Isolation of High Quality Urinary DNA Using Norgen's Urine DNA Mini Slurry Kit

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INTRODUCTION

Urine has been known for centuries to reflect pathological changes. It offers a great opportunity for the development of novel, non-invasive assays for the diagnosis, monitoring and early detection of cancer. Its impact has yet to reach its full potential, especially for clinical applications; however, initial studies have revealed its significant potential for clinical diagnosis (1).

It has been reported that some plasma-circulating DNA crosses the kidney barrier and appears in urine. This DNA is then referred to as "transrenal DNA" (2). In fact, K-ras mutations have been detected in urine of patients with pancreatic or colorectal carcinoma (2). In addition, Y chromosome–specific sequences were also detected in the urine of a pregnant woman with a male fetus as well as in a woman who previously received blood from a male donor (3).

Many advantages favor the use of urinary DNA for cancer biomarker discovery over blood and tissue samples: (1) urine-based tests are noninvasive; (2) urine is noninfectious for HIV and less infectious for many other pathogens; (3) the concentration of urinary DNA is similar to that in plasma; (4) additional urine can be easily collected for analysis and; (5) DNA isolation from urine is technically much easier because of its low protein concentration (1000-fold lower than blood) (2).

Norgen's Urine DNA Isolation Mini Kit (Slurry Format) provides a fast, reliable and simple procedure for isolating DNA from various amounts of urine ranging from 5 mL to 25 mL. For urine samples which are less than 2 mL, it is recommended that Norgen's Urine DNA Isolation Kit is used, which uses spin columns. The procedure for Norgen's Urine DNA Isolation Mini Kit (Slurry Format) is based on using Norgen's proprietary resin as the separation matrix in the form of slurry. DNA found in urine can be divided into 2 basic categories. The larger species (genomic DNA) is generally greater than 1 kb in size, and appears to be derived mainly from cells shed into the urine. The second species is smaller, generally between 150 and 250 bp (apoptotic DNA), and derives, at least in part, from the circulation. The second species is also considered as an RNA/DNA hybrid as reported by Halicka *et al.* (2000). Both types of DNA can be isolated reliably using this kit.

Typical yields of DNA will vary depending on the input sample, with more concentrated samples tending to yield more DNA. Preparation time for a single sample is less than 30 minutes. The purified urine DNA is compatible with Real time PCR, Methylation-sensitive restriction enzyme digestion PCR and many other downstream applications.
Despite the current battery of advanced and powerful molecular tools, the isolation of genetic materials from bodily fluids, especially urine, remains difficult. This is due to the presence of different inhibitors that are found naturally in urine (i.e. urea and salts). Norgen's Urine DNA Isolation Mini Kit (Slurry Format) has been developed to specifically target the isolation of high quality urinary DNA free from such inhibitors.

In this application note, Norgen's Urine DNA Isolation Mini Kit (Slurry Format) is used to isolate total urinary DNA from urine samples of various volumes. The quality of the isolated urinary DNA is demonstrated through different downstream applications including real-time PCR of the 5S gene using different input volumes. In addition, the urinary DNA isolated using Norgen's Urine DNA Isolation Mini Kit (Slurry Format) was used to detect the aberrant promoter hypermethylation of the tumor suppressor gene RASSF1A among Hepatocellular Carcinoma Patients.

METHODS AND MATERIALS

Urine DNA Isolation

Total DNA was isolated from 5 mL of urine using Norgen's Urine DNA Isolation Mini Kit (Slurry Format) as per the provided protocol (**Figure 1**). Briefly, 700 µL of Binding Buffer I was added to the 5 mL urine sample and mixed well by inversion. The tubes were centrifuged for 5 minutes at 2,000 *x g*, then the supernatant was discarded. Next, 20 µL of both Proteinase K and Pronase were added to the precipitated slurry pellet and vortexed for 10 seconds. The mixture was then incubated at 60°C for 20 minutes. Following the incubation, 260 µL of Binding Buffer II was added, mixed well by pipeting and then the slurry mixture was transferred into a Mini Filter Spin column. The tubes were centrifuged for 2 minutes at 6,700 *x g*, and the flow-through discarded. Next, 450 µL of Wash Solution I was added to the column and centrifuged for 1 minute, the flowthrough was discarded and the spin column reassembled with its collection tube. Next, 450 µL of Wash Solution II was added to the column and centrifuged for 1 minute. The flow-through was again discarded and the spin column reassembled with its collection tube. The column

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was spun for 1 minute in order to thoroughly dry the resin, and the collection tube was discarded. The spin column was transferred to a fresh 1.7 mL Elution tube and 150 µL of Elution Buffer was applied to the column and centrifuged for 2 minutes at 200 *x g* (~2,000 RPM), followed by a 1 minute spin at 14,000 *x g* (~14,000 RPM). For the second elution the spin column was transferred to a fresh 1.7 mL Elution tube and 100 µL of Elution Buffer was applied to the column and centrifuged for 1 minute at 14,000 *x g* (~14,000 RPM). Purified DNA was then stored at -20°C for several days or at -70°C for long term storage.

Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to visualize the purified urine DNA. Generally, one tenth of the eluted DNA was run on a 1.2% agarose gel in Tris-Acetate-EDTA buffer at 100V. Pictures of the gel were taken after 15 minutes, in order to visualize the different species of DNA that had been isolated.

Real time PCR Assay

DNA isolated from the urine was used as template for Real Time PCR with primers specific to the *S15* housekeeping gene (5S-F 5'-GCCATACCACCCTGAACG-3' and 5S-R 5'-AGCCTACAGCACCCGGTATT-3').

Methylation-sensitive restriction enzyme digestion PCR

To assess levels of CpG methylation of the RASSF1A by methylation-sensitive restriction enzyme PCR, DNA isolated from a Hepatocellular carcinoma (HCC) patient, a Hepatitis C virus (HCV infected patient) and a control individual was subjected to *DpnI* digestion. *DpnI* will only digest methylated DNA sequences. *DpnI* was chosen, in particular, based on the number of their recognition sites within the amplicon (i.e. the availability of 4 or more *DpnI* recognition sites was the criteria upon which *DpnI* digestion was used). Each gene was amplified before and after digestion. The gene was designated as hypermethylated if there was no amplification in case of *DpnI* digestion. GAPDH, as an internal control, was also amplified to exclude possible false positive results. The isolated urine DNA was digested with 20 U of *DpnI* (NEB, New England Biolabs). The digestion was performed at 37°C for 16 hours, heat-inactivated at 65°C for 15 minutes, and purified using Norgen's Enzymatic Reaction Cleanup Kit. The purified digestion was then amplified using primers that can specifically recognize the promoter of the RASSF1A.

RESULTS AND DISCUSSION

Norgen's Urine DNA Isolation Mini Kit (Slurry Format) has been optimized to isolate high quality DNA from urine. Total urinary DNA was isolated from various volumes of a single urine sample (7, 10, 14 and 25 mL urine, respectively). The time of sample collection was optimized to be the first midstream voided urine. By observing the DNA isolated from the four urine samples, it can be observed that the total DNA content in urine gradually increases by increasing the volume of the urine used as an input for isolation (**Figure 2**). The different urinary DNA species are very well demonstrated in **Figure 2**. The highest band is the high molecular weight DNA isolated from the exfoliated cells found in urine, whereas the low molecular band represents the apoptotic DNA that results from apoptosis. An additional band was found in the range of 750 -1000 bp, which corresponds to the circulating DNA. The apoptotic band agrees with the literature in many characteristics, as it has a size of 150-200bp and contains a DNA/RNA hybrid (Halicka *et al.,* 2000).

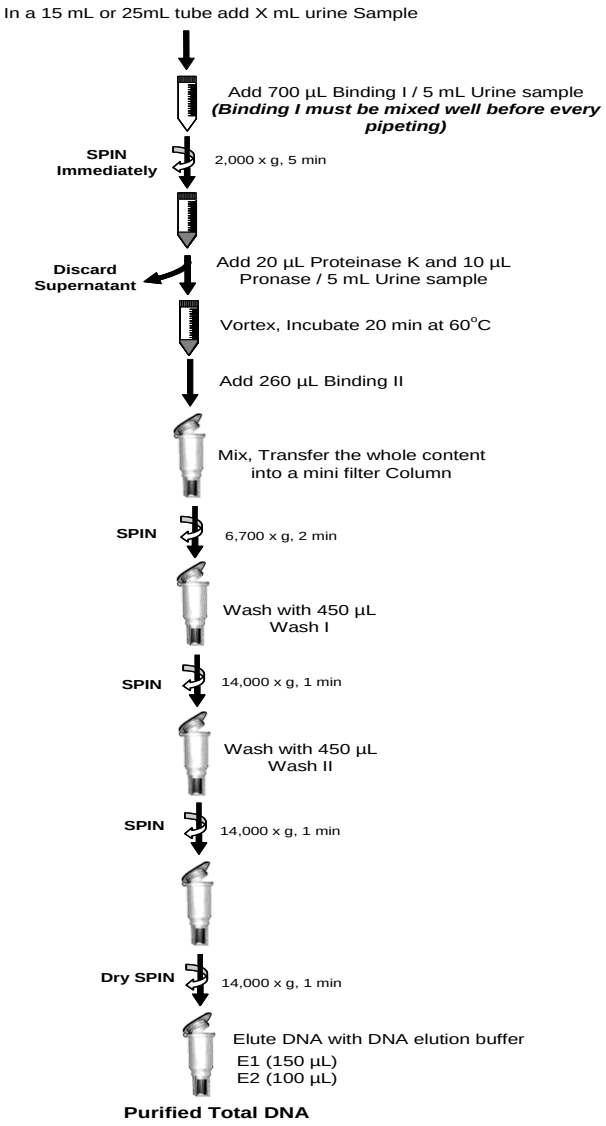

Figure 1: A flowchart for the purification of DNA using Norgen's Urine DNA Isolation Mini kit (Slurry Format).

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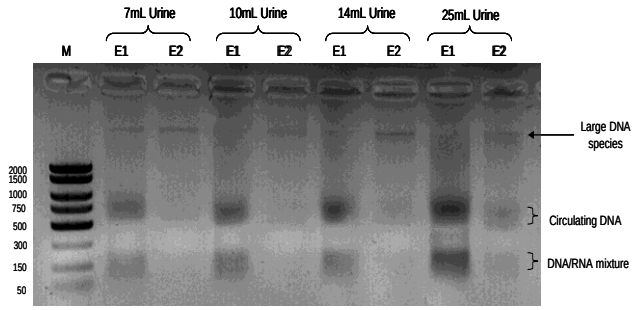

Figure 2: Isolation of High Quality Urinary DNA from Different Urine Volumes. A typical 1.2% agarose gel showing total urinary DNA isolated from different urine volumes using Norgen's Urine DNA Isolation Mini Kit (Slurry Format). Each lane shows one tenth from each elution (E1: 15 µL out of 150 µL were loaded on the gel, E2: 10 µL out of 100 µL were loaded on the gel). Lane M is 10 µL of Norgen's FastRunner DNA Ladder.

This DNA/RNA hybrid was confirmed when one of the samples was subjected to both *DNase* and *RNase* digestion (**Figure 3**). In **Figure 3**, the high molecular weight genomic DNA as well as the circulating DNA was completely digested when it was subjected to *DNase* treatment, whereas the low molecular weight apoptotic DNA was partially digested. In contrast, the high molecular weight gDNA as well as the circulating DNA were not affected by *RNase* digestion, whereas the low molecular weight apoptotic DNA was partially digested. This confirms that the low molecular weight band is a mix between the DNA and RNA. This agrees with and confirms the previous reports that these DNA/RNA mixtures were generated as a result of apoptosis.

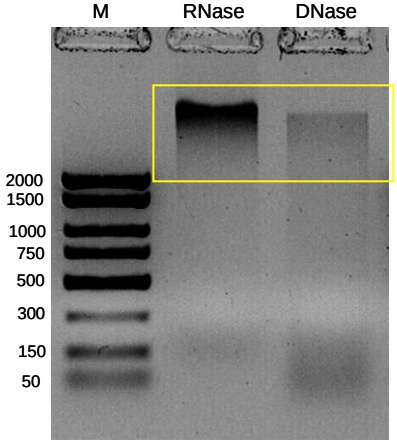

Figure 3: Treatment of the Total Urinary DNA Isolated from 5 mL of Urine with *DNase* and *RNase*. DNA isolated from a 5 mL urine sample was subjected to *DNase* and *RNase* treatment. The high molecular weight gDNA was completely digested when treated with DNase but was not affected by the RNase treatment (yellow rectangle). On the contrary, the low molecular weight band was partially digested when treated with both *DNase* and *RNase*. Lane M is Norgen's FastRunner DNA Ladder.

The quality of DNA isolated by Norgen's kit was further demonstrated by real time PCR amplification of the 5S rRNA gene using the DNA isolated from urine as a template (**Figure 4**).

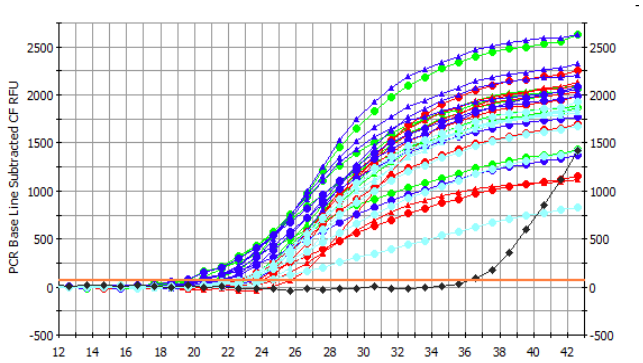

Figure 4: Isolation and Detection of DNA from Different Volumes of Urine. Total genomic DNA was isolated from 7, 10, 14 and 25 mL urine samples using Norgen's Urine DNA Mini Isolation Kit (Slurry Format). The isolated DNA was then subjected to real time PCR using primers specific for the human 5S gene. The red lines in the PCR baseline graph above correspond to the DNA isolated from the 7 mL urine sample, the green lines correspond to the DNA isolated from the 10 mL sample of urine, the blue lines correspond to the DNA isolated from the 14 mL of urine, whereas the orange lines correspond to the DNA isolated from the 25 mL of urine. The isolated DNA was eluted into two separate elutions of 150 µL and 100 µL, respectively. To test the absence of PCR inhibitors usually accompanying DNA isolated from urine, an increasing amount from each elution (1, 3, 6 and 9µL) was used as the template in the PCR reactions.

The quality of DNA isolated by Norgen's kit was further demonstrated by real time PCR amplification of the 5S gene using the DNA isolated from urine as a template (**Figure 4**). Increasing volumes of the DNA isolated from different volumes of urine input were used as the template for the real time PCR reactions. This was done to test if there are any PCR inhibitors that are contaminating the isolated DNA. Therefore, 1, 3, 6 and 9 µL of each elution (E1 and E2) were used from the DNA isolated from 7, 10, 14 and 25 mL urine as a template for the amplification of the 5S gene. The 5S rRNA gene was successfully amplified from both elutions (E1 and E2) of each urine input volume, indicating the high quality of the isolated urinary DNA. Furthermore, no PCR inhibition was observed when using an increasing amount from the isolated DNA as a template in the real time PCR reaction. **Figure 4** shows the high quality of the isolated urinary DNA from various urine volumes using Norgen's Urine DNA Isolation Mini Kit (Slurry Format).

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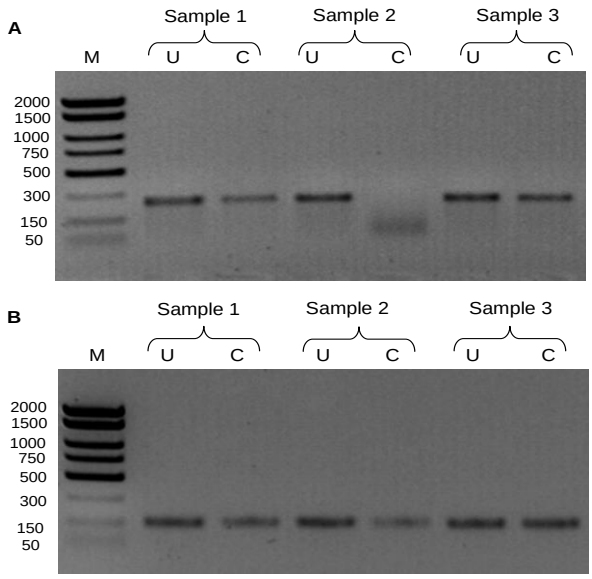

Figure 5: Detection of aberrant promoter methylation of the RASSF1A tumor suppressor gene. Total urinary DNA was isolated from 5mL urine samples collected from a control healthy individual (sample 1), an HCC patient (sample 2) and HCV patient (sample 3). The isolated DNA was digested with the *DpnI* restriction enzyme. Panel A shows the amplification of the RASSF1A promoter region before and after digestion with *Dpn*I. Panel B shows the amplification of the GAPDH gene used as an internal control for the PCR amplification. U: Uncut with *DpnI*, C: cut with *DpnI*.

In order to demonstrate the usefulness of the DNA isolated from urine using Norgen's Urine DNA Isolation Mini Kit (Slurry Format), the aberrant promoter methylation of the RASSF1A tumor suppressor gene was analyzed in a control healthy individual, an HCV infected patient and an HCC patient (Figure 5). RASSF1A tumor suppressor gene is known to be hypermethylated in HCC patients but in neither HCV infected patients nor in healthy individuals. The aberrant promoter methylation of the RASSF1A was studied using the Methylation-Sensitive Restriction Enzyme Digestion PCR. The *DpnI* enzyme was chosen to digest the DNA isolated from the control, HCC and HCV patients since the promoter region of the RASSF1A contains more than three recognition sites for the *Dpn*I enzyme. Furthermore, *DpnI* is sensitive to any methylation in the CpG islands (i.e. the *DpnI* enzyme will only cut if the CpG islands were methylated). Therefore, if the promoter region of the RASSF1A was methylated, the RASSF1A promoter will be digested with the *DpnI* enzyme and will not yield any PCR product. On the contrary, if the RASSF1A promoter region was not methylated, the *DpnI* will not digest the RASSF1A region and hence it will be amplified. A specific set of primers was designed to be flanking the promoter region that contains the *DpnI* recognition sites.

Furthermore, a specific set of primers was designed to amplify a region from the GAPDH gene that does not contain any *Dpn*I recognition sites. In this way, the GAPDH should be amplified in all cases and hence can be used as control for the efficiency of the amplification. **Figure 5** shows that the RASSF1A promoter region was not amplified after being digested with the *DpnI* enzyme but it was amplified before being digested with the *Dpn*I enzyme in only the DNA isolated from the HCC patient. This indicates the aberrant promoter hypermethylation and hence the silencing of the RASSF1A in the patient with liver cancer. On the contrary, the RASSF1A region was amplified before and after treatment with *Dpn*I from the DNA isolated from the control healthy individual and that from the HCV infected patient. This indicates that RASSF1A gene is active as it was not found to be hypermethylated.

CONCLUSIONS

Through analysis of the performance of Norgen's Urine DNA Isolation Mini Slurry Format Kit in the isolation of total DNA from various urine volumes, a number of conclusions regarding Norgen's kit can be made:

1. Norgen's kit allows for the isolation of high quality total circulating urinary DNA within 30 minutes from various urine volumes, making the DNA isolation rapid and convenient.

2. Norgen's kit also demonstrated that the DNA isolated from increasing urine volumes is linear, indicating the efficiency and the robustness of the isolation method.

3. In addition to the isolation of total urinary DNA with a high quantity, Norgen's Urine DNA Isolation Kit (Slurry Format) was able to isolate urinary DNA with a high quality and free from the contaminants usually found in urine. The quality of the isolated DNA was demonstrated by the amplification of the 5S gene from an increasing template input in the real time PCR reaction. There was no PCR inhibition observed, indicating the high quality of the isolated urinary DNA from various urine volumes.

4. It was further demonstrated that the DNA isolated from urine using Norgen's Urine DNA Isolation Mini Kit (Slurry Format) can be used to study the aberrant promoter methylation of tumor suppressor genes using the Methylation-Sensitive Restriction Enzyme Digestion PCR. This method requires a high quantity and a high quality DNA, and this was provided by using Norgen's Urine DNA Isolation Mini Kit (Slurry Format).

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