Accepted 8 April 2003)

ABSTRACT. One-step RT-PCR procedure without initial RNA extraction step is tested for laser microdissected tissue sample. Unfixed cryosections of liver and kidney tissue of male SD rats were cut using laser microdissection system and directly used as templates for RT-PCR study. To check the sensitivity, 5, 25, 125, and 625 hepatocytes were cut and put in PCR-tube. After DNase treatment and cDNA synthesis with pd(N)6 random primer, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNAs were amplified by 60 thermal cycles. GAPDH-specific bands were observed at as few as 25 hepatocytes. Specificity of this procedure was tested for hepatocytes, renal tubular epithelium and glomerular tissue using albumin PCR primers. Approximately 250 cells were cut and albumin cDNA was amplified as described above. Albumin specific band was observed only in hepatocytes sample. To apply this approach to quantitative PCR, various numbers of hepatocytes were cut and put in 0.2 mL PCR tube. After reverse transcription and 10 cycles of GAPDH cDNA amplification by regular thermal-cycler, PCR solution was transferred to 96-well plate designed for real-time PCR system, and further 40 cycles were performed. As a result, GAPDH cDNAs were successfully amplified with a good correlation between the number of template hepatocytes and the intensity of PCR signal. From these results, we concluded this approach would be very useful for the expression analysis of microdissected pathology samples.

KEY WORDS: laser microdissection, one-step RT-PCR.

*J. Vet. Med. Sci.* 65(8): 917–919, 2003

Laser microdissection (LMD) is now well established as a tool for facilitating the enrichment of cells of interest from tissue sections, overcoming the problem of tissue heterogeneity. LMD has been used extensively in combination with analysis at the DNA and RNA levels [2, 3, 8]. Since in most basic and clinical studies, procurement of several hundred to several thousand cells is necessary to provide enough genetic material for reliable amplification, laser-microdissected tissue sometimes lacks enough amounts cells [1]. As for RNA analysis, furthermore, sample RNA can be lost during RNA extraction steps, i.e., removal of protein, precipitation, and purification, and it makes following molecular analysis difficult or impossible [4]. In this study, we tried a one-step RT-PCR protocol without initial RNA isolation step for laser-microdissected tissue sample and its application to real-time PCR technique for quantitative analysis.

Male SD rats weighing –100 g were purchased from Charles River Japan (Yokohama, Kanagawa), kept in an air-conditioned animal room and fed basal diet. The animals were necropsied under ether anesthesia and liver and kidney tissue were obtained and embedded in OCT compound (Sakura Finetech, Tokyo) and kept at –20°C until sectioning. Cryosections at 10 $\mu$m thick were cut by cryostat and placed on slide glass designed for laser microdissection microscope (LMD, Leica Microsystems Japan, Tokyo). The samples were briefly rinsed with RNase-free water, stained by hematoxilin, air-dried, and cut using LMD (Fig. 1). To test the sensitivity of one-step RT-PCR procedure, 5, 25, 125, and 625 hepatocytes were cut and put in 0.2 mL PCR-tube and incubated with DNaseI (Takara, Kyoto) according to the manufacturer's instruction. After adding glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific primers (Table 1) and PCR solution containing SYBR Green® double strand DNA binding dye (QuantiTect SYBR Green(r) RT-PCR Kit, QIAGEN), the cDNA was synthesized at 42°C for 30 min. After incubation at 95°C for 5 min to inactivate the reverse transcriptase, 60 time thermal cycles was performed. All these procedures were done using a thermal cycler (Takara Thermal cycler MP, Takara, Kyoto, Japan). PCR condition was as follows; denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 30 sec. Final extension was done at 72°C for 5 min. PCR products were electrophoresed on a 1.5% agarose gel containing 0.1% ethidium bromide. The bands were visualized using Epi-Light UV500 (Taitec, Nagoya, Japan). To examine the specificity of this procedure, approximately 250 hepatocytes, proximal tubular epithelial cells, or glomerular cells were cut and albumin cDNA was amplified as described above using albumin-specific primer set (Table 1). Albumin primer set covered several exons [8], so that cDNA-specific amplification could be distinguished from non-specific one derived from genomic DNA or hnRNA based on the product size. To confirm the ability to apply this protocol to real-time PCR technique and its quantitative potential, 10, $10^2$, $10^3$, $10^4$, and $10^5$ hepatocytes were cut and placed in 0.2 mL PCR tube with GAPDH-specific primers and PCR solution as described. Prior to PCR reaction in the

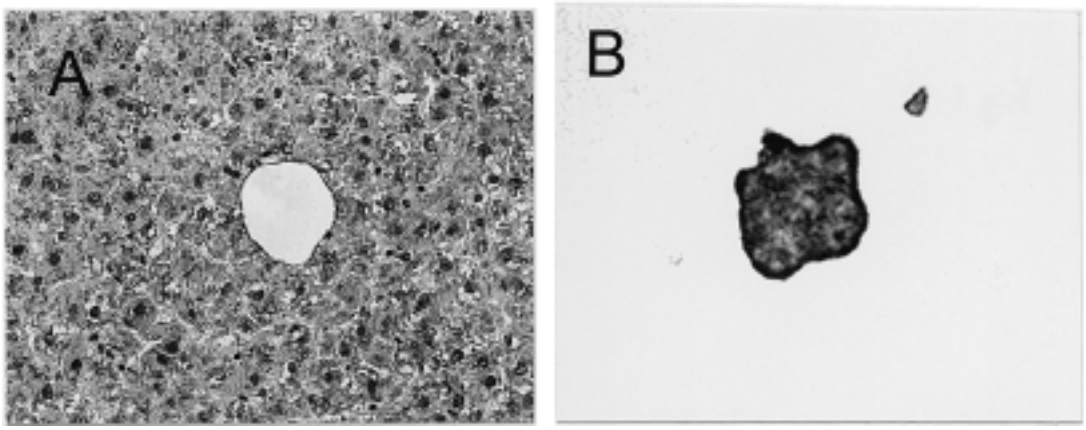


Fig. 1. Microdissection of hepatocytes. (A) Unfixed liver tissue microdisected using LMD. Hematoxylin stain. 100×. (B) Microdissected hepatocytes in the cap of 0.2 mL PCR tube × 150.

Table 1. Sequence of primers

| Gene | Sequence (5'–>3') |
|---|---|
| GAPDH | 5' primer TAAAGGGCATCCTGGGCTACACT<br>3' primer TTACTCCTTGGAGGCCATGTAGG |
| Albumin | 5' primer TTGCCAAGTACATGTGTGAG<br>3' Primer GGTTCTTCTACAAGAGGCTG |

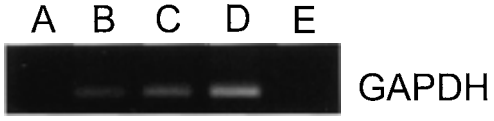


Fig. 2. Sensitivity of one-step RT-PCR from microdissected tissue sample. Approximately 5 (lane A), 25 (lane B), 125 (lane C), and 625 (lane D) hepatocytes were microdissected. After reverse transcription, GAPDH cDNAs were amplified with 60 thermal cycles. GAPDH was detected as few as 25 hepatocytes. Lane E: non-reverse transcription control without pd(N)6 random primer.


Fig. 3. Specificity of one-step RT-PCR from microdissected tissue sample. Approximately 250 hepatocytes (lane A), proximal renal tubular epithelial cells (lane B), glomerular cells (lane C) were microdissected. After reverse transcription, albumin cDNAs were amplified with 60 thermal cycles. Albumin-specific band was detected only in hepatocytes sample. Lane E: non-reverse transcription control. 250 hepatocytes without pd(N)6 random primer.

quantitating multiple fluorophores equipment (real-time PCR system, ABI PRISM® 7700, Applied Biosystems, Foster City, CA, U.S.A.), cDNA synthesis and 10 cycles of amplification were performed using a regular thermal cycler, then PCR solution was transferred to ABI PRISM(r) 7700. Further 40 cycles of PCR reaction was done and amplification signals were analyzed. Thermal condition of PCR was consistent as described before. PCR signals were detected as the fluorescence emissions of SYBR Green® dye. Amplification signals were analyzed by Sequence Detection System Analysis Software v. 1.7. (Appied Biosystems), and relative amount of template mRNA was calculated based on the difference of cycle number to obtain a certain intense signal.

In sensitivity assay, GAPDH specific band was observed at and above 25 cells and the intensity of the band increased depending on the number of template hepatocytes. No non-specific amplification was detected in negative control sample in the absence of reverse transcription (Fig. 2). As for specificity examination, albumin cDNA-specific band was observed only in hepatocytes sample, and non-specific band was not detected (Fig. 3). Since we couldn't confirm whether microdissected tissue sample was completely transferred from 0.2 mL PCR tube used in LMD system to 96-well plate designed for real-time PCR equipment, cDNA synthesis and 10 cycles of PCR reaction was performed using regular 0.2 mL PCR tube and thermal-cycler prior to real-time PCR. Then, PCR solution was moved to 96-well plate and following 40 cycles of amplification was done by ABI PRISM® 7700. Quantitative data were average values of duplicate experiments and they were almost comparable. Amplification signal of GAPDH-cDNA was first detected after 6 cycles in the $10^5$ cell sample, and all samples reached to the plateau level by 28 thermal cycle. Quantitative data was obtained at 20 cycle with clear linearity between the amount of template hepatocytes and amplification signals

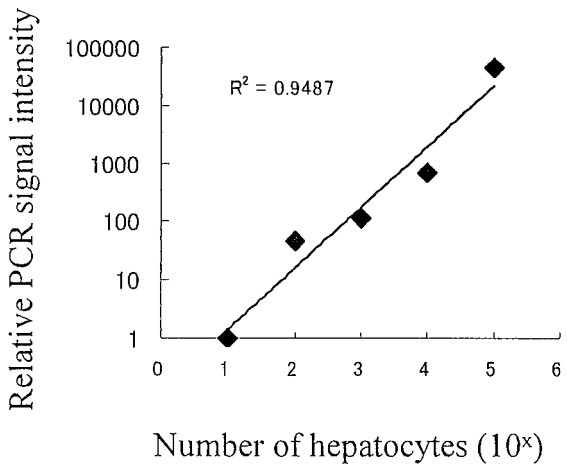


Fig. 4. Verification of quantitative RT-PCR from microdissected tissue sample. Linearity of cDNA amplification was evaluated using various number of hepatocytes cut by LMD and GAPDH PCR primer. 10, $10^2$, $10^3$, $10^4$, and $10^5$ hepatocytes were cut and placed in 0.2 mL PCR tube. After cDNA synthesis and 10 cycles of amplification in a regular thermal cycler (Takara Thermal cycler MP) PCR solution was transferred to ABI PRISM(r) 7700, and additional 40 cycles amplification was performed. A stringent correlation ($R^2$=0.9487) of amplification signal and the number of template hepatocytes is observed. Data are average value of duplicate experiments obtained at 20 cycle.

($R^2$=0.9487, Fig. 4).

In the present study, RT-PCR was successfully done in spite of skipping of RNA extraction step even under the coexistence of protein, DNA, and other cellular components. We speculate that RNA was accessible for the first strand cDNA synthesis because microdissected tissue was extremely small and cytoplasm of cut cell was directly exposed to RT-PCR solution. Further, since the cryosections were processed immediately after sectioning, we might avoid the internal RNase and other factors that interfere with the RT-PCR reaction. As for quantitative RT-PCR, feasibility of this technique in combination with LMD has been first demonstrated by detecting up-regulation of TNF-alpha gene in LPS-stimulated rat lung tissue [5]. Meanwhile, expression profiling in combination with LMD technique is rapidly becoming popular. Although it is clear that LMD enables us to procure selected tissue and subsequent tissue-specific RNA from heterogenous cell populations, it would be very difficult to obtain sufficient RNA for following molecular analysis because of very limited amount of cells. To overcome this problem, RNA extraction from microdissected tissue and its linear amplification using T7 RNA polymerase have recently been reported [6, 7]. Specht *et al.* [10, 11] assessed the influence of several RNA extraction methods on RNA quantification and demonstrated possible RNA recovery from LMD samples and its application to quantitative RT-PCR. These approaches, however, need a complicated procedure and still have limitations of sample amount. In the present study, we demonstrated a simple and rapid one-step RT-PCR procedure skipping the RNA isolation steps to avoid RNA loss and successfully amplified GAPDH and albumin cDNA from microdissected tissues samples with good sensitivity and specificity. In combination with regular thermal-cycler, this protocol could be simply applied to real-time PCR technique. In conclusion, this experimental protocol is useful for expression analysis for laser-microdissected pathology samples.

ACKNOWLEDGEMENT. We thank Hiroko Ito for her expert technical assistance.

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