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TECHNICAL ADVANCE

RNA Detection in Urine

From RNA Extraction to Good Normalizer Molecules

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RNA detection in liquid urine biopsy specimens could be an optimal method for noninvasive diagnostic and prognostic procedures in urologic disorders; however, there are no standardized procedures for implementing it in the clinic. We present a systematic evaluation of the best storage conditions and purification methods using four commercially available extraction kits to purify RNA from void urine. We measured different RNA molecules to select good and stable biomarkers and normalizers for analyses in liquid urine biopsy specimens. We have established a new combined procedure for RNA isolation from urine and found good performance in 25 urine samples from healthy volunteers of both sexes. Associations were tested using the *t*-test for paired samples, and miRNA specimens were selected as the more stable molecules. Stability analysis was performed, and we found miR193a and miR448 as the best normalizers to be used in this biofluid. This is a highly reproducible method that could be used to evaluate urine samples for diagnostic and prognostic purposes. *(J Mol Diagn 2016, 18: 15—22; http://dx.doi.org/10.1016/j.jmoldx.2015.07.008)*

Comprehensive profiling studies, aimed at understanding the complexity of gene alterations within tumors, have a great effect on the ability to detect, characterize, and treat malignant tumors. Early detection has been proven to improve outcomes, but screening approaches are costly and frequently invasive for patients. In bladder cancer, the lifetime cost per patient is among the highest of all tumor types,[1] with clinical surveillance and recurrence treatments representing an economic burden for the public health system. Urine-based methods are especially interesting for urogenital cancers and have been implemented for the detection of proteins, such as metalloproteinases,[2] and chromosomal alterations (UroVysion, Abbott Laboratories, Abbott Park, IL),[3] but their sensitivity is low. Urine collection is no-invasive and relatively time and cost efficient compared with other clinical samples, such as blood and cerebrospinal fluid. The main issue in applying the advances in genomic profiling to develop urine-based detection systems is RNA degradation, which makes it difficult to implement such diagnostic and prognostic procedures in clinical settings.

In the past decade, miRNAs have been selected as good and stable biomolecules, able to differentiate various pathophysiologic conditions, including differentiation,[4,5] inflammation,[6] diabetes,[7] and several types of cancers.[8,9] Unlike other biomolecules, miRNAs are very homogeneous among individuals and reveal restricted tissue distribution.[10] In addition, miRNAs are very stable in biofluids, such as serum, plasma, and urine,[11] which makes them good candidates to be explored as noninvasive early disease clinical biomarkers. In the present study, we compared four different RNA isolation protocols, two of them specific for small RNA and two for total RNA (including miRNAs), to establish the best purification protocol for urine samples. We evaluated mRNA, snRNA, and miRNA isolation to identify those with the best

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Table 1 Primers Used for cDNA and qPCR from mRNA Templates

Name	Sequence
RT-qPCR-GUSB	5′-CTTCTGTACTTCTTATATAC-3′
F-GUSB	5′-CGCCCTGCCTATCTGTATTC-3′
R-GUSB	5′-TCCCCACAGGGAGTGTGTAG-3′
RT-qPCR-ACTB	5′-GCATTACATAATTTACAC-3′
F-ACTB	5′-CCAACCGCGAGAAGATGA-3′
R-ACTB	5′-TCCATCACGATGCCAGTG-3′
RT-qPCR-GAPDH	5′-TACTTTATTGATGGTACA-3′
F-GAPDH	5′-AGCCACATCGCTCAGACAC-3′
R-GAPDH	5′-GCCCAATACGACCAAATCC-3′

F, forward; R, reverse.

stability and reproducibility in quantitative RT-PCR (RT-qPCR) studies. Finally, we selected an RNA isolation procedure that combines two of the four protocols initially evaluated and applied it to reveal that miRNA analysis from urine preserved at room temperature could be used as a routine clinical diagnostic and prognostic system for urologic malignant tumors.

Materials and Methods

Urine Collection and Preservation

Urine was collected in 100-mL sterile flasks (Deltalab, S.L.U., Barcelona, Spain) and delivered to the laboratory in <2 hours. All samples were preserved by immediately adding Norgen preservation solution. Specimens were divided, 30 mL was filtered by 0.45 μmol/L (Millipore, Merck KGaA, Darmstadt, Germany) to the resin-containing tube from Norgen (Norgen Biotek Corp, Thorold, Ontario, Canada), 15 mL was filtered to a clean tube for storage at room temperature, and 2 mL was filtrated to a 2-mL tube and frozen at −20°C.

RNA Purification

RNA was purified with the four selected RNA extraction kits: Norgen Total RNA kit (catalog no. 29600), Norgen miRNA kit (catalog No. 29000; Norgen Biotek Corp), miRCURY kit (catalog No. 300113; Exiqon A/S, Vedbæk, Denmark), and Qiagen miRNeasy serum/plasma kit (Qiagen GmbH, Venlo, the Netherlands). RNA extraction was performed following the manufacturer's instructions, except for the Qiagen assay for which 200 μL of plasma was substituted with 200 μL of filtered urine. For the fifth mixed protocol, 30 mL of urine was incubated with the Norgen slurry resin, and after sedimentation the liquid supernatant was eliminated and the resin-containing volume (approximately 3 mL) was preserved with the preservation solution (Norgen Biotek Corp. For RNA purification, the 200 μL of plasma recommended by Qiagen was substituted by 200 μL of the preserved urine-slurry resin (Norgen Biotek Corp).

RT-qPCR

Reverse transcription for the mRNA assays was performed using the Omniscript Reverse Transcription Kit (Qiagen) and a primer mix specific for all genes of interest, using 10 ng of total RNA. PCR was performed in a 7500 Fast Real Time PCR System using Go Taq PCR master mix (Promega Corp, Madison, WI) and 1 μL of cDNA as a template. Melting curves were performed to verify specificity and absence of primer dimerization. The sequences of the specific oligonucleotides used are listed in Table 1.

To measure miRNAs and snRNAs, reverse transcription was performed from 10 ng of total RNA along with miRNA-specific primer using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). PCR assays were performed using TaqMan Gene Expression Master Mix and 7500 Fast Real Time PCR System (Applied Biosystems).

Normalizer Analysis

The expression data were exported and analyzed using two different reference gene stability analysis software packages: geNorm (Biogazelle, Zwijnaarde, Belgium)[12] and NormFinder (Microsoft Excell add-in provided by MDL-Molecular Diagnostic Laboratory, Department of Clinical Biochemistry, Aarhus University Hospital, Skejby Sygehus,

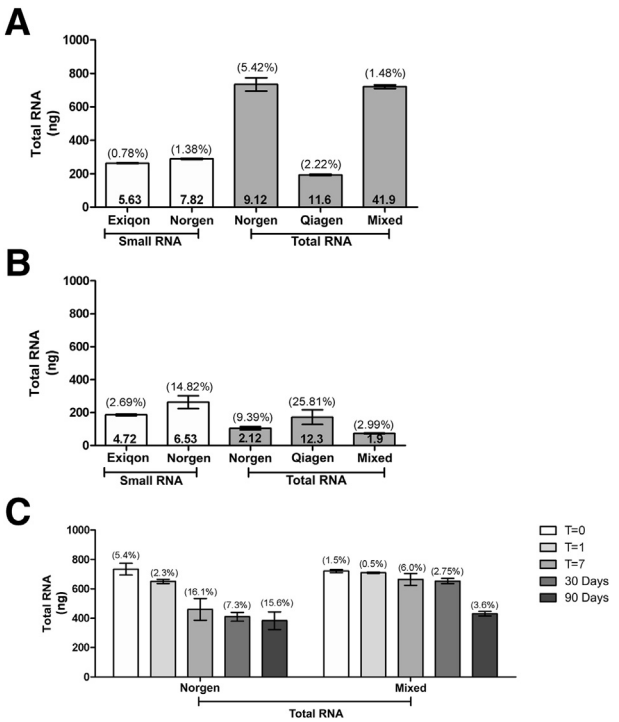

Figure 1 Total RNA mean yield for each of the three conditions from a single urine sample. **A:** Fresh urine samples. **B:** After 24 hours frozen at −20°C. **C:** After storage at room temperature from 0 to 90 days. The mean of each concentration obtained measured by Nanodrop absorbance at 260 nm is inside each bar and percentage coefficient of variation in parentheses.