Molecular markers in renal transplant biopsies

Groningen, M.C. van

Chapter 2

Improvement of extraction and processing of RNA from renal biopsies

M.C. Roos-van Groningen, M. Eikmans, H.J. Baelde, E. de Heer, and J.A. Bruijn

Department of Pathology, Leiden University Medical Center, Leiden, The Netherlands

Kidney International 65(1):97-105, 2004

Abstract

Assessment of mRNA levels has the potential to predict renal outcome. The objective of this study was to optimize several steps in the protocol for obtaining cDNA from routine clinical kidney biopsy material.

RNA integrity was compared between different methods for extraction of RNA, synthesis of cDNA, and storage of renal tissue. Hereby, RNA*later*, an RNA-preserving compound, was tested for implementation in a protocol for renal biopsies that combines routine histology and RNA expression studies. Gel electrophoresis and real-time polymerase chain reactions were outcome parameters for assessment of RNA integrity.

The Trizol method rendered higher RNA yields from fresh renal tissue than the NP40 method and RNeasy spin columns. RNA yields were not affected when renal tissue was stored at -70°C for up to 2 months in PBS. cDNA levels obtained using Avian Myeloblastosis Virus (AMV) reverse transcriptase (RT) were at least twice as high as those obtained with Sensiscript and Superscript RT. RNA*later* maintained RNA integrity in whole renal cortex stored at 4°C for up to 3 months. Dissection of small biopsies in RNA*later* rendered similar RNA yields in comparison with dissection in PBS, but the yield of glomeruli from the cortices was 50% lower ($P < 0.005$). Integrity of RNA*later*-treated tissue, evaluated by light microscopy and immunofluorescence, was diminished.

This study shows optimization of several steps in the protocol for extraction and handling of RNA in renal cortical tissue. RNA extraction and cDNA synthesis can be optimized by the use of the Trizol method and AMV RT, respectively. RNA*later* is beneficial for preserving RNA integrity in whole renal cortex during storage and processing, but is not suitable for implementation in routine diagnostic histological stainings combined with RNA expression studies in dissected biopsy material.

Introduction

Histology and renal function are used for diagnostic and prognostic purposes in nephrological practice. Assessment of mRNA levels has the potential to predict renal outcome [1-6]. The possibility of the use of mRNA levels as diagnostic and prognostic tools might lead to early intervention strategies. In routine clinical practice only minute quantities of renal tissue are available, which makes implementation of mRNA assessment difficult. In addition, potential contamination by RNase molecules during the RNA extraction procedure leads to degradation of the RNA [7].
We have previously described an improved method for obtaining RNA from microdissected glomeruli from fresh kidney biopsies [8]. We intend to implement improved methods of RNA processing in future protocols. Recent studies have shown that RNA preserving compounds have the potential of maintaining the integrity of the RNA contained in neuroblastoma xenografts and renal tissue during storage and microdissection [9;10]. Studies in tumor biology have shown that the preserving compound RNA*later* (Ambion, Austin, Texas) maintains RNA integrity whilst also preserving histological integrity of human skin and breast tissues [11;12].
Quantification of RNA levels for diagnostic and prognostic purposes requires that each step in the protocol for obtaining cDNA from renal biopsy material be optimized. In the current study, we intended to improve several steps in this protocol. We also examined the feasibility of the use of RNA*later* in routine diagnostic practice and the effect of RNA*later* on the integrity of both RNA and histology.

Materials and Methods

Kidney tissue
A cadaveric donor kidney was used to optimize the protocols for RNA extraction and cDNA synthesis from glomerular samples (kidney 1). This kidney was obtained from a 70-year-old male. Cold ischemia time was 33 hours. Another cadaveric donor kidney (kidney 2) was used for investigating the effects of RNA*later* on RNA integrity and renal morphology. This kidney was obtained from a 45-year-old female. Cold ischemia time was 17 hours. Both kidneys were obtained from Eurotransplant (ET) and were unsuitable for transplantation due to technical reasons. Tissue histology and RNA integrity was evaluated for both ET kidneys before commencing the experiments in this study. Quantified ratio of the 28S to 18S rRNA bands for both kidneys was approximately 2.

Real-time PCR
As a read-out system for RNA and cDNA yields, we applied real-time PCR by using a Prism 7700 Sequence Detector System (Perkin Elmer Biosystems, Foster City, CA). Measurements in each sample were performed in duplicate. This procedure is described in detail elsewhere [13]. Primers were obtained from Gibco BRL (Breda, The Netherlands) and TaqMan probes were obtained from Perkin

Elmer Biosystems. Collagen α1(IV) primer sequences were: forward 5´-ACT CTT TTG TGA TGC ACA CCA-3´ and reverse 5´-AAG CTG TAA GCG TTT GCG TA-3´, and the probe sequence was 5-AAT GGC GCA CTT CTA AAC TCC TCC AGG CAG G-3´. TGF-β primer sequences were: forward 5´-CCC AGC ATC TGC AAA GCT C-3´ and reverse 5´-GTC AAT GTA CAG CTG CCG CA-3´, and the probe sequence was 5´-ACA CCA ACT ATT GCT TCA GCT CCA CGG A-3´. The 5´ ends of the probes for TGF-β and collagen α1(IV) were labelled by the reporter dye tetrachloro-6-carboxyfluorescein (TET). The 3´ ends were labeled by the quencher dye 6-carboxy-tetramethyl-rhodamine (TAMRA). The PCR reaction contained 7.5 µM of probe, 7.5 µM of primer, 1 x EuroGentech mix, 0.5 U AmpliTaq Gold DNA polymerase, 0.8 mM dNTP, 5 mM $MgCl_2$, and 5 µL cDNA sample (diluted 1:50 for tubulointerstitial samples and diluted 1:10 for glomerular samples). Reactions took place in optical 96-well reaction plates covered with optical caps (Perkin Elmer). Amplification cycles were performed at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and at 60°C for 1 minute. As a standard in each PCR run we used a 5-fold dilution range of 2 pg of plasmid containing the appropriate template.

Optimization of RNA extraction from glomerular samples

Glomeruli from kidney 1 were obtained by sieving of cortical tissue consecutively through a 212 µm and 150 µm mesh as described previously [14]. To compare different methods of RNA extraction, we extracted RNA from batches of 15 glomeruli using either an NP40-based solution [15], Trizol reagent (Invitrogen, Breda, The Netherlands) [16] or RNeasy mini columns (Qiagen, Valencia, CA) [17]. Total RNA was used for cDNA synthesis, and cDNA yields were measured by real-time PCR for collagen α1(IV) as described above.

Effect of storage of glomerular samples on RNA integrity

We wanted to assess to what extent the duration of storage of isolated glomeruli at a temperature of -70°C affects RNA integrity. The three different RNA extraction methods mentioned above were applied in duplicate on 5 batches of 15 glomeruli, either immediately after glomeruli had been isolated or after glomeruli had been stored at -70°C for 1, 2, 4 or 8 weeks. For each time period, glomerular batches were stored in an NP40-based solution, phosphate-buffered saline (PBS), or RTL buffer (Qiagen, RNeasy kit), and further processed by the NP40-method, the Trizol method, and RNeasy mini columns, respectively. RNA extraction with the latter method was also performed in duplicate on 5 batches of 15 glomeruli that had been stored in PBS for the various time periods mentioned above.

Optimization of cDNA synthesis

RNA was extracted from three batches of 15 glomeruli with the Trizol method. To optimize the cDNA synthesis protocol, cDNA reactions were performed in triplicate using either Superscript® (Invitrogen, Breda, The Netherlands) at 42°C, AMV RT (Roche, Mannheim, Germany) at 42°C or Sensiscript+ at 37°C (Qiagen, Valencia, CA). cDNA synthesis was performed in triplicate for each transcript according to the manufacturer's manual using Oligo dT(15) primers.

As a read-out system for cDNA yields, real-time PCR was performed for collagen α1(IV) and TGF-β .

Effect of RNAlater on RNA integrity in cortical tissue stored at 4°C
Sixteen pieces of renal cortex, approximately 5 x 5 x 2 mm in size, were obtained from cadaveric donor kidney 2. Eight pieces were treated with 0.5 ml of RNA*later* as described in the manufacturer's manual, and 6 pieces were placed in 0.5 ml of PBS. RNA was extracted immediately from two fresh, untreated samples (t = 0). Three samples (duplicates) were stored at 4°C for either 1 week, 2 weeks or 3 months in PBS. Four samples (duplicates) were stored at 4°C for either 1 week, 2 weeks, 1 month, or 3 months in RNA*later*. The Trizol method was used for RNA isolation. To assess the integrity of the RNA samples, we applied 1 mg of RNA to a 1% agarose-formalin gel.

Effect of RNAlater on microdissected tissue: RNA yields and glomerular morphology
We wanted to compare the effect that RNA*later* and PBS had on RNA stability during microdissection of fresh renal tissue and in renal tissue that had been frozen and thawed prior to microdissection. Twenty-seven pieces of cortex (1 x 1 x 10 mm) were taken from cadaveric donor kidney 2. Figure 1 demonstrates the different treatments of these pieces. Nine samples were used for incubation and subsequent microdissection in either three compounds: PBS, RNA*later*, or RNA*later* 1:8 (diluted in PBS). Microdissection of the renal tissue was performed as described in a previous paper [18]. For each treatment condition, three fresh pieces of cortical tissue (triplicates), which had not been frozen before, were microdissected, three pieces (triplicates) were microdissected after storage for 1 hour at a temperature of -70°C, and three pieces (triplicates) were microdissected after storage for 1 week at -70°C. Each of the 27 cortical samples was weighed before incubation (mean 11.5 ± 3.9 mg). We counted the glomeruli obtained through microdissection of each sample to assess glomerular yields. Tissue morphology of microdissected cortex and glomeruli was visually evaluated under a Zeiss stereomicroscope at 6.5x and 25x magnification, respectively. All glomerular and tubulointerstitial samples were further processed for RNA extraction with the Trizol method. RNA pellets were dissolved in such a volume of RNase-free water that 2 µl was added for each mg of cortical tissue originally used for RNA extraction. Two µl of RNA (equivalent to 1 mg of cortical tissue) was used for cDNA synthesis, and cDNA yields were measured by real-time PCR for collagen α1(IV). Data represent the relative collagen α1(IV) transcription level per glomerulus (glomerular samples) or per mg tissue (tubulointerstitial samples).

Effect of RNAlater on tissue integrity
Three pieces of cortex were obtained from cadaveric donor kidney 2. One piece was immediately fixed in 4% formalin and embedded in paraffin tissue. The two remaining pieces were treated overnight at a temperature of 4°C with RNA*later* or RNA*later* 1:8 and then fixed in 4% formalin for paraffin embedding. Sections of 4 µm were cut from the three differently embedded samples and

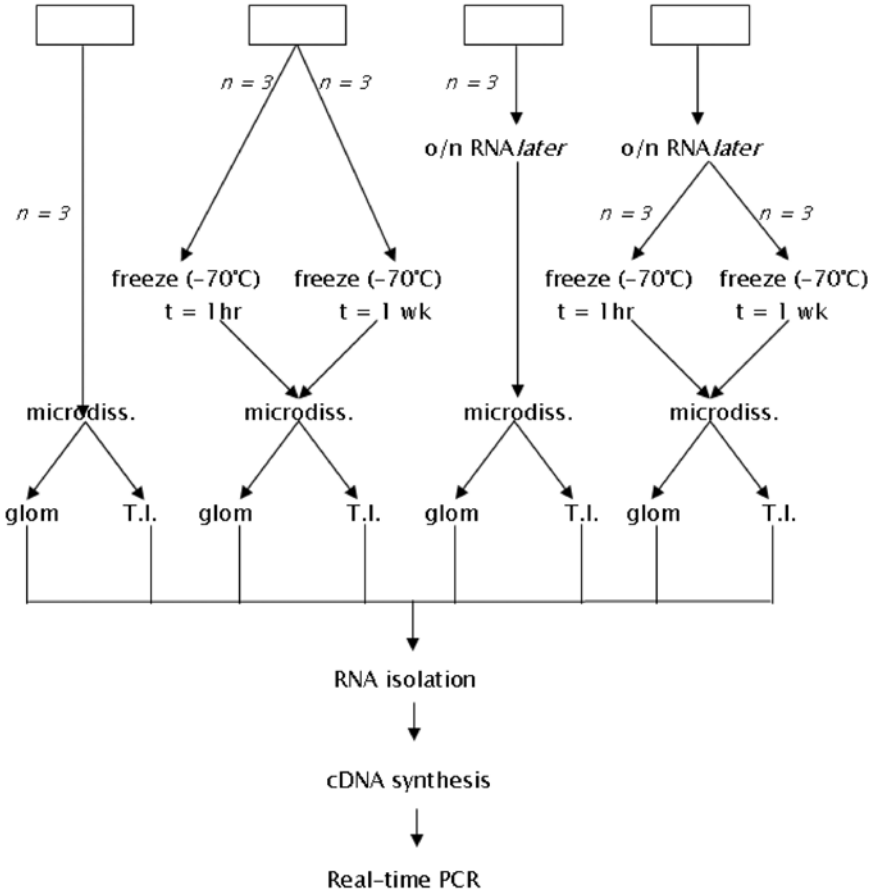

Figure 1. RNA*later* study protocol. Renal cortex was obtained from a cadaveric donor kidney and each step was applied in triplicate. RNA*later* 1:8 as incubation compound was tested in the same manner as RNA*later*, depicted in the two trees on the right.

subsequently stained with Periodic-acid Schiff. Histology was evaluated with light microscopy.

To determine the effect of RNA*later* on the results of immune fluorescence stainings, we treated a portion of cortex with RNA*later*. The provenance of this cortex sample was a renal biopsy (2 mm in length) from a patient diagnosed with lupus nephritis. After treatment with RNA*later*, the sample was washed twice in PBS for 15 minutes [19], after which the biopsy was snap frozen in liquid nitrogen. The tissue was embedded in OCT medium (Sakura Finetek, Zoeterwoude, The Netherlands), and 2 mm-sections were cut in a Leica CM3050 S cyrostat. Washing of tissue with PBS after RNA*later* treatment was necessary because tissues that had been treated with RNA*later* without ensuing PBS washing could not be sectioned at -20°C due to property of RNA*later* to lower the freezing point. Immune fluorescence for IgA, IgG, IgM, C1Q, and C3 was performed on the 2 mm-sections according to standard diagnostic routine protocols.

Stainings on the sample treated with RNA*later* were compared with the same stainings performed on the untreated core biopsy from the patient.

Statistical Analysis
Data are presented as the means of duplicate measurements, and the differences between groups were tested with independent-samples *t* tests. Analyses were performed with SPSS version 10.0 software. For all tests $P < 0.05$ was considered statistically significant.

Results

Optimization of RNA extraction from glomerular samples and effect of tissue storage on RNA
Different methods of RNA extraction were compared. Figure 2 demonstrates that Trizol and RNeasy spin columns render better results than the NP40 method. cDNA yields were not affected when glomerular tissue was stored for up to 56 days and at a temperature of -70°C in PBS or the RNase inhibiting compound RLT (Fig. 2). Based on the observation that the Trizol method rendered higher RNA yields from fresh renal tissue than the RNeasy method, we decided to use Trizol for RNA extraction in subsequent experiments.

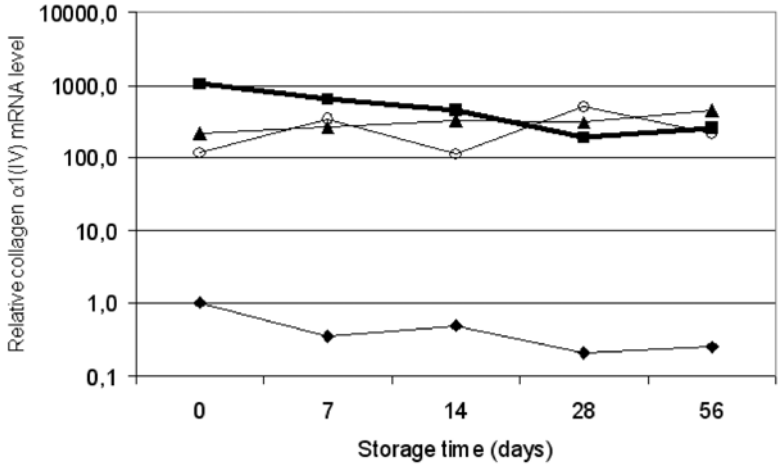

Figure 2. Optimization of a method for RNA extraction and effect of tissue storage on RNA yields. Three methods of extraction were compared:(■), Trizol extraction (storage in PBS); (▲) RNeasy extraction (storage in PBS); (○) RNeasy extraction (storage in RLT); (◆) NP40 extraction (storage in NP40). Collagen α1(IV) mRNA levels were assessed with real-time PCR. Data represent the mean of duplicate measurements. Trizol and RNeasy spin columns for RNA extraction lead to better results than use of the NP40 method.

Optimization of cDNA synthesis
To optimize the protocol for cDNA synthesis we compared the efficiency of Superscript®, AMV-, and Sensiscript+ reverse transcriptase (RT) enzymes. cDNA yields, which were measured for collagen α1(IV) and TGF-β by real-time PCR,

were obtained with the three methods described above and are depicted in Figure 3. Usage of AMV RT resulted in higher yields of cDNA than usage of Sensiscript+ and Superscript® for collagen α1(IV) ($P < 0.005$ and $p < 0.001$, respectively) and for TGF-β ($P < 0.005$ and $P < 0.001$, respectively).

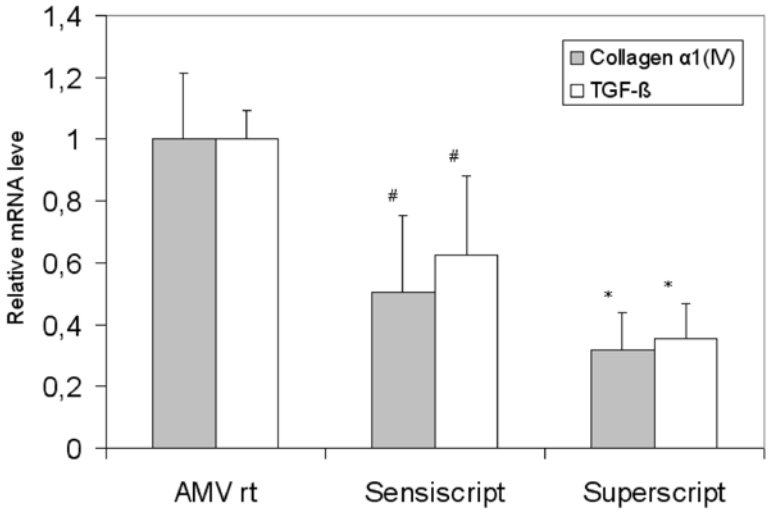

Figure 3. Optimization of cDNA synthesis. cDNA yields were compared with different reverse transcriptase enzymes for cDNA synthesis. As a read-out system, collagen α1(IV) and TGF-β mRNA were assessed with real-time PCR. cDNA yields were significantly higher through the use of AMV RT in comparison with Sensiscript+ and Superscript®. *, $P < 0.001$; #, $P < 0.005$ versus results in AMV RT.

Effect of RNAlater on RNA integrity in cortical tissue stored at 4°C

We wanted to determine the effect of RNA*later* on RNA stability in the renal cortex that had been stored at 4°C. Figure 4 shows that integrity of RNA from tissue incubated in RNA*later* for one week was not different from that of RNA obtained from fresh cortical tissue ($t = 0$). In fact, RNA integrity from tissue treated with RNA*later* and stored at 4°C was maintained over a time period of at least 3 months. In contrast, RNA from renal tissue stored in PBS at 4°C already showed signs of degradation, according to the lower ratio between the 28S and 18S rRNA band, after storage for 1 week (Fig. 4).

Effect of RNAlater on microdissected tissue: glomerular morphology and yields

Glomeruli could easily be removed from cortical tissues microdissected in PBS solution. In general, microdissection of the cortex required less than 10 minutes, and no glomeruli were detected in the tubulointerstitium after visual inspection. Glomeruli had typical round-shaped appearances (Fig. 5B). Disruption of tissues in the RNA*later* compound proved to be more difficult because the cortical tissue was more coherent, which lengthened the process of microdissection to a minimum of 20 minutes. Glomeruli were difficult to remove from the cortex, missed their typical round-shaped appearances (Fig. 5D), and were difficult to

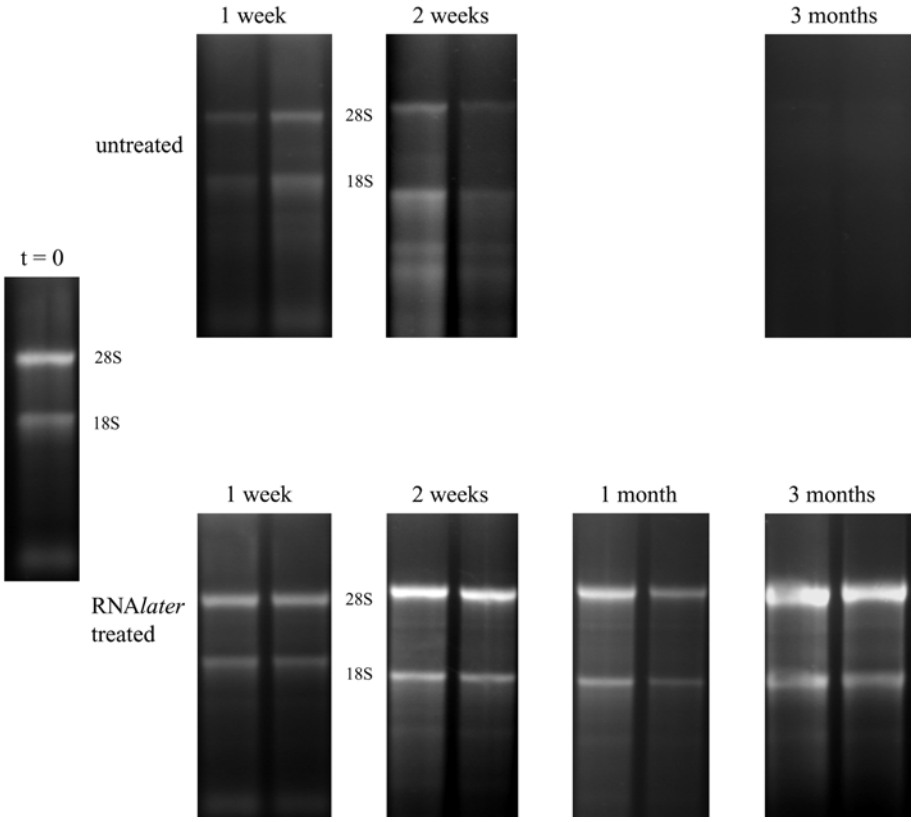

Figure 4. RNA integrity in whole renal cortex stored at 4°C. Comparison of the effect of PBS and RNA*later* on RNA integrity in whole renal cortex stored at 4°C was assessed by RNA gel electrophoresis. Incubation of human renal cortex in RNA*later* solution maintained RNA integrity over a period of three months. The photograph shows a representative result of triplicate measurements.

distinguish from tubulointerstitial debris. The number of glomerular yields (per mg tissue) obtained through microdissection in RNA*later* was approximately twice as low as the number of yields obtained through microdissection in PBS (Fig. 6; bars 4–6 *versus* bars 1–3, $P < 0.005$). Because of the viscous nature of the RNA*later* compound, glomerular and tubulointerstitial samples that had been collected in reaction tubes could not be pelleted by centrifugation. We wanted to test whether the altered tissue morphology and the relatively low amount of glomerular yields obtained with RNA*later* could be avoided if a 1:8 dilution of RNA*later* (in PBS) was used for microdissection. The 1:8 dilution of RNA*later* indeed maintained glomerular morphology, although the microdissection procedure and removal of glomeruli from the surrounding tubulointerstitium (Fig. 5F) remained problematic. Usage of RNA*later* 1:8 resulted in glomerular yields that were comparable to those obtained with PBS (Fig. 6; bars 7–9 *versus* bars 1–3).

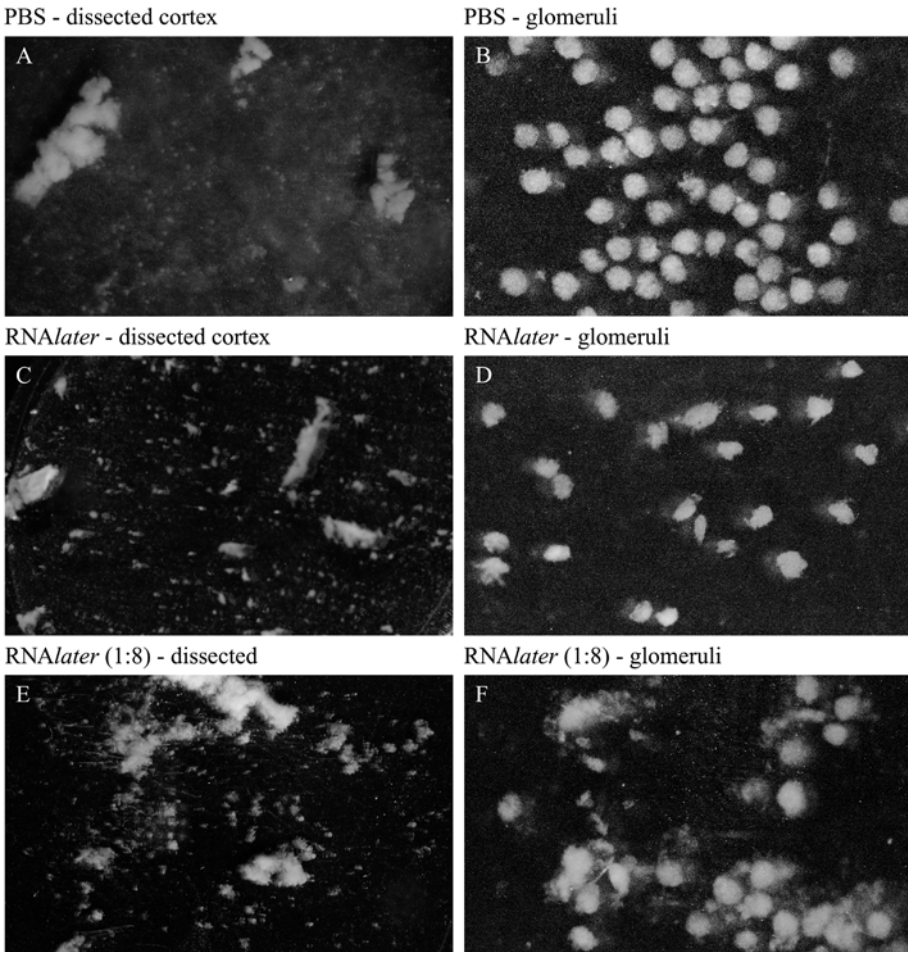

Figure 5. Microdissection of renal tissue in PBS and RNA*later*. Renal cortex microdissected in PBS, RNA*later* or RNA*later* 1:8 is depicted in the left column (magnification 6.5x). Microdissection in tissues incubated in either RNA*later* or RNA*later* 1:8 presented problems. Glomeruli obtained through microdissection in RNA*later* had lost their round-shaped appearances (Figure D, magnification 25x) and were difficult to distinguish from surrounding tubulointerstitial debris.

Effect of RNAlater on microdissected tissue: RNA yields

RNA integrity of cortical tissues that were either incubated and microdissected in PBS, RNA*later* or RNA*later* 1:8 was compared. Assessment of RNA yields in glomerular and tubulointerstitial samples through real-time PCR for collagen α1(IV) are depicted in figure 7A and 7B, respectively. In both glomeruli and their tubulointerstitial counterparts, which were obtained through microdissection of fresh cortical tissue (t = 0), no significant differences in RNA yields were found between samples microdissected in PBS, RNA*later* or RNA*later* 1:8 (Fig. 7A and 7B; bar 1, 4, and 7). Cortical samples that had been incubated in PBS or in RNA*later* 1:8 and were subsequently stored at -70° C,

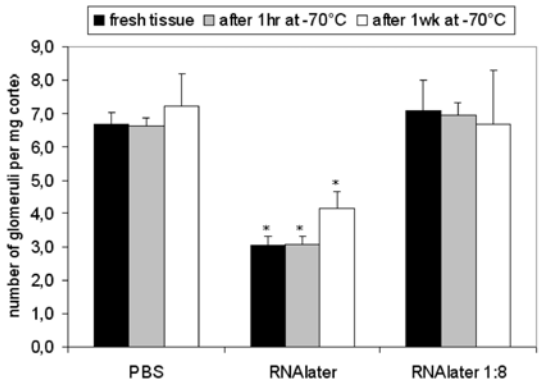

Figure 6. Glomerular yields after microdissection in PBS, RNA*later* or RNA*later* 1:8. A significantly lower number of glomeruli could be obtained from tissues dissected in RNA*later*. *, $P < 0.005$ versus microdissection in PBS.

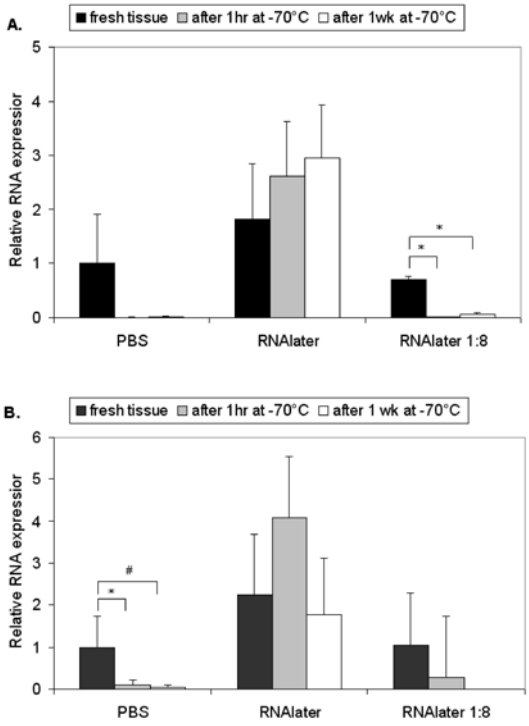

Figure 7. RNA yields were obtained from microdissected renal biopsies. (A) Glomerular samples and (B) tubulointerstitial samples obtained through microdissection of tissue treated with PBS, RNA*later* or RNA*later* 1:8 were assessed using real-time PCR for collagen α1(IV). The graphs display relative mRNA levels which have been corrected for the number of glomeruli (glomerular samples) or mg tubulointerstitial tissue (tubulointersitial samples). *, $P < 0.01$; #, $P < 0.05$.

thawed, and microdissected, rendered lower RNA yields in tubulointerstitial and glomerular compartments than freshly microdissected tissues without prior freezing and thawing (Fig. 7A and 7B; bars 2 and 3 *versus* bar 1, and bars 8 and 9 *versus* bar 7). In contrast, RNA*later* preserved the integrity of glomerular RNA that had been obtained through microdissection of renal cortex after freezing and thawing. The time period (1 hour or 1 week) during which the tissue was stored, at a temperature of -70°C, did not affect these results (Fig. 7A; bar 5 and 6 *versus* bar 4). This trend was also observed in the corresponding tubolointerstitial compartments (Fig. 7B; bar 5 and 6 *versus* bar 4; $p > 0.05$).

Effect of RNAlater on tissue integrity

We compared the results of PAS-stained paraffin sections of cortical tissue that had either been fixed in formalin immediately, after incubation in RNA*later* or after incubation in RNA*later* 1:8 (Fig. 8). The histology of the tissues incubated in RNA*later* and in RNA*later* 1:8 seem to be affected negatively. Histology of tissues in RNAlater 1:8 had dispersed cytoplasma, and nuclei in the tubuli had disappeared.

In addition, tissue integrity was evaluated by immune fluorescence (IF) staining of tissue from a patient diagnosed with lupus nephritis. Stainings are presented in Figure 9. The panels on the left show the IF stainings on biopsy tissue for IgA, IgG, C1Q, and C3. This tissue was processed through routine diagnostic protocols in our hospital center. As shown in the right panels, pre-treatment of the tissue with RNA*later* resulted in a dispersed staining pattern and a decreased intensity of the fluorescent signal.

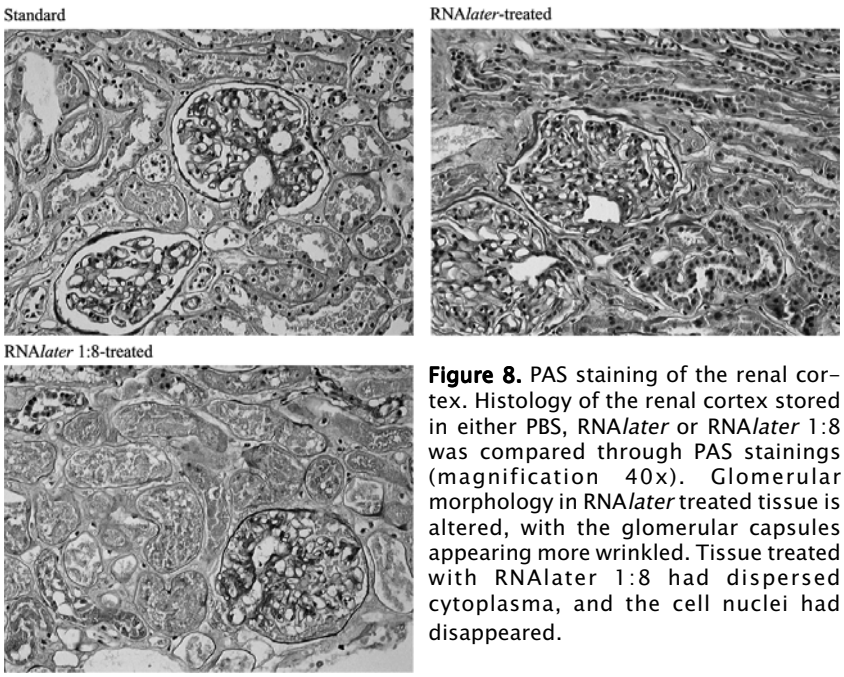

Figure 8. PAS staining of the renal cortex. Histology of the renal cortex stored in either PBS, RNA*later* or RNA*later* 1:8 was compared through PAS stainings (magnification 40x). Glomerular morphology in RNA*later* treated tissue is altered, with the glomerular capsules appearing more wrinkled. Tissue treated with RNAlater 1:8 had dispersed cytoplasma, and the cell nuclei had disappeared.

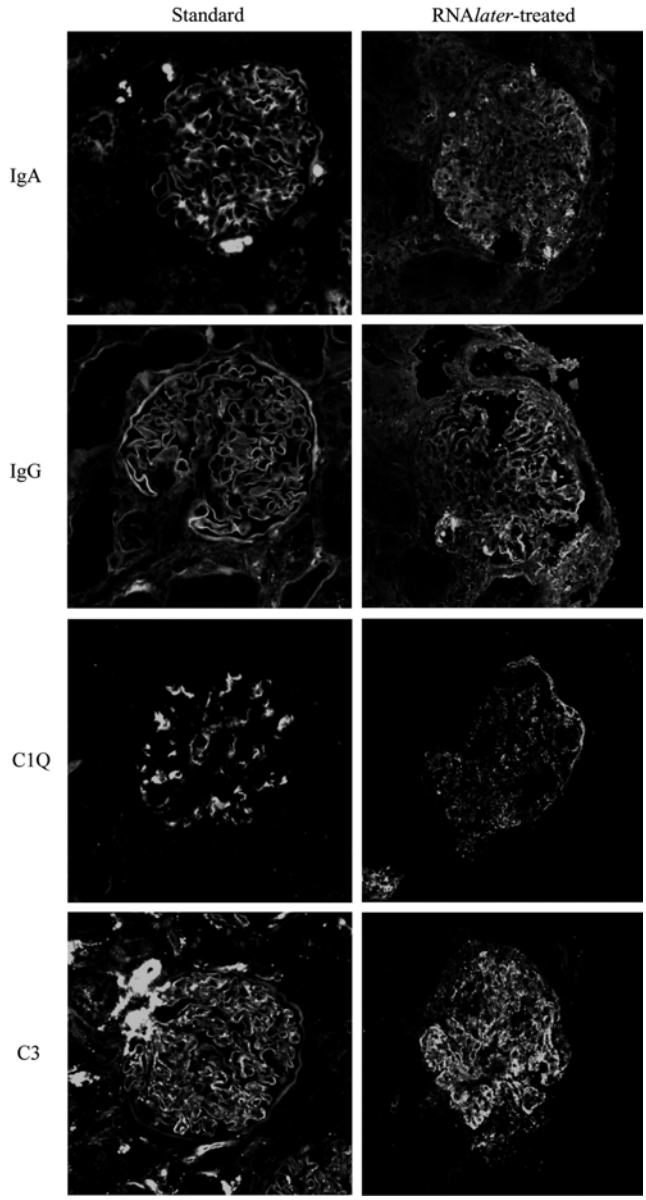

Figure 9. Immunofluorescence stainings for IgA, IgG, C1Q, and C3. The left panels represent stainings on renal cortex from a patient with lupus nephritis. Stainings were performed according to standard diagnostic practice. The renal cortex from a patient with lupus nephritis, which was stored in RNA*later* (right panels), displayed a dispersed staining pattern and a decreased intensity of the fluorescent signal.

Discussion

Assessment of mRNA levels in kidney tissue might be used as a diagnostic and prognostic tool in renal disease [20-24]. Our first objective was finding a method with which the quality and quantity of RNA from renal biopsies can be improved. This is necessary because only minute amounts of RNA are obtained from a biopsy specimen and loss of intact RNA might occur through degradation by RNase molecules [25]. The findings presented in the current study are a prolongation of an earlier study [26]. Our second objective was integrating RNA expression studies and routine diagnostics. This requires a reagent that maintains RNA integrity while it does not affect tissue histology. RNA preserving compounds such as guanidine/phenol affect tissue integrity, while reagents used for tissue fixation such as formalin, ethanol, and xylene are detrimental for RNA integrity [27;28]. Recent studies have reported that RNA*later* maintains both RNA and histological integrity. This feature of RNA*later* was discovered in experiments on several non-renal tissues [29-31]. In the current study we tested this compound in renal tissue.

We found that Trizol and RNeasy spin columns for RNA extraction rendered better results than the NP40 method. In practice, acquired renal tissue is processed for RNA extraction after a storage period of no longer than 14 days. The Trizol method gives better RNA extraction than the RNeasy spin columns from tissue that has been stored for a period up to 14 days. This combined with the aspect of cost effectiveness, led to subsequent experiments being performed with the Trizol method. No RNA degradation was observed when glomerular tissue was stored at -70°C for up to 6 weeks. Remarkably, we did not observe a difference in RNA yields between glomerular tissue stored in PBS and in the RNase inhibiting compound RLT. We also found that cDNA yields can be significantly improved by using AMV RT instead of Sensiscript+ and Superscript®.

Incubation of the human renal cortex in RNA*later* solution at 4°C maintained RNA integrity over a period of three months. These findings are in accordance with those found in other studies, in which RNA integrity was maintained in human skin and tumor tissues during storage at 4°C in RNA*later* [32;33]. Our findings also confirm observations from an earlier study by Cohen *et al* [34]. Subsequently, we investigated the effect of RNA*later* on renal biopsies by combining routine diagnostics and RNA expression studies. The use of RNA*later* as storage and microdissection compound proved beneficial with respect to maintenance of RNA integrity. RNA yields from renal tissues dissected in RNA*later* were approximately twice as high as those from renal tissues dissected in PBS. This difference was, however, not significant. The observation that usage of RNA*later* on fresh renal tissue leads to slightly higher RNA yields than when it is used on fresh tissues processed in PBS might be explained by the fact that RNA*later* protects against RNA degradation during microdissection [35]. The capacity of RNA*later* to preserve RNA has also been demonstrated in studies performed on breast core needle biopsies and xenograft CNS tumor cell samples [36;37]. During freeze-thaw cycles of tissues embedded in OCT medium, the RNA contained in the glomeruli and the tubulointerstitium is subject to degradation

[38]. This observation was confirmed in the current study when renal cortex was microdissected in PBS after the tissue had been subjected to a freeze-thawing procedure. Storage of the tissues in RNA*later* during freeze-thawing maintained RNA integrity. Despite usage of RNA*later* for procurement of RNA yields being more advantageous than usage of PBS, we achieved a two fold lower yield of glomeruli per mg of cortical tissue after microdissection in RNA*later*. Especially in the small biopsy specimens available for microdissection, from which around 10 glomeruli are normally obtained, glomerular sample size is critical. A reduction of 50% in glomerular yield, as was the case with RNA*later*, would be disadvantageous if it is the objective to measure mRNA levels in the biopsy specimen that are representative of those in the whole kidney. In addition, several technical problems arise when glomerular and tubulointerstitial compartments in RNA*later* compound are separated. Probably due to the high concentration of salt and the viscous nature of this reagent, glomeruli had lost their typical round-shaped appearances and were difficult to distinguish from tubulointerstitial debris. Because of the reagent's viscosity, the RNA*later* fluid could not easily be separated from the renal compartment through pelleting in a centrifuge.

In a previous study it was shown that RNA*later* preserved histological integrity of various formalin fixed human tissues such as lung, liver, heart, and skin [39]. These findings are in contrast with those from the current study, in which we found that RNA*later* negatively affected tissue integrity in renal tissue, which was evaluated by light microscopy on sections from formalin fixed material. Processing of renal tissue, after RNA*later* treatment, for immunohistochemical assessments caused complications. Due to capacity of the RNA*later* compound to reduce the freezing point, frozen tissues could not be sectioned at -20°C. A reduction of temperature in the cryostat device to -40°C solved this problem, but this is not feasible in routine diagnostic practice. Staining patterns for IgA, IgG, C1Q, and C3 in renal tissue treated with RNA*later* from a patient with lupus nephritis were severely altered compared to staining patterns in untreated tissue of the same patient. Presumably, the combination of RNA*later* and the subsequent washing of the tissue in PBS resulted in disruption of the antibody epitopes.

In this study we have shown how RNA extraction can be optimized with the Trizol method. Furthermore, optimal yields of cDNA were obtained through the use of AMV RT. Renal samples can be stored in PBS at -70°C for at least 6 weeks without degradation of RNA. Preservation of RNA integrity of the renal cortex can be improved through the use of RNA*later*. However, even though overall RNA*later* renders higher, yet not significant, RNA yields after microdissection than PBS, several technical drawbacks limit the implementation of RNA*later* in standard diagnostic protocols. Until a solvent is established that protects both renal tissue morphology and RNA integrity from whole tissue and microdissected tissue, the use of PBS medium appears to be a suitable solution.

Reference List

1. Delarue F, Hertig A, Alberti C, Vigneau C, Ammor M, Berrou J, Akposso K, Peraldi MN, Rondeau E, Sraer JD: Prognostic value of plasminogen activator inhibitor type 1 mRNA in microdissected glomeruli from transplanted kidneys. Transplantation 72:1256-1261, 2001
2. Eikmans M, Sijpkens YW, Baelde HJ, de Heer E, Paul LC, Bruijn JA: High transforming growth factor-beta and extracellular matrix mRNA response in renal allografts during early acute rejection is associated with absence of chronic rejection. Transplantation 73:573-579, 2002
3. Eikmans M, Baelde HJ, Hagen EC, Paul LC, Eilers PH, de Heer E, Bruijn JA: Renal mRNA levels as prognostic tools in kidney diseases. J Am Soc Nephrol 14:899-907, 2003
4. Lee SK, Goyal M, de Miguel M, Thomas P, Wharram B, Dysko R, Phan S, Killen PD, Wiggins RC: Renal biopsy collagen I mRNA predicts scarring in rabbit anti-GBM disease: comparison with conventional measures. Kidney Int 52:1000-1015, 1997
5. Munaut C, Bergijk EC, Baelde JJ, Noel A, Foidart JM, Bruijn JA: A molecular biologic study of extracellular matrix components during the development of glomerulosclerosis in murine chronic graft-versus-host disease. Lab Invest 67:580-587, 1992
6. Striker LJ: Modern renal biopsy interpretation: can we predict glomerulosclerosis? Semin Nephrol 13:508-515, 1993
7. Lee KH, McKenna MJ, Sewell WF, Ung F: Ribonucleases may limit recovery of ribonucleic acids from archival human temporal bones. Laryngoscope 107:1228-1234, 1997
8. Eikmans M, Baelde HJ, de Heer E, Bruijn JA: Processing renal biopsies for diagnostic mRNA quantification: improvement of RNA extraction and storage conditions. J Am Soc Nephrol 11:868-873, 2000
9. Grotzer MA, Patti R, Geoerger B, Eggert A, Chou TT, Phillips PC: Biological stability of RNA isolated from RNAlater-treated brain tumor and neuroblastoma xenografts. Med Pediatr Oncol 34:438-442, 2000
10. Cohen CD, Frach K, Schlondorff D, Kretzler M: Quantitative gene expression analysis in renal biopsies: a novel protocol for a high-throughput multicenter application. Kidney Int 61:133-140, 2002
11. Florell SR, Coffin CM, Holden JA, Zimmermann JW, Gerwels JW, Summers BK, Jones DA, Leachman SA: Preservation of RNA for functional genomic studies: a multidisciplinary tumor bank protocol. Mod Pathol 14:116-128, 2001
12. Ellis M, Davis N, Coop A, Liu M, Schumaker L, Lee RY, Srikanchana R, Russell CG, Singh B, Miller WR, Stearns V, Pennanen M, Tsangaris T, Gallagher A, Liu A, Zwart A, Hayes DF, Lippman ME, Wang Y, Clarke R: Development and validation of a method for using breast core needle biopsies for gene expression microarray analyses. Clin Cancer Res 8:1155-1166, 2002
13. Eikmans M, Baelde HJ, de Heer E, Bruijn JA: Effect of age and biopsy site on extracellular matrix mRNA and protein levels in human kidney biopsies. Kidney Int 60:974-981, 2001
14. Baelde JJ, Bergijk EC, Hoedemaeker PJ, de Heer E, Bruijn JA: Optimal method for RNA extraction from mouse glomeruli. Nephrol Dial Transplant 9:304-308, 1994
15. Klimecki WT, Futscher BW, Dalton WS: Effects of ethanol and paraformaldehyde on RNA yield and quality. Biotechniques 16:1021-1023, 1994
16. Vincek V, Nassiri M, Knowles J, Nadji M, Morales AR: Preservation of tissue RNA in normal saline. Lab Invest 83:137-138, 2003