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Quantitative gene expression analysis in renal biopsies: A novel protocol for a high-throughput multicenter application

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Quantitative gene expression analysis in renal biopsies: A novel protocol for a high-throughput multicenter application.

Background. Recent advances in gene expression analysis may add the quantification of mRNA species in renal biopsies to routine diagnostic procedures in nephrology.

Methods. A systematic evaluation was performed on the relevant steps required to efficiently obtain cDNA from renal biopsies for high-throughput reverse transcription-polymerase chain reaction (RT-PCR) based mRNA quantification.

Results. The protocol preserves mRNA integrity by a novel RNase inhibitor and allows meticulous microdissection followed by maximal RNA recovery from tissue samples. Reverse transcription was optimized to give the best yield from minimal starting material. RNA quantity and quality were systematically investigated by real-time RT-PCR and electrophoresis on a microfluidic system, respectively. The reported procedure offers high RNA preservation and increases the yield of cDNA significantly compared to former protocols.

Conclusion. The simplicity of biopsy material acquisition combined with the centrally performed processing makes this protocol suitable for a wide spectrum of expression analysis in diverse clinical settings.

Percutaneous biopsy of the kidney has become the gold standard to establish a diagnosis in renal diseases. Further, it serves to guide proper management and gives information concerning the extent of injury and the prognosis of disease [1, 2]. Immunofluorescence and electron microscopy added over the last decades as adjunctive techniques to light microscopic analysis represent the three approaches used for routine diagnostic of renal biopsies [3].

These techniques evaluate structural changes and detect variations on the protein level. Quantitative analysis of a series of mRNA species would add a new dimension to routine biopsy analysis [4]. The rapid degradation of cellular mRNA and the labor intensive techniques required for analysis, such as in situ hybridization and quantitative reverse transcription-polymerase chain reaction (RT-PCR) on microdissected renal compartments [5], have precluded wide spread application of mRNA analysis. Interest in gene expression analysis has been fueled by rapid developments in molecular biology that could offer novel approaches in clinical medicine. The study of gene expression should provide valuable information about the nature and prognosis of disease processes. New techniques in mRNA quantification are applicable to routine analysis. The real-time PCR technique allows highly accurate template quantification from minimal tissue samples [6, 7]. The cDNA array technology displays gene expression patterns of thousands of mRNAs in a single reaction [4]. Moreover, it is now possible to study individual mRNA species in single cells (for example, glomerular podocyte [8]).

Reverse transcription-polymerase chain reaction based approaches have been used in the past for gene expression analysis of microdissected specimen in human renal disease and animal models [7, 9–15]. Unfortunately, most studies had inherent shortcomings including limitation in mRNA quality and quantity, available quantification systems and biopsy population for analysis.

To address these problems, the following strategies were employed in this study: Starting from existing methods [7, 9, 10], a new protocol was systematically designed and evaluated taking advantage of novel developments and techniques in the field of gene expression analysis. RNA preservation was achieved in former studies by immediate snap freezing in liquid nitrogen and storage at −80°C. Hence, the variation in time to freezing caused significant problems in the yield and quality of mRNA. We used a novel RNase inhibitor that allows efficient preservation of mRNA during storage and microdissection. Next, RNA isolation procedures were evaluated. For minute amounts of starting material, direct RT on the cellular lysate without prior RNA isolation has been shown to be superior to former isolation techniques [9]. This in situ RT prevents the loss of RNA during isolation,

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but introduces cytoplasmic proteins into RT causing RNA degradation and RT inhibition. A high throughput RNA isolation technique is now introduced that provides a complete lysis of cells and a significant increase in cDNA yield. For determination of mRNA levels, the real-time RT-PCR technique was found to offer a highly accurate quantification of minimal amounts of mRNA and allowed the quantitative analysis of the RT reaction. RNA integrity from microdissected samples could be determined by a microfluidic system [16]. This systematic evaluation has produced a novel protocol that combines a high throughput analysis with reproducibility and clinical applicability.

METHODS

Human biopsies

Human kidney biopsies or renal samples from a tumor-free part of a nephrectomy specimen were obtained from patients after informed consent and with acknowledgment of the local ethical committee. The material was handled as described below following the different protocols tested.

Murine tissue

For testing species independent steps of the protocol murine renal tissue was used. CD 1 mice were sacrificed and the kidneys were removed immediately. Further handling went along the respective protocols.

Microdissection

Microdissection was performed manually under a stereomicroscope using two dissection needle holders. If not otherwise stated the material was microdissected in an ice-cold solution. In previous studies microdissection was performed in phosphate-buffered saline (PBS) with or without vanadyl ribonucleoside complex (VRC) [9, 17]. For the present study three sets of four isolated glomeruli were microdissected in PBS with or without VRC (10 mmol/L; Life Technologies, Karlsruhe, Germany). No significant difference in cDNA yield was found comparing the two methods. Threshold cycle (Ct) values for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) on 1% of the respective cDNA were comparable after microdissection in VRC or pure PBS [VRC, 30.7 ± 3.5 (± SD); VRC-PBS, 30.6 ± 2.2; quantification methods are in the section, "**Real-time quantitative RT-PCR**"). Consequently, microdissection were performed in PBS if not otherwise stated. Effective tissue separation was verified by nephron segment specific gene expression pattern.

RNA quality control by microfluid electrophoresis

Total RNA was isolation by silica gel columns and diluted in 30 μL RNase-free water. 2 μL of this solution was analyzed using the RNA 6000 LabChip (Agilent Technologies, Waldbronn, Germany) on a 2100 Bioanalyzer (Agilent Technologies). This system performs electrophoretic separation of total RNA by means of a microfluidic system [16]. Experiments were performed as described by the manufacturer. The high quality of RNA was confirmed by circumscribed ribosomal peaks in the electrophoresis read-out, with no additional signals below the ribosomal bands and no shift to shorter fragments.

Reverse transcription

If not otherwise stated, RT was performed in a 45 μL volume, containing 9 μL buffer, 2 μL dithiothreitol (DTT; both from Life Technologies), 0.9 μL 25 mmol/L dNTP (Amersham Pharmacia, Freiburg, Germany), 1 μL RNase inhibitor (Rnasin; Promega, Mannheim, Germany) and 0.5 μL Microcarrier (Molecular Research Center, Cincinnati, OH, USA), 1 μg random hexamers (2 mg/mL stock; Roche, Mannheim, Germany) and 200 U reverse transcriptase (Superscript, Life Technologies) for one hour at 42°C. No DNase treatment was performed as cDNA-specific primers are available for most targets, the contamination by genomic DNA was low (**Results** section), and a contamination of the cDNA solution by DNase may lead to the loss of the template during prolonged storage.

Real-time quantitative RT-PCR

Real-time RT-PCR was performed on a TaqMan ABI 7700 Sequence Detection System (PE Biosystems, Weiterstadt, Germany) using heat-activated TaqDNA polymerase (Amplitaq Gold; PE Biosystems). After an initial hold of two minutes at 50°C and ten minutes at 95°C, the samples were cycled 40 times at 95°C for 15 seconds and 60°C for 60 seconds. For all quantitative analyses the cDNA content of each sample was compared with another sample following the ΔΔCt technique [6]. This procedure uses the formula $A_0/B_0 = (1 + E)^{(Ct,B-Ct,A)}$, where A_0 is the initial copy number of sample A; B_0 is the initial copy number of sample B; E is the efficiency of amplification; Ct,A is the threshold cycle of sample A; and Ct,B is the threshold cycle of sample B. The amplification efficiency was defined as 1, as all analyses were performed during the same run, including control dilution series. Following the formula above, the cDNA content of sample A compared to sample B was calculated by subtracting the mean Ct (triplicates) of sample A from the mean Ct of sample B (=ΔCt). Copies of the individual transcripts in sample A were defined as $2^{\Delta CT}$ copies of transcripts in sample B. Initially, the accuracy of this approach was tested in 21 samples with different cDNA contents (Ct values for GAPDH 23 to 28, mean 26 ± 1.5 SD). Dilutions were calculated along the above protocol to give an expected Ct value of 28. Real-time

RT-PCR of the diluted samples gave the predicted Ct of 28, with a standard deviation below 0.33.

GAPDH and β-actin served as housekeeping genes to assess the overall cDNA content. The following oligonucleotide primers (300 nmol/L) and probes (100 nmol/L) were used: human Wilms tumor antigen (WT-1; gb X51630, bp 1155 to 1221), sense primer 5′-AAATGGACAGA AGGGCAGAGC-3′, antisense primer 5′-GGATGGGC GTTGTGTGGT-3′, fluorescence labeled probe (FAM) 5′-ACCACAGCACAGGGTACGAGAGCGA-3′; human Synaptopodin (gb NM 007286, bp 1655 to 1720), sense primer 5′-CCCAAGGTGACCCCGAAT-3′, antisense primer 5′-CTGCCGCCGCTTCTCA-3′, fluorescence labeled probe (FAM) 5′-ACTTGCTGGATCTG GTACAGACAGCGG-3′, murine GAPDH (gb M 32599, bp 730 to 836): sense primer 5′-CATGGCCTTCCGT GTTCCTA-3′, antisense primer 5′-ATGCCTGCTTC ACCACCTTCT-3′, fluorescence labeled probe (VIC) 5′-CCCAATGTGTCCGTCGTGGATCTGA-3′. Commercially available pre-developed TaqMan reagents were used for human GAPDH and β-actin. Similar amplification efficiencies for all targets were demonstrated by analyzing serial cDNA dilutions showing a slope value of log input cDNA amount versus (Ct target A − Ct target B) of <0.1. The primers for GAPDH and WT-1 were cDNA-specific, not showing any amplification signal tested on 10,000 copies genomic DNA. All primers and probes were obtained from PE Biosystems.

Statistics

Data are given as mean ± SD. Statistical significance ($P < 0.05$) was analyzed using ANOVA and the Student t test, respectively.

RESULTS

Biopsy and storage

To standardize storage conditions a novel, commercially available RNase inhibitor (RNAlater, Ambion, Austin, TX, USA) was evaluated. mRNA stability was tested in murine renal tissue samples stored at room temperature, +4°C, −20°C and −80°C in PBS or RNase inhibitor, respectively. After five days the tubulointerstitial tissue was microdissected to samples of ~0.1 mm^3 and GAPDH levels quantified by real-time RT-PCR. In PBS, only storage at −80°C gave a significant protection of mRNA, demonstrated by a 10.4 ± 5.5 (SD)-fold higher cDNA yield compared to room temperature (Fig. 1). In contrast, the RNase inhibitor offered satisfactory mRNA preservation even at room temperature (8.4 ± 3.1-fold compared to PBS). Storage at −20°C and −80°C was comparably efficient with a 12.5 ± 3.1 and 14.3 ± 6.7-fold increase in RNA yield compared to PBS at room temperature, respectively (Fig. 1).

To analyze the long time efficiency of RNA protection

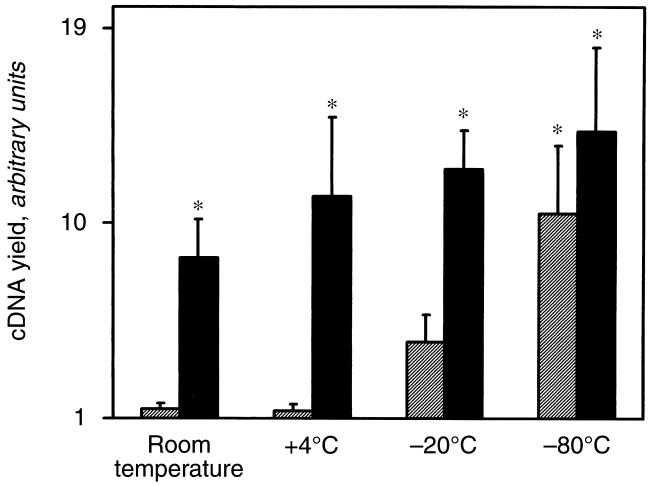

Fig. 1. RNA is protected by storage in RNase inhibitor. Real-time RT-PCR for GAPDH on renal tissue incubated at different temperatures with (■) or without (▨) RNase inhibitor for five days. The RNase inhibitor shows a significant RNA protection even at room temperatures. No significant difference is seen between the storage at −20°C and −80°C in RNase inhibitor. $N = 3$ for each condition, results are mean ± SEM, $^*P < 0.05$ vs. storage at room temperature without RNase inhibitor.

three sets of three to four isolated glomeruli were microdissected from renal biopsies either stored for six months or less than two weeks in RNase inhibitor. No difference was found in Ct values for GAPDH (28.9 ± 1.5 for samples stored for less than 2 weeks and 28.3 ± 0.5 for samples stored for up to 6 months, using 1% of the respective cDNA).

RNA degradation can be studied by the analysis of ribosomal RNA signals in routine gel analysis, but the amount of total RNA from microdissected samples is to small to be visible on a routine gel. To determine the quality of RNA on samples stored for six months in RNase inhibitor at −20°C, a microfluidic system was employed. Tubulointerstitial fragments (~1 mm^3) were microdissected from six different renal biopsies and total RNA isolated as described below. Freshly isolated total RNA from tissue culture lysate with circumscribed ribosomal RNA bands on a 1% 3[N-morpholino]propane sulfonic acid (MOPS) gel served as control. All microdissected samples showed intact ribosomal RNA (28S/18S ratio >2). No signs of RNA degradation as small RNA fragments, additional peaks below ribosomal bands or loss of the overall RNA signal were detected by capillary electrophoresis (Fig. 2).

Microdissection

Manual microdissection was performed under a stereomicroscope. This offers a reliable and fast dissection of glomeruli and tubulointerstitial fragments, as evaluated by high power phase contrast microscopy and analysis for nephron segment specific markers by real-time RT-PCR.

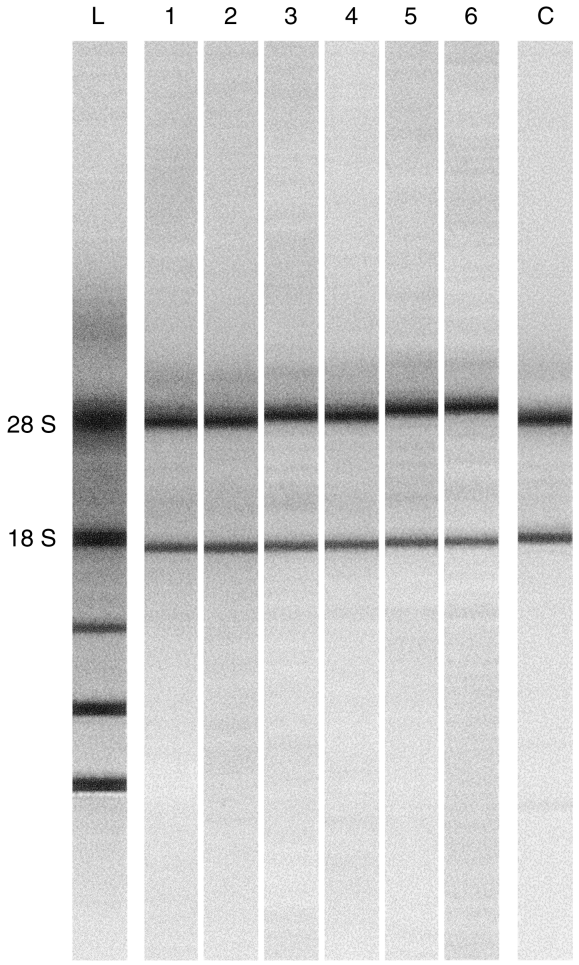

Fig. 2. RNA integrity after six months storage in RNase inhibitor. Six microdissected human tubulointerstitial fragments from renal biopsies were stored for up to 6 months in RNase inhibitor at −20°C, total RNA isolated by silica gel columns and 6% of the isolated RNA analyzed by electrophoresis on a microfluidic chip. All six show good preservation of 18 S and 28 S ribosomal RNA band on the gel-like view, comparable to control RNA (C). L = molecular size marker. Drifts due to migration times were eliminated by alignment of the 18 S band (see [16]).

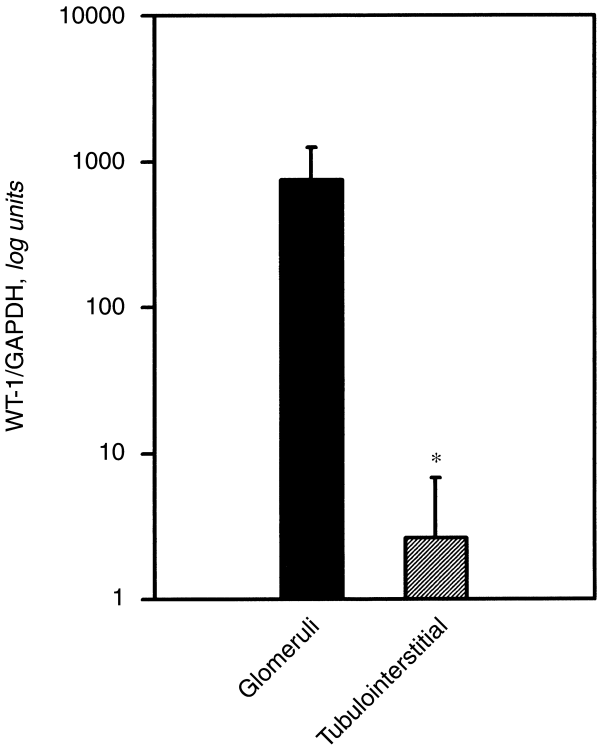

Fig. 3. Analysis of nephron segment-specific mRNA markers in microdissected samples. Expression of GAPDH and WT-1 determined in human glomerular (■) and tubulointerstitial (▨) fragments. The expression of WT-1, a podocyte marker, is 300-fold higher in the glomerular samples, confirming effective tissue separation by microdissection. Data are $N = 10$ and 8, respectively; results are mean ± SD, $^*P < 0.05$.

Samples containing only glomerular structures gave a 300-fold higher signal for WT-1, a marker for glomerular epithelial cells, than tubulointerstitial samples (Fig. 3).

Frozen tissue samples suffer membrane damage leading to leakage of cytoplasm and RNA. In addition, RNases can degrade mRNA even in ice-cold PBS. Both mechanisms could induce a significant loss of mRNA during microdissection. To increase the RNA yield we tested microdissection of murine renal tissue in 100, 80, 60, 40, 20% RNase inhibitor or pure PBS. The duration for microdissection of tubulointerstitial samples was standardized to ten minutes for all solutions. As shown in Figure 4A, a dose-dependent increase in mRNA was found in solutions containing RNase inhibitor. The microdissection in 100% RNase inhibitor offered the highest yield in cDNA, 60-fold above microdissection in PBS.

Meticulous microdissection requires a sufficient time window. Therefore, mRNA stability was compared between immediate microdissection and after a 90 minute delay in RNase inhibitor at room temperature. Analyzing the real-time RT-PCR signal for GAPDH showed no significant difference in the cDNA yield between both procedures (Fig. 4B). The RNase inhibitor allows detailed and time-consuming microdissection of specific organ structures for mRNA experiments.

To analyze the total loss of mRNA during microdissection, renal tissue was either directly snap-frozen in liquid nitrogen without microdissection or microdissected either in PBS or 100% RNase inhibitor ($N = 4$ each, analyzed as above). Snap-frozen and RNase inhibitor treated samples gave comparable cDNA yields, whereas the yield from samples microdissected in PBS was significantly reduced (Fig. 4C).

Cell lysis and homogenization

Several lysis protocols were systematically compared, including lysis in 2% Triton-X 100 combined with freeze-thaw cycles in liquid nitrogen, denaturing lysis buffer, ultrasound treatment or shredder columns for fragmen-

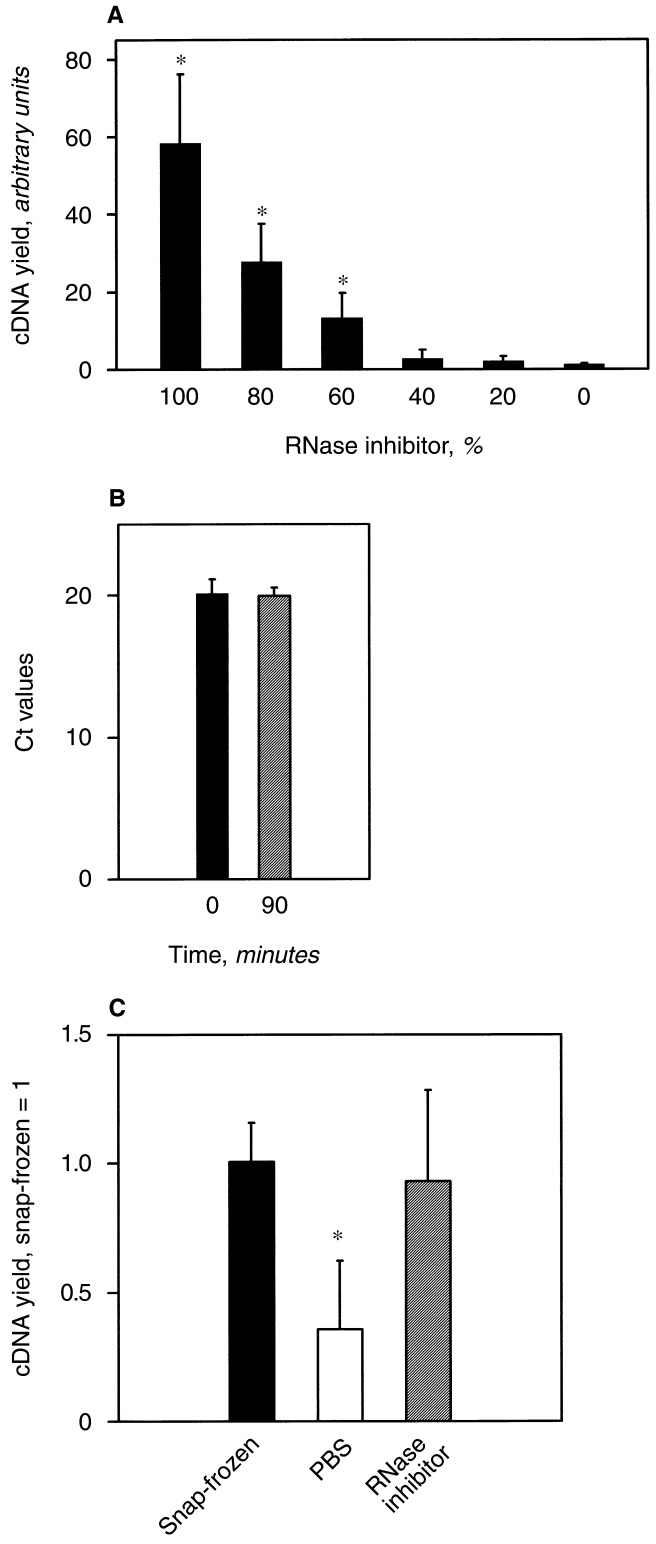

Fig. 4. Evaluation of microdissection conditions. (*A*) RNA yield after microdissection in RNase inhibitor. Human renal tissue was microdissected in 100, 80, 60, 40, 20, and 0% ice-cold RNase inhibitor (diluted in PBS). Bar graphs represent the expression ratio for GAPDH normalized to the lowest signal detected. A concentration dependent increase of RNA yield can be seen. All experiments were performed in duplicate, and results are mean ± SD; $*P < 0.05$. (*B*) The mRNA is stable during microdissection in RNase inhibitor for at least 90 minutes. Tubulointerstitial samples were analyzed at baseline (■) or after 90 minutes in RNase inhibitor at room temperature (▨). No difference in the Ct values was found with cDNA-specific GAPDH primers. (mean 20.1 ± 1.0. and 19.5 ± 0.6, respectively, $N = 3$ for each condition). (*C*) Loss of mRNA during microdissection in PBS. Renal tissue was either directly snap-frozen in liquid nitrogen (■) or microdissected in PBS (□) or 100% RNase inhibitor (▨). Direct snap-freezing without microdissection and microdissection in RNase inhibitor gave comparable cDNA yields. Samples microdissected in PBS showed significantly reduced amounts of cDNA, compared to both snap-frozen and RNase inhibitor treated samples. Data are $N = 4$ for each condition, results are mean ± SD, and $*P < 0.05$.

tation of microdissected tissue. The highest cDNA yield was achieved by denaturation of the sample with β-mercaptoethanol and guanidine thiocyanate containing lysis buffer for ten minutes at room temperature. Tissue fragmentation with ultrasound or shredder columns did not improve the yield (data not shown).

RNA isolation

Using microdissected renal tissue, in situ RT was compared to a silica gel-based isolation protocol (RNeasy-Mini; Qiagen, Germany) followed by RT of the isolated total RNA. A 75-fold higher cDNA yield was found after RNA isolation compared to in situ RT. This technique gave reproducible RNA yields in more than 1000 RNA isolations from minimal tissue samples down to a single human or murine glomerulus.

To test for contaminating genomic DNA total RNA was isolated from microdissected glomeruli by the above procedure ($N = 3$). Half of each sample was reverse transcribed, the other half processed without addition of reverse transcriptase. All samples were analyzed for Synaptopodin, as the respective reagents show similar amplification efficiencies on cDNA and genomic DNA. Reverse transcribed samples showed Ct values of 25 to 26, whereas only one not-reverse transcribed sample containing only genomic DNA gave any amplification signal below cycle 45 (that is, Ct 35). This demonstrates a contamination by genomic DNA below 0.1%.

Reverse transcription

Three different concentrations of random hexanucleotides and reverse transcriptase were tested on 10 ng human total RNA in 45 μL RT volume. As shown in Figure 5A the highest yield of cDNA was found with the lowest amount of primer (1 μg/45 μL). For reverse transcriptase a concentration of 200 U per reaction was sufficient. Of note, an interference with real-time PCR could be observed in reactions containing more than 10% of the above RT mix (data not shown). This could be circumvented by diluting the RT product 1:10 in Tris and EDTA (TE) buffer prior to the amplifications.

Linearity of RT reaction was evaluated using fivefold

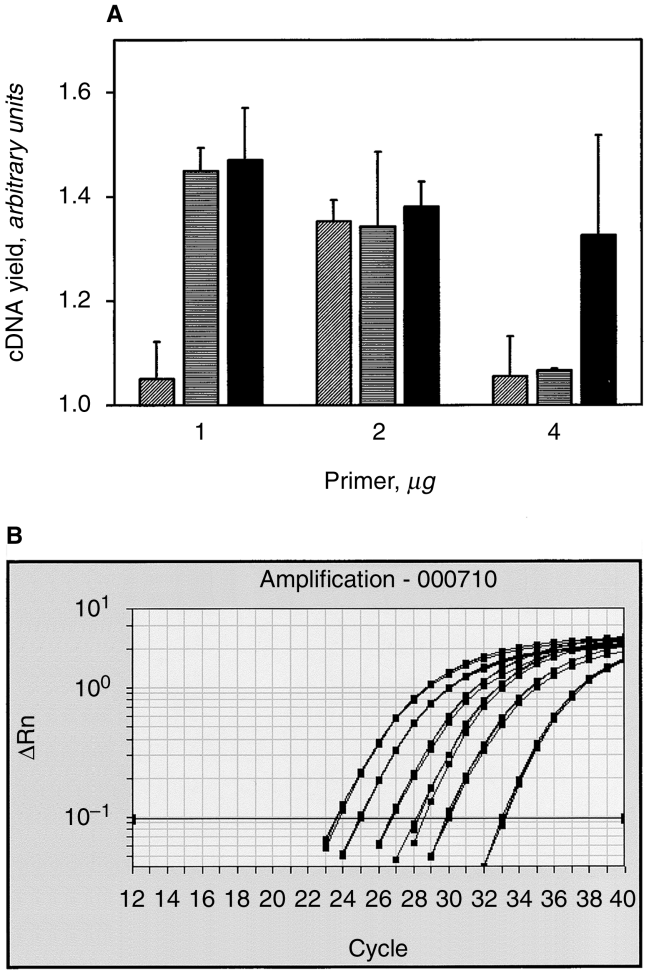

Fig. 5. Evaluation of reverse transcription conditions. (*A*) Comparison of different enzyme and random primer concentrations. To optimize the RT reaction different primer and enzyme concentrations were tested on 10 ng human total RNA. A decrease of random primer concentration showed a 50% increase in cDNA yield determined by real-time RT-PCR using 200 U RTase. $N = 2$ for each condition, results are mean ± SD (RTase = reverse transcriptase). Symbols are: (▨) 100 U RTase; (▤) 200 U RTase; (■) 400 U RTase. (*B*) Verification of quantitative real time RT-PCR. Linearity of RT reaction was evaluated using fivefold dilutions of human total RNA in a range mirroring the amount found in microdissected renal material (400, 80, 16, 3, 0.6, and 0.12 ng). After RT under the above conditions, RT efficiency was evaluated by quantification of GAPDH and β-actin via real-time RT-PCR. The six dilutions were analyzed by real-time RT-PCR in triplets; the corresponding amplification curves for β-actin are shown. (ΔRN = fluorescence reporter signal minus baseline signal). A stringent correlation of PCR product quantity with starting RNA concentration could be shown for both mRNAs ($R^2 = 0.98$ and 0.95 for GAPDH and β-actin, respectively), indicating linear, quantitative RT over a 3 log range.

dilutions of 400 ng to 120 pg human total RNA, a range found in microdissected renal samples. After RT under the above conditions RT efficiency was evaluated by quantification of GAPDH and β-actin via real-time RT-PCR. A positive correlation of PCR product quantity with starting RNA concentration was found for both templates ($R^2 = 0.98$ and 0.95, respectively), demonstrating linearity of the RT over a 3 log range (Fig. 5B).

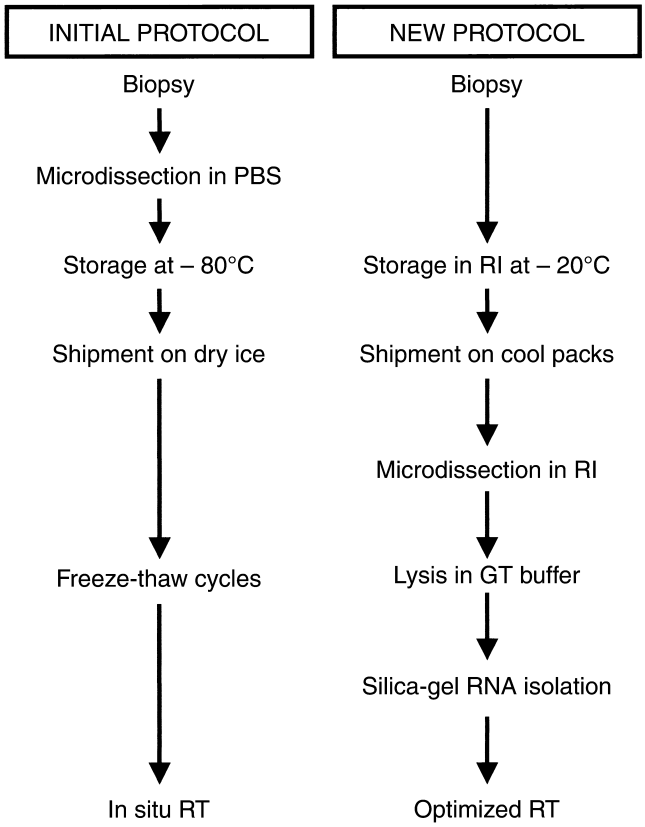

Fig. 6. Comparison of the initial and the newly developed protocol for renal biopsy handling. Both protocols are shown in a schematic overview. Abbreviations are: RI, RNase inhibitor; GT, guanidine thiocyanate; RT, reverse transcription.

Direct comparison of protocols

Processing cortical segments of murine renal tissue either following the previously described (initial) or the optimized protocol allowed direct comparison of cDNA yield (Fig. 6). Following the initial protocol, tissue was directly microdissected in PBS, lysed by three freeze–thaw cycles in Triton X-100 and random-primed in situ reverse transcribed. In a second set of samples the material was harvested and microdissected in 100% RNase inhibitor, total RNA isolated using silica gel-based columns and random-primed reverse transcribed. cDNA levels obtained by the two protocols were quantified using GAPDH. A 75 ± 15-fold higher cDNA yield was obtained with the newly developed protocol as compared to the initial procedure ($N = 4$, Fig. 7).

DISCUSSION

Routine gene expression analysis is likely to be introduced as a novel instrument in biopsy interpretation [4, 5]. The expectations are that these new tools will add quantitative parameters to conventional biopsy diagnostic procedures, thus improving disease classification, prog-

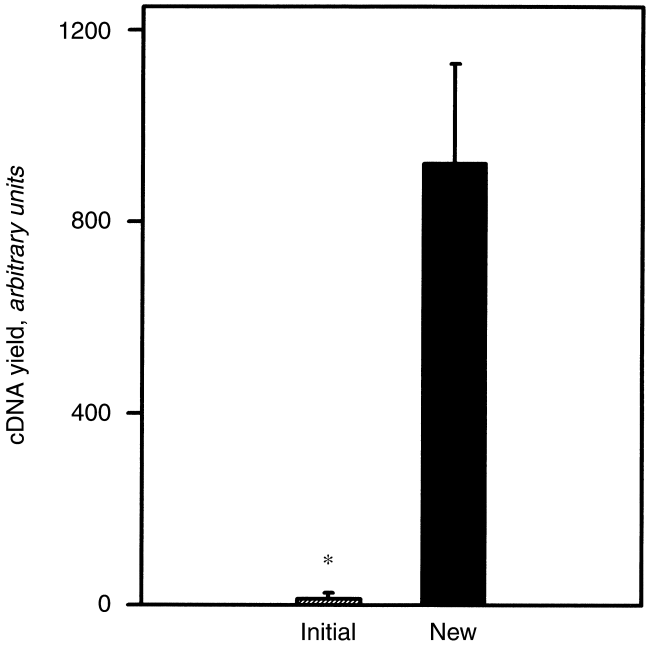

Fig. 7. Comparison of the initial (▨) with the improved (■) protocol. Renal tissue was processed using microdissection in PBS combined with in situ RT (initial protocol) or storage and microdissection in RNase inhibitor, RNA isolation and optimized RT (new protocol). An increase in cDNA yield of 2 log steps was demonstrated. Data are $N = 4$ for each condition, mean ± SD; $*P < 0.05$.

nostic prediction and information concerning the response to the different treatments available. First results reported in the diagnosis and classification of hematological diseases show great promise [18].

The introduction of molecular analysis of renal biopsies requires the development of a robust and reproducible protocol for tissue processing. Our study performed a systematic evaluation of all relevant steps required to obtain cDNA from renal biopsies for gene expression analysis. Special care was taken to address the critical problems of biopsy processing relevant in the setting of a multicenter study. We were able to establish a protocol that not only improves the reproducibility of mRNA quantification via the RT-PCR approach, but also increases the amount of mRNA available for expression screening analysis. Most of the procedures described for renal tissue should also be applicable for other material obtained by fine needle biopsy.

We addressed the loss or degradation of RNA by testing a new RNase inhibitor [19, 20]. The ability to store and ship tissue in a solution preserving RNA allows a standardized microdissection in a central core facility. This approach significantly decreases the workload of the participating clinical centers and thereby facilitates patient recruitment. In addition, it adds a further level of security to RNA protection, as an interruption in keeping the tissue frozen does not lead to an immediate loss of mRNA. Using this procedure over 500 biopsies have been sampled, sent to the core facility, and stored for up to eight months without any observed degradation of RNA.

A significant increase in RNA yield was achieved by the addition of a new silica gel-based RNA isolation procedure. In previous studies in situ RT without prior RNA isolation showed a higher yield of cDNA compared to standard RNA isolation procedures [9]. Consequently, protocols were established that lacked an aggressive cell lysis step [9–12]. Such protocols could generate biased RNA samples by the preferential lysis of superficial cell layers (such as podocytes from glomeruli). The procedure used in our study combines aggressive cell lysis and RNA isolation, yields a significant increase in cDNA, and also minimizes genomic DNA contamination. Eikmans et al recently reported reproducible RNA isolation by the silica gel-based technique using tubulointerstitial fragments, but not glomeruli with their minute amount of RNA [17].

Analysis of the reverse transcription showed an increased cDNA yield when the random primer concentration in the reaction was decreased. This counterintuitive observation may be due to an increase in cDNA length secondary to fewer binding events of random primers per mRNA molecule. The resulting longer cDNAs may serve as more effective templates in RT-PCR.

Finally, the employment of real-time RT-PCR for mRNA quantification allowed systematic evaluations with exquisite sensitivity, using 1% from starting material as little as one isolated glomerulus. Thus, analyses could be performed on up to 100 targets from microdissected glomeruli. In addition, the real-time RT-PCR quantification could be applied for high-throughput analysis.

The considerable increase in RNA yield and integrity obtained by the protocol described should be helpful also for extending cDNA microarray analysis to small biopsy material. In fact, RNA obtained from microdissected tubulointerstitial compartment could be already sufficient as starting material for highly sensitive expression arrays. However, for microdissected glomeruli or single nephron segments a quantitative and reproducible way to amplify the messages in a truly linear manner will be necessary [21]. Optimal RNA recovery will require fewer cycles of linear amplification and thereby decrease amplification bias [22]. In the future, the reported protocol may facilitate a screening for molecular markers in kidney diseases, whose determination could be added to pathology procedures even on routine formaldehyde-fixed renal biopsies [23].

In summary, the protocol presented in this study allows the easy processing of renal biopsy material for molecular analysis in diverse clinical settings. It offers a high degree of mRNA protection during transportation and storage and an increased yield and quality of the cDNA obtained for expression studies. This approach

should allow multicenter studies investigating gene expression screening in human renal biopsy material. A better definition of the molecular pathways activated in human renal disease can lead to novel molecular diagnostic tools and new therapeutic targets. The introduction of diagnostic gene expression analysis into clinical practice should improve the predictive strength of biopsy analysis, giving clinicians pathophysiologically defined diagnostic categories, reliable prognostic information, and prediction for response to the available treatment options.

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