

Quantification of Gene Expression in Mouse and Human Renal Proximal Tubules

Jun-ya Kaimori, Masaru Takenaka, and Kousaku Okubo

Summary

The kidney consists of many functional modules called nephrons. Each nephron has a tubular structure made up of several structurally and functionally distinct segments. The analysis of individual segments requires the use of microdissection techniques. We describe protocols that have been used to successfully isolate messenger RNA from proximal tubules of both freshly prepared and archival samples using laser capture microdissection and laser-manipulated microdissection.

Key Words: Proximal tubule; laser capture microdissection; real-time PCR.

1. Introduction

1.1. Kidney Structure

The human kidney consists of approx 1 million functional units called nephrons. Each nephron has a convoluted tubular structure made up of five morphologically and functionally distinct segments. Various manipulative techniques have been developed for the selective isolation of these segments (1). Laser microdissection techniques (2) enable isolation of specific cell types from renal biopsy specimens that are routinely generated for laboratory diagnosis (3,4). In this chapter, we summarize the protocols that have been successfully employed in recovering messenger RNA from proximal tubules of both freshly prepared and archival samples (5) using laser capture microdissection (LCM) (3) and laser-manipulated microdissection (LMM) (4).

1.2. Tissue Preservation

It is easy to isolate RNA from kidney tissues compared with other tissues that are rich in RNase (pancreas), fibrous proteins (muscular tissue), or mucus

From: *Methods in Molecular Biology*, vol. 293: *Laser Capture Microdissection: Methods and Protocols*
Edited by: G. I. Murray and S. Curran © Humana Press Inc., Totowa, NJ

secretions (airways, alimentary tracts). On the other hand, it has a complex histological structure. In general, microscopic structure and RNA are both preserved either by ethanol, formalin, or RNA-preserving solutions such as RNAlater. Ethanol dehydrates the tissue and stops the action of nuclease and protease without any deleterious effect on RNA even after prolonged incubation.

The fixative action of formalin is via inactivation of degradation enzymes by methylol addition or methylene bridge formation within or across protein molecules. It similarly affects bases in nucleic acids and makes them a poor template for molecular biological applications (6). Moreover, prolonged incubation of tissues in formalin results in extensive protein crosslinking that subsequently makes tissue resistant to solubilization. RNAlater is a widely used reagent for tissue RNA preservation. Its action seems to be precipitation of proteins by high ion concentration similar to the action of ammonium sulfate. It preserves RNA in renal specimens but tissue morphology is not well maintained. Accordingly, for laser microdissection of renal tissue, ethanol is the preferred fixative, especially as it can be perfused through the tissue. For biopsy specimens we treat tissue blocks briefly with RNAlater and then store them in ethanol. We find that prolonged digestion with proteinase K is necessary to obtain a satisfactory yield of RNA from archived tissue samples (6).

1.3. Messenger RNA Content

The average epithelial cell contains 0.4 pg of mRNA (200,000–300,000 molecules). In a differentiated cell, about 20,000 genes are expressed. There is wide variation in expression of individual genes: the most abundant mRNA amounts to 10% of total mRNA and the least abundant occurs at only a few copies per cell. As an empirical rule, the fraction of transcripts from one gene is approximately equal to $0.1/r$, where r stands for activity rank of the gene in a cell (7). For example, mRNA from the hundredth most abundant gene exists at 200 copies ($0.1/100 \times 200,000$) in a cell. Assuming the sensitivity of the following RNA detection assay as 100 copies and the yield of viable RNA at 10%, it is necessary to collect 1000 cells for detection of all active transcribed genes, 50 cells for the 1000 most abundant genes, and 5 cells for the 100 most common genes. In mice, the most abundant mRNA is for the androgen-regulated protein (NM_010594 in NCBI RefSeq) in proximal tubules (8) and α -B crystallin (NM_001885) in collecting ducts (9).

2. Materials

2.1. RNA Isolation From Mouse Proximal Tubules

1. Phosphate-buffered saline (PBS), pH 7.4.
2. 99.5% Ethanol (RNA grade).
3. 30% Sucrose/PBS (RNA grade).

4. Tissue-Tek optimum cutting temperature (OCT) compound (Sakura Company, CA, USA).
5. Glass slides.
6. 0.1% poly-L-lysine solution (Sigma Diagnostics, St. Louis, MO).
7. 1.35- μ m polyethylene foils (Laser Pressure Catapulting (LPC) membrane; P.A.L.M. Bernried, Germany).
8. Cryostat (JUNG CM3000, Leica, Germany)
9. Carrazzi's hematoxylin solution (RNA grade) (Wako Pure Chemical Industries, Osaka, Japan).
10. Laser capture microdissection (LCM) system (Arcturus Engineering Inc. Mountain View, CA)
11. LCM transfer film (CapSure TF-100; Arcturus Engineering).
12. Superscript preamplification System (Invitrogen, Carlsbad, CA).
13. Microcon YM-10 (Millipore, Bedford, MA).
14. The TSA-1 TaqMan probe 5'-CTGTGGCCAGTTTCATGCCAGGAGAAAGA-3' (accession no. U47737, 3373 bp-3401 bp) with FAM as reporter and TAMRA as quencher, the TSA-1 forward primer sequence 5'-GATGTGCTTCTCATGTACCGATCAG-3' (3330 bp-3354 bp), and its reverse primer sequence 5'-CAGCGGCAGATAACGTGATACAG-3' (3408 bp-3430 bp).
15. ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA).
16. TaqMan Universal PCR Master Mix (Applied Biosystems).
17. TaqMan ribosomal RNA Control Reagents (Applied Biosystems).

2.2. RNA Isolation From Human Proximal Tubules

1. RNAlater (Ambion Inc., Austin, TX).
2. 99.5% ethanol (RNA grade).
3. Xylene.
4. Wax (Paraplast+56°C, Oxford Lab Ware).
5. Microtome: Reichert-Jung, Hn 40 (Cambridge Instrument, Germany).
6. 1.35- μ m polyethylene foils (Laser Pressure Catapulting, LPC membrane; PALM).
7. 76 \times 26 mm glass cover slips (Deckglaser, Germany).
8. Laser-manipulated microdissection (LMM) system (PALM system) (Carl Zeiss, Oberkochen, Germany).
9. Laser capture microdissection (LCM) system (Arcturus Engineering).
10. Proteinase K solution: 200 mM Tris-HCl pH 7.5, 200 mM NaCl, 1.5 mM MgCl₂, 2% SDS, 500 μ g/mL proteinase K, 50 μ g/mL tRNA, RNA grade.
11. TRIzol reagent (Invitrogen).
12. Superscript preamplification system (Invitrogen).
13. Microcon YM-10 (Millipore).
14. The TaqMan probe for human osteopontin (OPN) sequence was 5'-CGA-AGTTTTCACTCCAGTTGTCCCCACA-3' (accession no. JO4765, 495 bp-522 bp) with FAM as reporter and TAMRA as quencher. The forward primer sequence of human OPN was 5'-TCACTGATTTTCCACGGACC-3' (464 bp-484 bp) and the reverse was 5'-CCTCGGCCATCATATGTGTCTA-3' (524 bp-545 bp).

Mouse kidney tissue

Perfusion of kidney
 ↓
 Fixation of tissues
 ↓
 Prepare snap frozen tissue specimens
 ↓
 Rapid staining by Hematoxylin
 ↓
 Laser-microdissection by LCM

Human kidney biopsy tissue

Fixation of tissue after biopsy
 ↓
 Prepare paraffin-embedded tissue specimens
 ↓
 Laser-microdissection by LMM and LCM
 Serial sections were stained by Hematoxylin and used to identify proximal tubules in the specimen

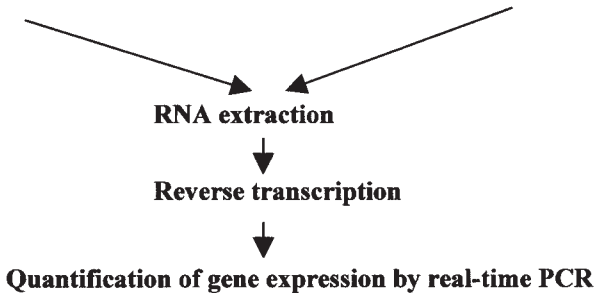


Fig. 1. Procedure for laser microdissection and real-time PCR. Mouse renal tissue and human renal biopsy tissue are processed differently. Mouse tissue is cut into frozen sections, while human renal biopsy specimens are embedded in wax. A combination of LMM and LCM is recommended to specifically collect proximal tubular epithelial cells.

15. TaqMan Human GAPDH Control Reagents (Applied Biosystems).
16. ABI Prism 7700 Sequence Detection System (Applied Biosystems).

3. Methods

The methods described below are RNA isolation from (1) mouse proximal tubules from histological tissue specimens and (2) human proximal tubules from biopsy specimens, followed by quantification of specific gene expression. The procedure is shown schematically in **Fig. 1**. Careful preparation of histological specimens is important for successful quantification.

3.1. RNA Isolation From Mouse Proximal Tubules

3.1.1. Mouse Kidney Tissue Processing and Laser Microdissection by LCM

1. Remove mice kidneys after perfusion with PBS and then with 99.5% ethanol (*see Note 1*).
2. Fix the removed kidneys for 4 h in 99.5% ethanol in Rnase-free glass (*see Note 2*).

3. Dehydrate with 30% sucrose/PBS overnight.
4. Embed the samples in OCT compound and freeze them on a small stainless board pre-chilled by liquid nitrogen (-196°C).
5. Store them in a deep freezer (-80°C) for later use.
6. Cut the frozen embedded sample with a cryostat into sections of $5\text{-}\mu\text{m}$ thickness.
7. Mount sections onto polyethylene foils on a glass slide (*see Note 3*).
8. Stain the membrane-mounted specimens rapidly with Carrazzi's hematoxylin solution for 10 s (*see Note 4*).
9. Wash with DEPC-treated water for 10 s.
10. Dehydrate with 99.5% ethanol for 5 min (*see Note 4*).
11. Dry at room temperature for 30 min.
12. Mount a glass slide with specimens onto an LM200 Image Archiving Workstation.
13. Cover sections with a transfer film (CapSure TF-100).
14. Microdissect the proximal tubules (PTs) with the laser beam set at $7.5\ \mu\text{m}$ diameter and $50\text{--}75\ \text{mV}$.
15. Approximately 50 PTs were collected for the following analysis.

Figures 2A and **2B** show examples of tubule isolation following the above protocol. Without laying polyethylene foils over glass slides (**step 7**), tubules resist microdissection.

3.1.2. RNA Extraction

1. In a 0.6-mL reaction tube, soak the collected PTs on a transfer film with $200\ \mu\text{L}$ TRIzol solution that includes $1\ \mu\text{g}$ of tRNA (*see Note 5*) for 5 min at room temperature.
2. Vortex for 10 s.
3. Add $40\ \mu\text{L}$ chloroform into reaction mixture.
4. Vortex for 15 s and leave the sample at room temperature for 5 min.
5. Centrifuge at 10,000 rpm for 15 min at 4°C .
6. Transfer the upper phase into another 0.6 mL tube.
7. Add $200\ \mu\text{L}$ isopropanol and $20\ \mu\text{g}$ glycogen and leave at room temperature for 10 min.
8. Centrifuge at 12,000 rpm for 15 min at 4°C .
9. Wash the pellet with $500\ \mu\text{L}$ of 99.5% ethanol.
10. Dry the pellet and resuspend with $10\ \mu\text{L}$ distilled water.

3.1.3. cDNA Synthesis

1. Add the following into the RNA solution: $0.5\ \mu\text{L}$ $50\ \text{ng}/\mu\text{L}$ random hexamers; $1.5\ \mu\text{L}$ DEPC-treated water.
2. Incubate at 70°C for 10 min.
3. Add the following into the reaction solution according to the manufacturer's instructions: $2\ \mu\text{L}$ 10X PCR buffer; $2\ \mu\text{L}$ $25\ \text{mM}$ MgCl_2 solution; $2\ \mu\text{L}$ $1\ \mu\text{L}$ $10\ \text{mM}$ dNTP mix; and $0.1\ \text{M}$ DTT.
4. Incubate for 5 min at 25°C .
5. Add $1\ \mu\text{L}$ SuperScript II reverse transcriptase ($200\ \text{U}/\mu\text{L}$) into reaction mixture.

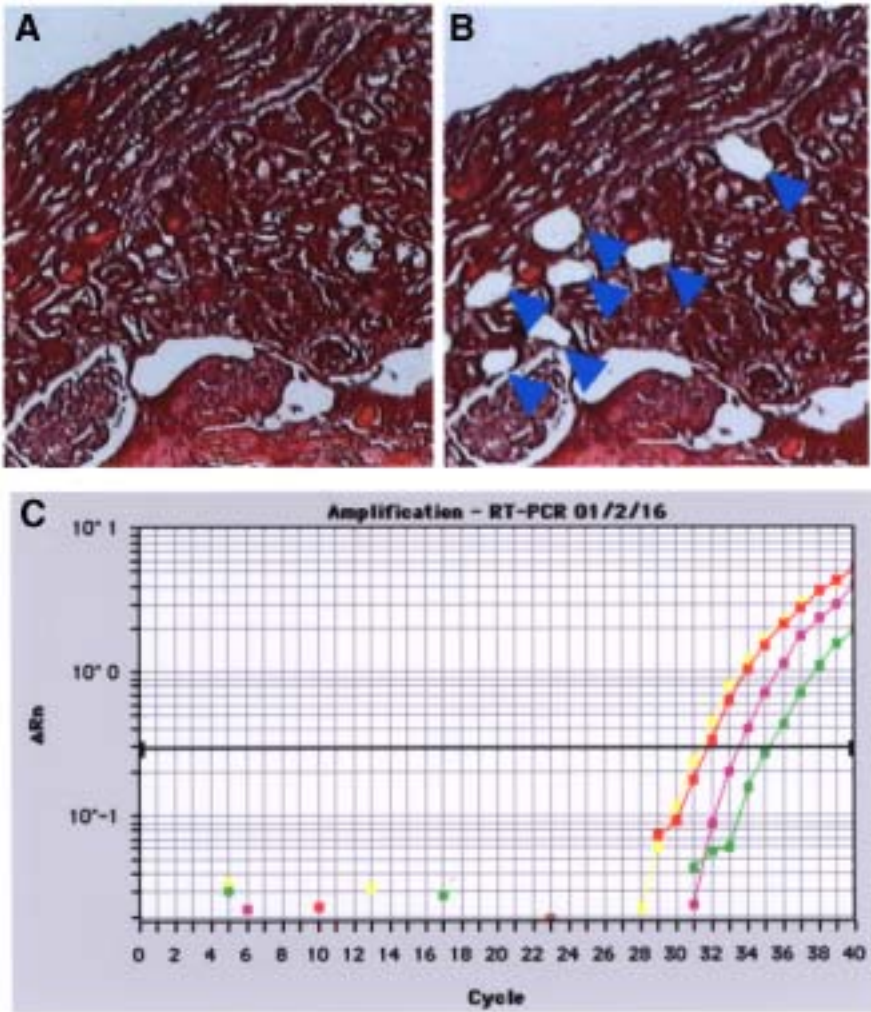


Fig. 2. Isolation of mouse proximal tubules by laser microdissection using LCM and detection of gene expression in microdissected tissue specimens. Figures show samples before (A) and after (B) laser microdissection of proximal tubules from 5- μ m-thick mouse renal tissue section. Arrows represent the original location of isolated proximal tubules. (C) Mouse TSA-1 gene expression was quantified in RNA from 50 proximal tubule segments isolated by LCM. The real-time PCR data are shown. Different colors in the amplification curve represent different experiments using different mice.

6. Incubate for 10 min at 25°C.
7. Incubate for 50 min at 42°C.
8. Incubate for 15 min at 70°C.
9. Chill on ice for 5–10min.
10. Add 200 µL distilled water into the reaction solution.
11. Transfer diluted reaction solution into Microcon YM-10 (*see Note 6*).
12. Centrifuge at 10,000 rpm for 15 min at 4°C (approx 40 µL of reaction mixture remained in YM-10).
13. Use 10 µL of the solution as a template for real-time PCR.

3.1.4. Quantification of mRNA by Real-Time PCR

1. Assemble the following reaction mixture in each tube for real-time PCR (*see Note 7*):
 - 25 µL TaqMan Universal PCR Master Mix (2X).
 - 5 µL 10 µM Mouse TSA-1 forward primer (*see Note 8*).
 - 5 µL 10 µM Mouse TSA-1 reverse primer.
 - 5 µL Mouse TSA-1 TaqMan probe.
 - 10 µL Sample.
 - 50 µL Total.
2. Perform real-time PCR using the following cycle profiles: Initial step 50°C for 10 min; 40 cycles 95°C for 2 min, then 95°C 15 s (denaturation) 60°C for 1 min (annealing/extension); final extension 70°C 10 for 10 min.
TaqMan ribosomal RNA Control reagents can be employed for standardization. In the example shown in **Fig. 2C**, the increased expression of TSA-1 mRNA in disease model PT was confirmed (*see Note 9*).

3.2. RNA Isolation From Human Proximal Tubules

3.2.1. Tissue Processing of Renal Biopsy Specimens

1. Immerse human renal biopsy specimens in RNAlater reagent for 5 s (*see Note 10*) as soon as they are obtained.
2. Fix in 99.5% ethanol overnight at 4°C.
3. Incubate in xylene for 1 h at room temperature and repeat this step three times.
4. Immerse in 100% wax for 1 h at 60°C and repeat this step three times.
5. Embed tissue samples in 100% wax.
6. Store in a deep freezer (–80°C).
7. Cut 10-µm sections using a microtome, taking appropriate precautions to ensure no contamination by ribonuclease.
8. Mount biopsy sections onto polyethylene foils as described above on 76 × 26 glass cover slips for LMM.
9. Dry biopsy sections on cover slips overnight at 37°C.
10. Dewax tissue sections by incubating in xylene for for three 5 min periods.
11. Dry specimens for 30 min at room temperature.
12. Stain several serial sections from each sample with freshly prepared Carrazzi's hematoxylin solution for 10 s.

3.2.2. Selective Tissue Collection by Combination of Laser-Mediated Microdissection and Laser Capture Microdissection Methods

1. Place the section mounted on a glass slide onto the laser-manipulated microdissection (LMM) system.
2. Identify as many proximal tubules as possible (*see Note 11*) by confirming their brush borders in the neighboring serial sections stained by hematoxylin (*see Note 12*).
3. Cut carefully inside the basement membrane of the proximal tubules with the laser beam to isolate the proximal tubular cells specifically.
4. Collect the samples by using LCM as described above (*see Subheading 3.1.1.*). Sections of proximal tubules are immediately processed for the following RNA extraction procedures.

Examples of human renal biopsy sections before and after dissection are shown in **Fig. 3A,B**. With the combination of LMM and LCM methods, only targeted cells were successfully isolated with virtually no contamination with other cells.

3.2.3. RNA Extraction From Collected PT Sections and Quantification of Osteopontin mRNA Expression by Real-Time PCR

1. Proximal tubules collected by the combined procedure of LMM and LCM were treated with 20 μ L of proteinase K solution including 1 μ g tRNA for 15 min at 45°C.
2. Add 200 μ L TRIzol reagent into the solution.
3. Extract RNA from TRIzol solution according to the manufacturer's instructions (*see Subheading 3.1.2.*).
4. Synthesize first strand cDNA (*see Subheading 3.1.2.*).
5. Perform real-time PCR of human OPN (**Fig. 3C**) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the ABI Prism 7700 Sequence Detection System (*see Subheading 3.1.2.*) (*see Note 14*).
6. Quantify the expression of human OPN/ GAPDH mRNA .

4. Notes

1. Perfusion with ethanol is the best fixative. Do not use a formalin-based fixative at any step.
2. The glassware used in fixation, dehydration, washing, and staining should be heat-sterilized for 3 h at 180°C to prevent contamination of RNase.
3. The thin polyethylene foil is attached onto a slide glass with nail polish. This foil allows us to isolate samples efficiently by the LCM method. The slide glasses are rinsed by a 0.1% poly-L-lysine solution.
4. Carrazzi's hematoxylin solution and 99.5% ethanol should be freshly prepared for each experiment. This is necessary to minimize degradation of RNA.
5. We usually add tRNA into the solution as a carrier that prevents loss of RNA by adhesion to surfaces of the tips and tubes.

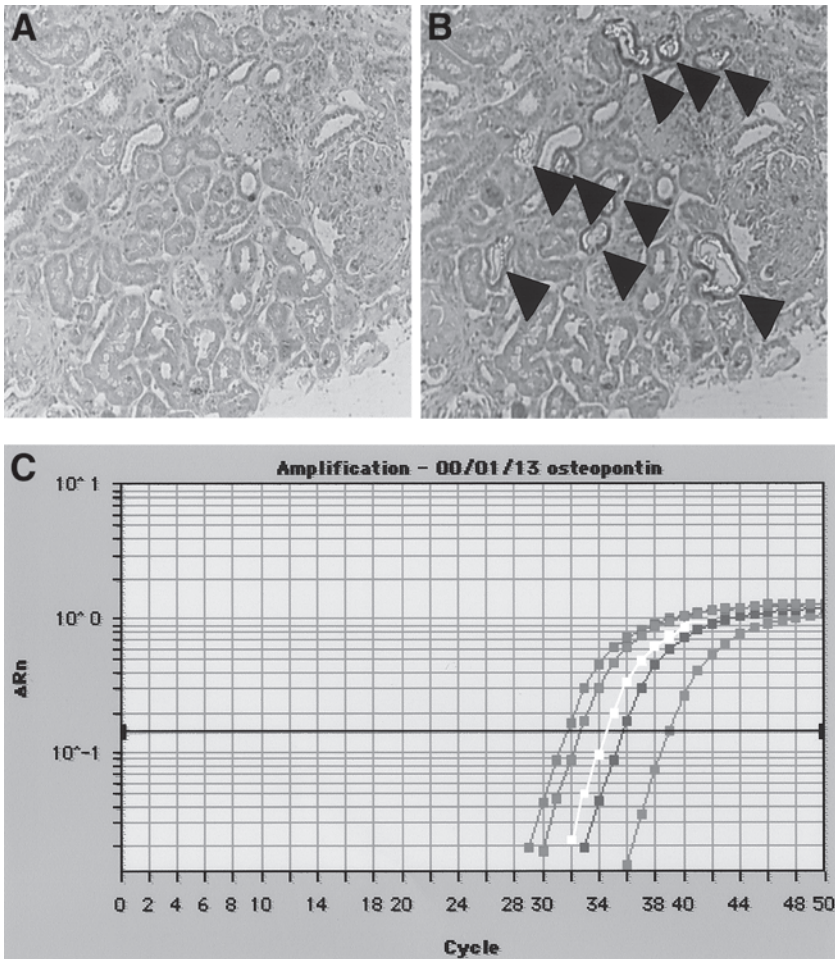


Fig. 3. Isolation of human proximal tubules by laser microdissection using LMM/LCM and detection of gene expression in microdissected tissue specimens. Figures show proximal tubules before (A) and after (B) laser-microdissection from 10- μ -thick human renal biopsy tissue sections. Arrows represent proximal tubules isolated from the specimen. (C) Human OPN gene expression was detected in RNA from 10 segments isolated by LMM/LCM. Each curve of a different color represents amplification kinetics of a sample from a different patient.

6. The excess random primer for reverse transcriptase reaction should be removed by column filter YM-10 before quantitative PCR.
7. The PCR experiment and RNA isolation procedure should be performed in separate laboratories in order to prevent contamination.
8. It is important to use a good primer set for real-time PCR. Preliminary PCR should be performed with several candidate primers selected by using primer design software, e.g. Primer Express. The set of primers that gives most efficient PCR should be chosen.
9. The quantitation of mRNA expression of TSA-1 was performed with standardization achieved by using rRNA representation. TaqMan ribosomal RNA control reagents (Perkin Elmer Applied Biosystems, Foster City, CA) were used as internal controls for mRNA expression (8).
10. Immersing the biopsy specimen in RNAlater reagent for more than 10 s usually results in disintegration of the tissue.
11. The number of cells required for mRNA detection depends on the target gene abundance (*see Subheading 1.3.*). 50 pulses of 10- μ m-thick PT cells from a well-preserved section seems to be adequate for detection of any mRNA expressed.
12. We identified proximal tubules by confirming their brush borders in sections stained with hematoxylin. The mRNA in mouse kidney fixed by perfusion remains intact after the staining procedure but in human biopsy specimens usually the RNA shows evidence of degradation.
13. The human GAPDH TaqMan probe and the forward and reverse primers were obtained from TaqMan Human GAPDH control reagents. To prepare positive controls for the quantification of mRNA, human GAPDH and OPN cDNA were cloned by means of PCR. The relative amounts of OPN and GAPDH mRNA in each sample should be calculated in terms of their own standard lines by using real-time PCR (5).

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