Methods for Separate Isolation of Cell-Free DNA and Cellular DNA from Urine-Application of Methylation-Specific PCR on both DNA Fractions

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Abstract: The analysis of genomic DNA is widely-used for research, forensic and diagnostic purposes. Here we describe reliable methods for isolation of cell-free DNA and cellular DNA from urine. Both DNA fractions are suitable for PCR and Methylation-Specific PCR (MSP) amplification, leading to consistent and reproducible results. A kinetics analysis describes the decline of efficiency of MSP performed with urinary DNA which had been stored at room temperature with and without proteinase K for various time periods.

Keywords: Cell-free DNA, Diagnosis, DNA methylation, Urine.

For research purposes, DNA is frequently required in order to determine genetic variability in defined populations and to identify genomic and epigenomic alterations in disease. In forensic science, DNA analyses aid enquiries associated with criminal offenses and missing persons investigations, often based on trace amounts of genetic material [1]. Forensic scientists frequently utilize repetitive DNA elements for gender and species identification or inference of human ancestry [2]. An emerging application of DNA-based assays is disease diagnostics, including the diagnosis of bladder and prostate cancers, the main urological causes of morbidity and mortality worldwide [3,4]. Novel potent biomarkers may detect these malignancies earlier and with greater accuracy and may even help to discriminate between various tumor subclasses, a step further towards individualized medicine. Thus, they could contribute as well to improve the therapeutic success.

Urinary DNA derives from either cell-free DNA originating from apoptotic or-, necrotic urothelial, tumor or blood-derived cells [5,6] or from intact cells shed into urine, such as exfoliated tumor cells [7], normal uroepithelial cells and leukocytes. Depending on the circumstances, tumor cell DNA could be enriched in either one or both DNA fractions. Therefore, proper differentiation between cell-free DNA released from disintegrated cells and DNA of intact cells in urine could add an additional value for diagnostic purposes. Basic prerequisites for use in diagnostics are an appropriate amount of each DNA fraction and sufficient quality for successful application in various PCR amplification procedures. In particular, altered DNA methylation is commonly found in urological malignancies [8,9] and should represent a useful source of beneficial biomarkers. DNA, and especially cell-free DNA isolated from urine should therefore be suitable for Methylation Specific PCR which includes bisulfite treatment, a routine technical step, which is inevitably associated with some chemically-induced degradation of the genetic material with diminished PCR efficiency as a consequence.

Voided native urine is transferred into a 50 ml sample tube and is immediately put on ice for short-term storage (5-10 min). Long-term storage should be done at -20°C or -80°C. Even after up to 18 months of storage at -20°C we observed that DNA can be isolated from urine and can be amplified even after bisulfite treatment. We suggest thawing of urine samples by placing the sample tube for 60 min in water at room-temperature. After thawing all cells are separated from one, still cold (0-4°C) 5 ml urine sample either by centrifugation at 4,000 rpm for 10 min or if cellular DNA will not be used anymore by a 0.22 µm filter. From the supernatant the cell-free DNA is either isolated by the NORGEN Urine DNA Isolation Kit (Biotek Corporation, Ontario, Canada) using one single column with 25 µl elution buffer or, alternatively, by conventional precipitation with 1 volume isopropanol from a 0.3 M sodium acetate solution (pH 5.2). DNA is sedimented by centrifugation at 15,000 rpm for 20 min and washed twice by 70% ice cold ethanol. Cellular DNA was isolated from the urinary precipitate using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) or NORGEN Urine DNA Isolation Kit according to the manufacturer´s protocol. The average amount of cell-free DNA and of cellular DNA, isolated by these procedures from healthy individuals and tumor patients was 200-550 ng and 400-1300 ng, respectively. In a third approach, the QIAamp Circulating Nucleic Acid Kit was used together with the QIAvac 24 plus Vacuum Manifold according to the manufacturer´s (Qiagen) protocol. It yielded almost double the amount of both DNA fractions in high quality from the same volume of urine.

On the left of Fig. (**1A**), urinary cell-free DNA is shown which is fragmented and covers a size range from 100 up to

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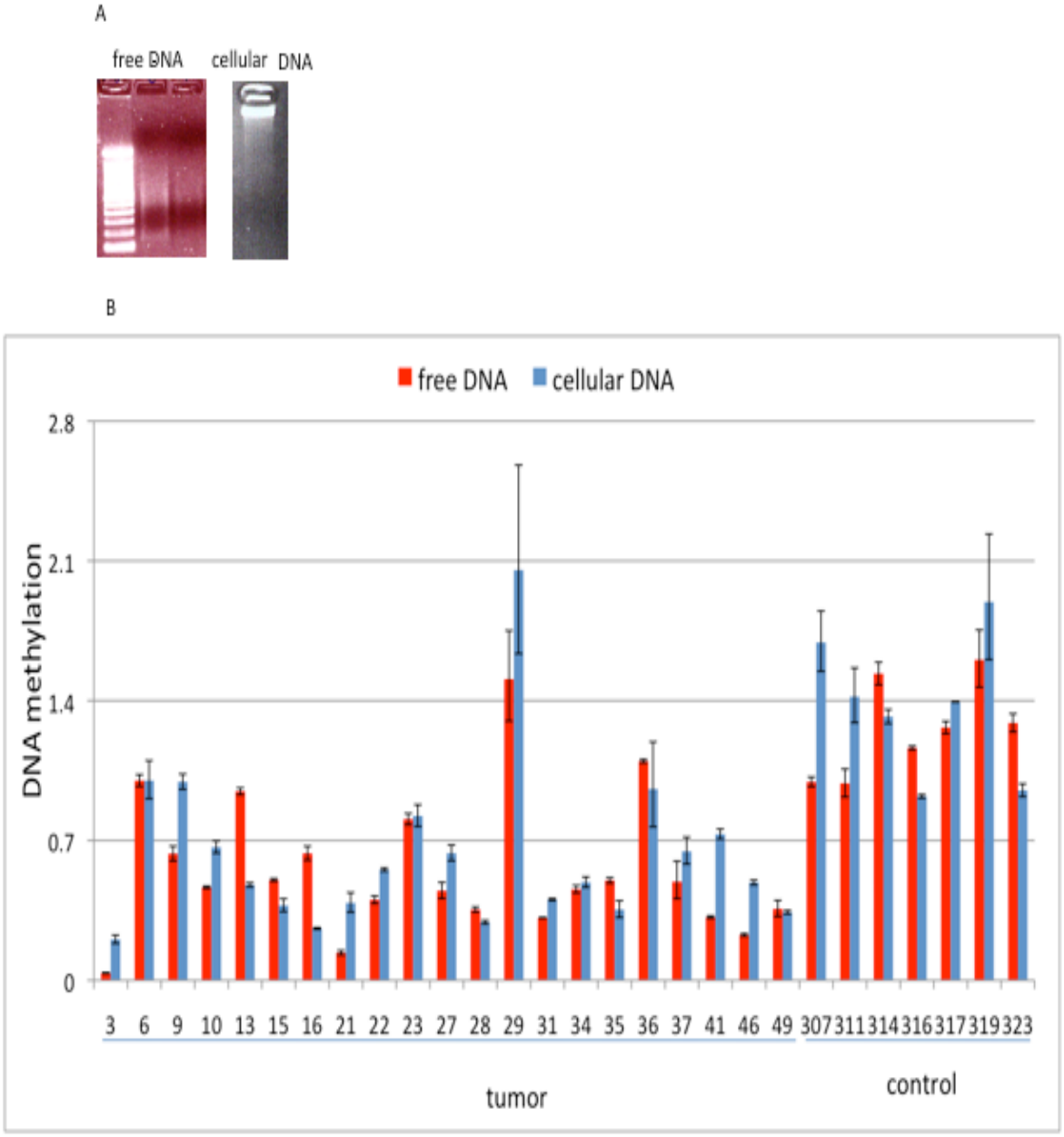

Fig. (1). Isolation of both urinary DNA fractions and MSP. Two samples of cell-free DNA and one sample of cellular DNA from urine are shown in **A**. They have been visualized by ethidium bromide staining on an agarose gel. The diagram below (**B**) shows the real time MSP result generated from urinary cell-free DNA (red bars) and cellular DNA (blue bars) from 21 tumor patient samples and 7 healthy donors (control), with primers specific for methylated LINE-1 promoter sequences.

2000 nucleotides. Both DNA fractions were subjected to bisulfite treatment by the EpiTect Kit (Qiagen). The converted DNA served as template for amplification of methylated LINE-1 sequences in a normalized real time MSP approach (Fig. **1B**). In particular in samples from healthy donors the degree of methylation should be nearly the same in both DNA fractions and differences should reflect differences in PCR efficiency. Indeed as shown in Fig. (**1B**) we reproducibly measured in both DNA fractions a similar degree of DNA methylation. Thus the presented methods allow the isolation of cell-free and cellular DNA from urine in sufficient amounts and quality. Both fractions gave reproducible results in MSP (Fig. **1B**) and conventional PCR (data not shown).

To test, how long the urine could be stored at room temperature before freezing, the urine was incubated with and without proteinase K (Qiagen) for 0, 4, 8, and 16 h at room temperature and total DNA was isolated by NORGEN Urine DNA Isolation Kit as recommended by the manufacturer, with the variation that 2 ml urine were used per sample for one column and 25 µl elution buffer. From these samples methylated LINE1 fragments were comparatively enriched by real time MSP. The time course in Fig. (**2**) shows that PCR efficiency is reduced at the 16 h time point and that proteinase K treatment maintains PCR efficiency, since at time point 8 and 16 the DNA methylation appears more pronounced in the proteinase-treated samples.

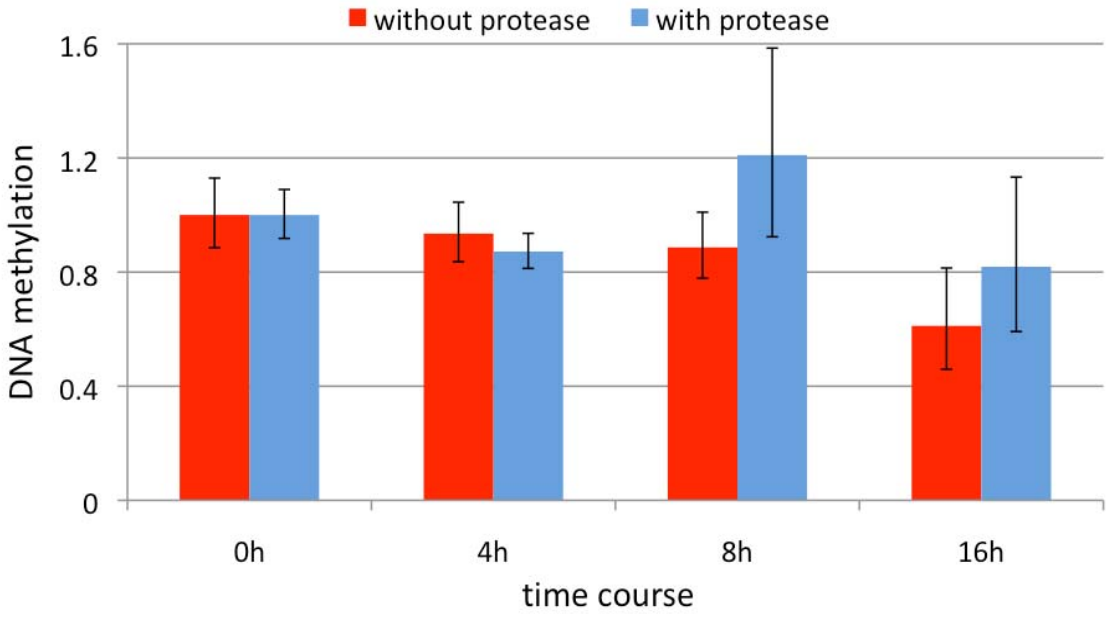

Fig. (2). Real time MSP amplification of methylated LINE-1 promoter sequences from total DNA isolated from urine samples which had been incubated with and without proteinase K for 0, 4, 8, and 16 h at room temperature.

Thus short term storage of urine, for up to 4-8 hours at room temperature does not impair MSP efficiency, suggesting that DNA is not significantly degraded by DNAses within this time window (Fig. **2**). For longer procedures at room temperature we suggest to use 0.15 AU of proteinase K per 2 ml urine to maintain PCR efficiency.

CONCLUSION

In conclusion with these protocols the proper separation of cell-free DNA from cellular DNA from urine samples can be achieved yielding both DNA fractions in a MSP/PCR ready quality. We consider that especially the cell-free DNA fraction from urine will gain in importance in the field of epigenetic biomarkers. In addition, our results suggest that urine should not be handled at room temperature for longer than 4-8 h without proteinase K if DNA should be preserved.

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CONFLICT OF INTEREST

The authors declare no competing interests.

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