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Optimization of RNA yield, purity and mRNA copy number by treatment of urine cell pellets with RNA*later*

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Abstract

Background: We have shown that measurement of mRNA for cytotoxic attack proteins perforin and granzyme B in urinary cells is a noninvasive means of diagnosing acute rejection of human renal allografts. Urinary cell mRNA studies have yielded useful information in other patient populations such as patients with cancer. The isolation of sufficient and high quality ribonucleic acid (RNA) from urinary cells however is problematic. RNA*later*, an RNA stabilization solution, has been reported to optimize RNA isolation from tumor tissues stored at room temperature and from pigment-rich ocular tissues. *Methods*: We explored whether the addition of RNA*later* to urine cell pellets improves RNA yield, enhances purity and facilitates measurement of low abundance mRNAs. We measured, with the use of real-time quantitative polymerase chain reaction (PCR) assay, levels of expression of a constitutively expressed gene 18S rRNA and mRNA for granzyme B and transforming growth factor-β_1 (TGF-β_1) in urine specimens and renal biopsies obtained from renal allograft recipients. *Results*: RNA yield ($P<0.01$, Wilcoxon signed rank test) and the *A*260/*A*280 ratio ($P<0.01$) were both higher with urine cell pellets treated with RNA*later* prior to snap freezing compared to cell pellets that were not treated with RNA*later* prior to snap freezing. Levels (copy number per 1 μg of total RNA) of 18S rRNA ($P<0.02$), granzyme B mRNA ($P=0.002$) and TGF-β_1 ($P=0.02$) were all higher with treated urine cell pellets compared to untreated cell pellets. Kruskall–Wallis one way analysis of variance and pair-wise comparisons with Student–Newman–Keuls test showed that the levels of mRNA for granzyme B ($P<0.05$) and TGF-β_1 ($P<0.05$) are significantly different between renal allograft biopsies and untreated urine cell pellets but not between the biopsy specimens and RNA*later*-treated urine cell pellets. *Conclusions*: The addition of RNA*later* to urine cell pellets improves RNA isolation from urinary cells and facilitates measurement of low abundance mRNAs.

Keywords: Urine PCR; Real time quantitative PCR; Transplantation; RNA*later*

1. Introduction

The search for diagnostic clues in the urine has a long history in medicine (Voswinckel, 2000) and a meticulous urine analysis provides invaluable insights into renal disease (Geyer, 1993). Advances in tech-

Abbreviations: RNA, ribonucleic acid; PBS, phosphate buffered saline; PCR, polymerase chain reaction.

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nology such as electron microscopy have enhanced the diagnostic utility of urine examinations (Fogazzi et al., 1994; Guder et al., 2000), and noninvasive diagnosis of acute rejection of renal allografts (Li et al., 2001) or BK viral nephritis (Ding et al., 2002) by mRNA profiling of urinary cells is now feasible.

Urine sediments from transplant recipients undergoing an episode of acute rejection have increased amounts of renal tubular cells, casts and lymphocytes (Simpson et al., 1989). It is well known that the integrity of the cells in the urine depends upon the physicochemical conditions of the urine itself such as pH, density, temperature and the time elapsed between the collection of the urine specimen and the processing of the sample (Lehmann, 1998; Haber and Ward, 2002). Given the high susceptibility of ribonucleic acid (RNA) to undergo degradation, isolation of adequate quantities of high quality RNA from urinary cells is a challenging process and the difficulties are further enhanced when the samples have to be shipped from clinical centers to a central molecular core laboratory.

An RNA stabilization solution, RNA*later*™, has been used to store tumor tissues at room temperature for 7 days (Grotzer et al., 2000). RNA*later* has also been used to preserve human ocular tissue (Wang et al., 2001). In the current investigation, we explored whether the addition of RNA*later* to the urine cell pellet optimizes RNA yield and facilitates amplification of low abundance mRNAs.

2. Materials and methods

2.1. Collection of specimens

A random midstream urine sample was collected from 15 stable pediatric renal transplant recipients aged 10–15 years. The protocols for urine and biopsy sample collection were approved by the IRB of the Hospital Infantil de Mexico Federico Gomez. A parent and patient written consent form was obtained in each instance.

Urine (about 100 ml) was collected from the renal allograft recipients and aliquoted into two 50 ml sterile conical tubes (Fig. 1). The tubes were centrifuged at $2000 \times g$ at room temperature for 30 min, and the supernatants were discarded. The urine cell pellets were then resuspended in 1 ml of phosphate buffered saline (PBS), and transferred to 1.8-ml cryotubes. The cryotubes were then centrifuged at $16{,}000 \times g$ for 10 min at room temperature and the supernatants were discarded. RNA*later* (150 μl) was added to one of the two cryotubes containing the urine cell pellet, mixed thoroughly and centrifuged at $16{,}000 \times g$ for 15 s at room temperature prior to snap freezing and storage at − 70 °C (RNA*later* treated group). The other cryotube containing the cell pellet was immediately frozen in liquid nitrogen (snap freezing) and stored at − 70 °C freezer (RNA*later* not treated group).

Ten of the 15 renal allograft recipients underwent protocol core needle biopsy of the allografts. The biopsy specimens were snap frozen and stored at − 70 °C until processed.

The cryotubes containing the urine cell pellets and allograft biopsy specimens were all shipped in dry ice to the Cornell molecular laboratory for RNA isolation and measurement of gene expression with the use of real-time quantitative polymerase chain reaction (PCR) assay.

2.2. RNA isolation

Total RNA was isolated with the use of a commercial kit (RNeasy mini kit, Qiagen, Valencia, California). In brief, 350 μl of RLT buffer (Qiagen) was added to the urine cell pellet and 450 μl of the buffer was added to the biopsy specimen. RNA*later* (50 μl) was added to the renal biopsy samples just prior to RNA isolation; 50 μl of RNA*later* was also added to the urine cell pellets that were not treated with RNA*later* prior to snap freezing. The urine cell pellets/renal tissues were homogenized using a 1-ml syringe and a 23-gauge needle (cell pellet) or rotor homogenizer (biopsy tissue). The homogenate was passed through a QIA Shredder column (Qiagen) to remove insoluble debris. RNA amount was measured with the use of a UV spectrophotometer and absorbance was measured at *A*260 and *A*280 nm and expressed as *A*260/*A*280 ratio (Manchester, 1995). RNA (1 μg) was reverse-transcribed to cDNA using TaqMan reverse transcription Kit (Applied Biosystems, Branchburg, NJ).

2.3. Real-time quantitative PCR assay

We designed gene specific primers and probes with the use of Primer Express software (PE Biosystems,

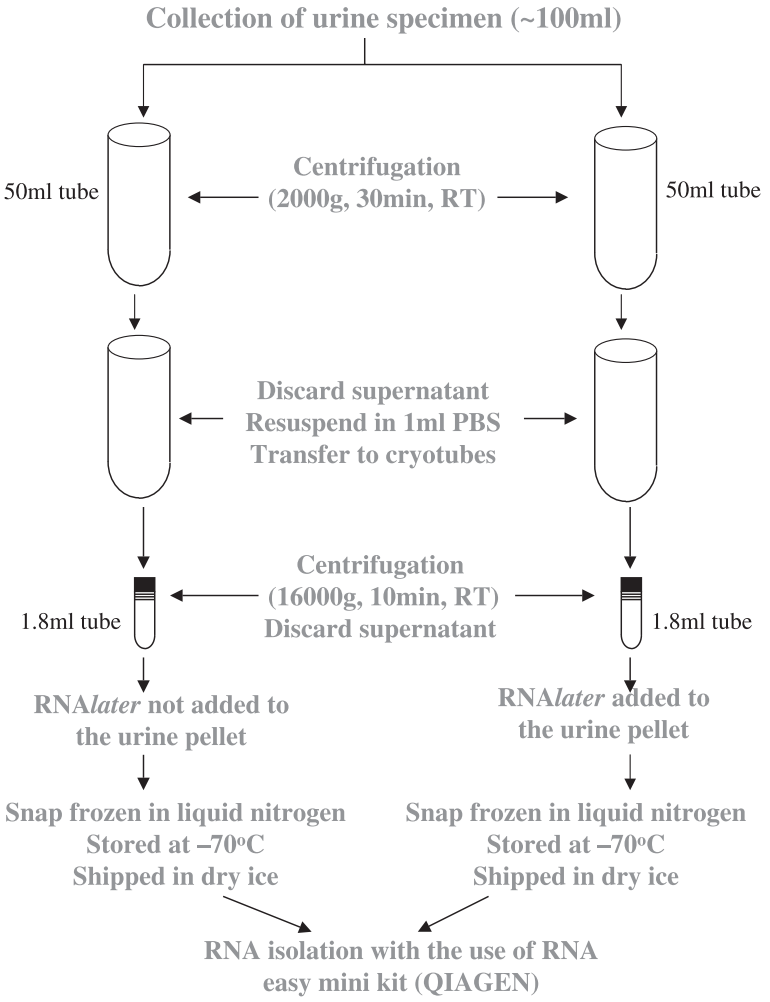

Fig. 1. Protocol for processing urine specimens. Urine (about 100 cm^3) was collected from renal allograft recipients and aliquoted into two 50 ml sterile conical tubes. The tubes were centrifuged and the supernatants were discarded. The urine cell pellets were then resuspended in 1 ml of PBS, and transferred to 1.8-ml cryotubes. The cryotubes were then centrifuged and the supernatants were discarded. One of the cryotubes was immediately frozen in liquid nitrogen (snap freezing) and stored at − 70 °C freezer. RNA*later* (150 μl) was added to the other cryotube, the tube was centrifuged at 16000 × *g* for 15 s at room temperature prior to snap freezing and storage at − 70 °C. The cryotubes were shipped in dry ice to the molecular laboratory for RNA processing. Total RNA was isolated from the urine cell pellets with the use of RNeasy mini kit.

Foster City, CA). In order to avoid genomic amplification, the primers for transforming growth factor β_1 (TGF-β_1) and for granzyme B were designed to span intronic sequences. The sequences and location of the oligonucleotide primers and the fluorogenic probes used in this investigation are depicted in Table 1. The probes were labeled at the 5′ end with 6-carboxy-fluorescein (FAM) and at the 3′ end with 6-carboxy-tetramethylrodamine (TAMRA). FAM functioned as the as the reporter dye and TAMRA as the quencher dye.

We measured levels of constitutively expressed 18S ribosomal RNA (rRNA) and levels of mRNA for granzyme B and TGF-β_1 with the use of ABI Prism 7700 Sequence Detector (PE Biosystems). Polymerase chain reaction was performed in a 25-μl reaction volume containing 2.5 μl of cDNA template, 12.5 μl of 2 × Universal Master Mixture (Applied Biosystems),

Table 1
Sequence and location of oligonucleotide primers and fluorogenic probes

Gene	Accession no.	Sequence (location)
18S rRNA	K03432	Sense: 5′-GCCCGAAGCGTTTACTTTGA-3′(929–948)
		Antisense: 5′-TCCATTATTCCTAGCTGCGGTATC-3′(1008–985)
		Probe: 5′FAM-AAAGCAGGCCCGAGCCGCC-TAMRA 3′(965–983)
TGF-β_1	XM_008912	Sense: 5′-CCCTGCCCCTACATTTGGAG-3′(1812–1831)
		Antisense: 5′-CCGGGTTATGCTGGTTGTACA-3′(1884–1864)
		Probe: 5′FAM-CACGCAGTACAGCAAGGTCCTGGCC-TAMRA 3′(1838–1862)
Granzyme B	J04071	Sense: 5′-GCGAATCTGACTTACGCCATTATT-3′(534–557)
		Antisense: 5′-CAAGAGGGCCTCCAGAGTCC-3′(638–619)
		Probe: 5′FAM-CCCACGCACAACTCAATGGTACTGTCGTAMRA-3′(585–559)

Gene-specific oligonucleotide primers were designed using Primer Express software (PE Applied Biosystems). The probes were labeled with 6-carboxy-fluorescein (FAM) at the 5′ end and with 6-carboxy-tetramethylrodamine (TAMRA) at the 3′ end. FAM functioned as the reporter dye and TAMRA as the quencher dye.

300 nM each of sense and antisense primers and 200 nM of the probe. The PCR amplification profiles included an initial incubation at 50 °C for 2 min, denaturation at 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. A standard curve using 18S ribosomal RNA amplicon was developed. 18S rRNA levels and the mRNA levels were expressed as number of copies per microgram of total RNA.

2.4. Statistics

We used Sigma Stat statistical software program for data analysis. Because the measured parameters deviated significantly from normal distribution, we used non-parametric tests. Wilcoxon signed rank test was used to compare results for two sample comparisons. Kruskall–Wallis one way analysis of variance on ranks was used for multiple sample comparisons and to control for type 1 error, we used Student–Newman–Keuls test for all pair-wise comparisons.

3. Results

3.1. RNA yield and A260/A280 ratios

Treatment of urine cell pellets with RNA*later* prior to snap freezing of the pellets in liquid nitrogen optimized RNA yield and improved the *A*260/*A*280 ratios (Table 2). Total RNA yields were higher in 12 of the 15 treated urine cell pellets compared to untreated cell pellets and the mean (± S.E.) RNA yield was 0.88 ± 0.13 μg with RNA*later* treatment and 0.65 ± 0.14 μg without treatment ($P < 0.01$, Wilcoxon signed rank test). Table 2 also shows that the purity of RNA ratios was improved by treatment of the cell pellet with RNA*later* and that the *A*260/*A*280 ratios were higher in 11 of 15 treated urine cell pellets compared to untreated cell pellets. The mean (± S.E.) *A*260/*A*280

Table 2
RNA yields and *A*260/*A*280 ratios

Renal allograft recipients	RNA yield (μg) from urine cell pellets		*A*260/*A*280 ratios	
	RNA*later* treated	RNA*later* not treated	RNA*later* treated	RNA*later* not treated
1	0.262	0.321	1.50	1.38
2	0.642	0.525	1.69	1.64
3	0.175	0.175	1.50	1.20
4	1.371	1.021	2.04	1.94
5	0.846	0.642	1.81	1.69
6	0.350	0.175	1.50	1.20
7	0.379	0.321	1.86	1.57
8	0.408	0.204	1.75	1.75
9	1.575	0.262	1.86	1.50
10	0.933	0.875	2.13	1.88
11	1.516	1.920	1.86	1.78
12	0.904	0.612	2.07	2.10
13	1.925	1.400	1.89	1.85
14	1.137	0.904	1.86	1.94
15	0.787	0.437	1.93	2.14
Mean	0.881	0.653	1.82	1.70
S.E.	0.129	0.138	0.05	0.07

Urine sediments were either treated with RNA*later* (RNA*later* treated) or untreated (RNA*later* not treated) prior to snap freezing in liquid nitrogen. The amount of total RNA isolated ($P < 0.01$) and the *A*260/*A*280 ratios ($P < 0.01$) were higher with treatment with RNA*later* prior to snap freezing. *P*-values were calculated with use of Wilcoxon Signed Rank Sum Test.

ratio was 1.82 ± 0.05 μg with RNA*later* treatment and was 1.70 ± 0.07μg without treatment ($P < 0.01$).

3.2. Levels of 18S rRNA, granzyme B mRNA and TGF-β_1 mRNA in urine cell pellets

Our studies, with the use of real-time quantitative PCR assay, demonstrated that the constitutively expressed 18S rRNA and mRNA for cytotoxic attack protein granzyme B and mRNA for multifunctional cytokine TGF-β_1 levels were all higher in the urine cell pellets treated with RNA*later* compared to the untreated urine cell pellets. In Fig. 2, a representative example illustrating the higher level of expression of 18S rRNA and mRNA for granzyme B and TGF-β_1 in the cell pellet treated with RNA*later* compared with the untreated pellet shows the earlier attainment of fluorescence detection threshold (Ct) with the treated sample compared to the untreated sample.

Fig. 3 illustrates data from all of the samples evaluated for the level of expression of 18S rRNA and mRNAs for granzyme B and TGF-β_1. The mean (± S.E.) level of expression of 18S rRNA increased from 1.41e + 010 ± 3.72e + 009 copies per μg of total RNA in the untreated cell pellets to 2.70e + 010 ± 6.79e + 009 copies per μg of total RNA in the pellets treated with RNA*later* ($P < 0.02$, Wilcoxon signed rank test). mRNA for granzyme B was detected only in 4 of 15 untreated urine cell pellets whereas granzyme B was measurable in 11 of treated pellets. The mean (± S.E.) level of expression of granzyme B mRNA increased from 1.36e + 003 ± 6.79e + 002 copies per μg of total RNA in the untreated cell pellets to 1.53e + 004 ± 5.72e + 003 copies per μg of total RNA in the treated pellets ($P < 0.006$). The mean (± S.E.)

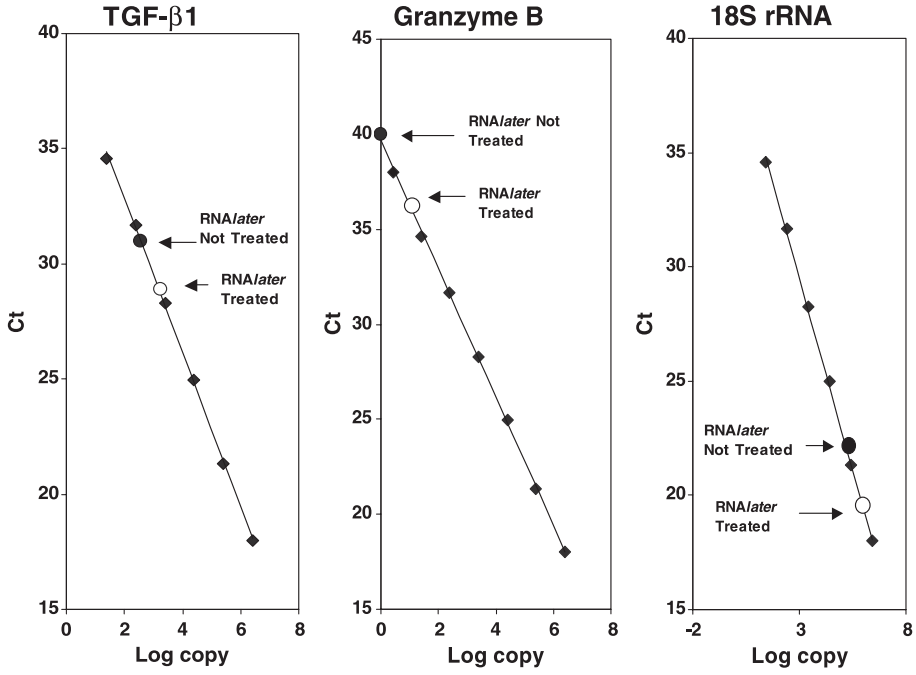

Fig. 2. 18S ribosomal RNA standard curve and Cts of 18S rRNA, granzyme B and TGF-β_1. A standard curve was generated with known copy numbers (10^1 to 10^6) of 18S rRNA (◆) in the real-time quantitative PCR assay. The correlation coefficient of the standard curve was 0.999, the slope was − 3.35 and the y-intercept value was 39.5. Ct is the threshold cycle (the cycle number at which the amplified copy number reaches a pre-fixed threshold). A representative example is shown for levels of expression of TGF-β_1, granzyme B and 18S rRNA in urine cell pellet that was either treated with RNA*later* (RNA*later* treated, ○) or not treated (RNA*later* not treated, ●). The PCR assays were established as duplicates. The higher amounts of TGF-β_1, granzyme B and 18S rRNA in the urine cell pellet treated with RNA*later* compared with untreated sample are reflected by a lower Ct with treated cell pellet compared to untreated cell pellet.

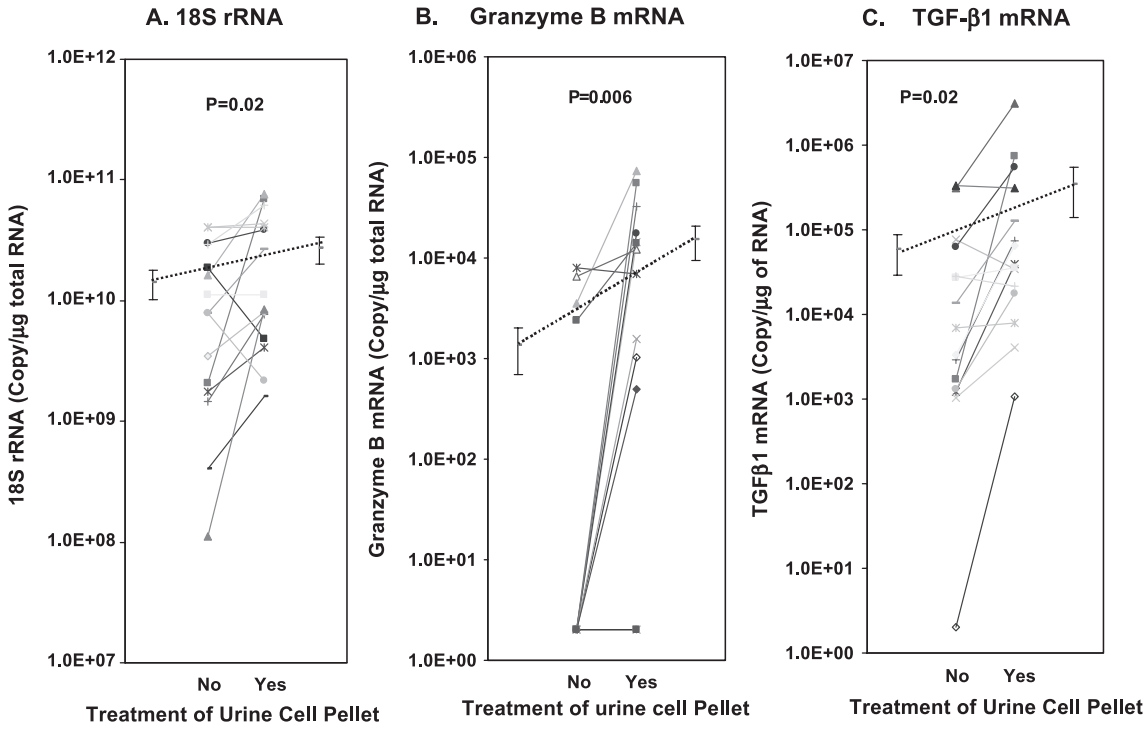

Fig. 3. 18S rRNA, granzyme B mRNA and TGF-β_1 mRNA levels in urine cell pellets. Urine specimens were collected from 15 recipients of renal allografts. The urine cell pellets were either untreated or treated with RNA*later* prior to snap freezing in liquid nitrogen. Total RNA was isolated from the cell pellets and levels of 18S rRNA and mRNAs for granzyme B and TGF-β_1 were measured with the use of gene specific primers and probes in real-time quantitative PCR assay. Individual values and mean ± S.E. values are shown. *P*-values were calculated with use of Wilcoxon Signed Rank Sum Test.

level of expression of TGF-β_1 mRNA increased from 5.79e + 004 ± 2.83e + 004 copies per µg of total RNA in the untreated urine cell pellets to 3.43e + 005 ± 2.05e + 005 copies per µg of total RNA in the treated ones ($P < 0.02$).

3.3. Comparison of levels of mRNAs in renal allograft biopsies with levels in urine cell pellets

We have reported that acute rejection of renal allograft is characterized by a heightened expression of granzyme B in renal allografts (Sharma et al., 1996a) and chronic rejection by intragraft hyperexpression of the fibrogenic cytokine TGF-β_1 (Sharma et al., 1996b).

Table 3 shows the levels of mRNA for granzyme B and TGF-β_1 in renal biopsy specimens and urine specimens collected at the time of allograft biopsies. Comparison of granzyme B mRNA levels showed that the mRNA levels among the biopsy specimens, urine pellets treated with RNA*later* and untreated urine pellets are significantly different ($P = 0.004$, Kruskall–Wallis one way analysis of variance). Pair-wise comparisons of mRNA levels with the use of Student–Newman–Keuls method showed that granzyme B mRNA levels in the treated urine sediment are not different from the levels found with allograft biopsy specimens ($P > 0.05$) whereas the levels in the untreated urine sediment are lower compared to the treated urine sediment ($P < 0.05$) and the allograft biopsy specimens ($P < 0.05$).

Comparison of TGF-β_1 mRNA levels showed that the mRNA levels among the three groups are also significantly different ($P = 0.006$). Pair-wise comparisons of mRNA levels showed that TGF-β_1 mRNA levels in the treated urine sediment are not different from the levels found with allograft biopsy specimens ($P > 0.05$) whereas the levels in the untreated urine

Table 3
Levels of mRNAs in renal allograft biopsy specimens and corresponding urine cell pellets treated or not with RNA*later*

Renal allograft recipients	Granzyme B mRNA copies/μg of RNA			TGF-β_1 mRNA copies/μg of RNA		
	Renal biopsy	Urine cell pellet		Renal biopsy	Urine cell pellet	
		Treated	Not treated		Treated	Not treated
1	ND	5.50e+04	ND	2.37e+04	7.51e+05	1.68e+03
2	9.30e+03	7.34e+04	3.57e+03	4.82e+05	3.10e+06	3.08e+05
3	3.49e+03	1.44e+04	ND	2.65e+05	3.43e+04	7.54e+04
4	3.43e+04	7.04e+03	7.95e+03	3.23e+05	3.99e+04	1.22e+03
5	8.17e+03	1.75e+04	ND	4.75e+05	5.50e+05	6.33e+04
6	3.12e+03	3.24e+04	ND	2.22e+05	7.39e+04	2.90e+03
7	4.57e+03	1.02e+03	ND	2.99e+05	1.06e+03	ND
8	1.01e+03	1.56e+03	ND	1.07e+05	1.26e+05	1.35e+04
9	1.00e+00	4.92e+02	ND	3.76e+05	6.43e+04	3.34e+03
10	1.83e+04	1.39e+04	2.36e+03	2.89e+05	3.53e+04	2.73e+04
Mean	8.23e+03	2.17e+04	1.39e+03	2.86e+05	4.78e+05	4.97e+04
SE	3.39e+03	7.84e+03	8.32e+02	4.58e+04	3.02e+05	3e+04

Renal allograft biopsies and corresponding urine specimens (cell pellets) were obtained from 10 recipients of renal allografts. Urine cell pellets were either untreated or treated with RNA*later* prior to snap freezing in liquid nitrogen. Total RNA was isolated from the biopsy specimens, and urine cell pellets and levels of mRNAs were measured with the use of gene specific primers and probes in the real-time quantitative PCR assay. mRNA copy number per μg total RNA was used as the dependent variable in Kruskall–Wallis one-way analysis of variance and Student–Newman–Keuls method was then used to compare the mRNA levels in the three groups. Levels of granzyme B and TGF-β_1 were significantly higher in urine cell pellet treated with RNA*later* compared to untreated cell pellets ($P<0.05$) whereas the levels of granzyme B and TGF-β_1 were not different between biopsy specimens and urine cell pellets treated with RNA*later* ($p>0.05$). ND = not detected.

sediment are lower compared to the treated urine sediment ($P<0.05$) and the allograft biopsy specimens ($P<0.05$).

4. Discussion

The new observation from the current investigation is that treatment of urine cell pellet with RNA*later* prior to snap freezing optimizes RNA yield and purity and facilitates measurement of low abundance mRNAs.

Grotzer et al. (2000) demonstrated that tumor tissues could be stored at room temperature for 7 days when RNA*later* is added to the tissues and snap freezing in liquid nitrogen and storage at −70 °C can be obviated. It was also shown that good quality RNA can be isolated from tumor tissue kept at 37 °C for 24 h and then stored for 6 days in the RNA stabilization solution.

Wang et al. (2001) found that preservation of ocular tissues in RNA*later* resulted in a 53–58% higher RNA yield compared to corresponding frozen tissues. Wang et al. also found that that RNA integrity was better preserved with RNA*later*.

Our studies confirm and extend in important ways earlier observations regarding the usefulness of RNA*later*. Data from the current investigation show that treatment of urine cell pellets with RNA*later* improves RNA yield and quality, and facilitates mRNA profiling of urinary cells from renal allograft recipients. In this regard, it is noteworthy that the levels of expression of mRNA for granzyme B and TGF-β_1 in the treated urine cell pellets were not only higher than the levels in the untreated urine cell pellet but also similar to the levels observed in renal allograft biopsy specimens. Our new observations lend support to the hypothesis that mRNA profiles of RNA*later*-treated urine cell pellets are a surrogate for mRNA profiles of renal allograft biopsies.

In our earlier single center study in which the diagnostic utility of measurement of levels of mRNA for granzyme B and perforin in urinary cells of renal allograft recipients was explored, the urine samples were processed within 4 h of collection and RNA was isolated from the urine cell pellets without freezing in liquid nitrogen and without storage at −70 °C (Li et al., 2001). Our current study suggests that the urine cell pellets can be snap frozen, stored at −70 °C and shipped long distance, and that RNA isolation from

such specimens is improved by treatment of the cell pellet with RNA*later* prior to snap freezing. As pointed out by Grotzer et al. (2000) with respect to tumor tissues, storage of urine cell pellets in RNA*later* may circumvent the logistical problems associated with snap freezing and storage at −70 °C. Furthermore, samples can be shipped without the need for dry ice and there may be sufficient time to ship samples from around the world.

The current investigation as well as our earlier study focused on mRNA profiling of urinary cells from renal allograft recipients. mRNA expression by urinary cells has been studied in other patient populations such as patients with cancer. Hotakainen et al. (1999) reported a significant association between histologically validated transitional cell carcinoma of the bladder and the detection of mRNA for the beta-subunit of chorionic gonadotropin in urinary cells. Smith et al. (2001) found survivin mRNA in the urinary cells from all 15 new patients with bladder cancer whereas survivin mRNA was not detected in those without bladder cancer. Isurugi et al. (2002) evaluated the expression of mRNA for the catalytic subunit of telomerase (hTERT) in the exfoliated cells in bladder washing and concluded that investigation of hTERT mRNA in exfoliated cells is useful in the diagnosis and management of patients with bladder cancer. Urinary cell mRNA profiling may also be useful in the diagnosis of renal infections. For example, we have shown that BK virus nephritis can be diagnosed with a high degree of specificity and sensitivity by measurement of levels of BK virus VP1 mRNA in urinary cells (Ding et al., 2002).

In summary, our investigation suggests a simple protocol for optimizing RNA yield from urinary cells. In view of the observations that urinary cell mRNA profiles are useful in a wide variety of clinical situations, the strategy of adding RNA stabilization solution RNA*later* to the urine cell pellet may have broad applications.

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