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PCR-ready human DNA extraction from urine samples using magnetic nanoparticles

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ABSTRACT

Urine-derived human genomic DNA (gDNA) has wide application in a variety of disciplines including clinical medicine, sports, and forensic science. We describe a novel method for gDNA extraction from urine samples using carboxylated magnetic nanoparticles (CMNPs) as solid-phase adsorbents. Sedimentation associated with freezing of urine samples significantly reduces cell capture by CMNPs. However, the addition of 10 mM EDTA and subsequent pH modification (pH 6.0–7.1) can re-dissolve urine sediments. Purified gDNA ranged from around 0.1 kb to more than 23 kb. PCR using specific primers targeting *K-ras*, *GAPDH*, *CYP3A4* and *GDF5* amplified 100% of varying sized gene fragments, verifying the high quality of the isolated DNA. Successful PCR amplifications using DNA isolated from urine samples as small as 50 μl were demonstrated. Enrichment of urine cells and subsequent adsorption of DNA can be achieved with the same CMNPs, greatly simplifying extraction procedures. The CMNP gDNA extraction technique proved to be simple, rapid, sensitive and environmentally friendly, with application for routine laboratory use and potentially within automated urine extraction platforms.

1. Introduction

Recently, the use of urine-derived human genomic DNA (gDNA) for genetic analysis has received considerable attention in medical, athletic and forensic contexts [1–4]. The isolation of DNA from urine, rather than traditional blood samples is advantageous, since it reduces the pain and risk of infection associated with venous blood collection, particularly during athletic competitions, where urine is the least invasive, and potentially the only source of DNA available for identifying individuals. The small amounts of nucleated cells (epithelial, leukocytes, and even exfoliated malignant cells) and cell-free DNA present in voided urine makes diagnosis (e.g., *K-ras* for cancer), genotyping and screening possible [5–10].

The rapid and efficient extraction of human urine gDNA suitable for molecular identification techniques (e.g., PCR) would be of great advantage, but is particularly difficult due to the complex composition of urine. Current gDNA extraction methods use centrifugation or filtration, which are very time-consuming and labor-intensive processes that cannot be automated. Further, existing approaches require large volumes of urine to obtain sufficient cells, and employ toxic organic reagents, enzymes, and spin columns to isolate DNA from proteins and other contaminants [1,8–11].

The separation of cells [12–14] and extraction of target biomolecules (e.g., protein, peptide, DNA, and RNA) [15–20] from biological samples using magnetic micro- or nanoparticles as solid phase adsorbents offer many benefits over conventional techniques, including faster processing times, fewer chemical reagents, and easier operation with potential for automation. Magnetic particles have been successfully used to extract genomic DNA from body fluids, such as blood, saliva, and semen [2,21–24]. However, reports of urine DNA extraction with magnetic particles are very limited. Recently, Siddiqui et al. [25] developed a solid-phase method for human gDNA isolation from urine using a variety of commercially available magnetic micro-beads (Genpoint AS, NorDiag ASA, Oslo, Norway). The urine used in this study however, was freshly prepared, but in many applications, urine samples need to be stored at low temperatures before processing. Low temperature storage is problematic as sediment may form within the urine, particularly under frozen conditions [26], which contains many physiological metabolites that may inhibit enzymes if not removed completely. The presence of sediment also strongly interferes with urine cell gDNA recovery using magnetic particles as solid-phase adsorbents. The elimination of urine sediment using

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Table 1
Gene location, PCR primers and predicted sizes of amplified products.

Assay	Primer (5′ → 3′)	Location	Product size
K-ras	Forward: GTACACATGAAGCCATCGTATA Reverse: CCACTTGTACTAGTATGCCTTAAG	chr12 (p12.1)	214 bp
GAPDH	Forward: ACCACA GTCCATGCCATCAC Reverse: TCCACCACCCTGTTGCTGTA	chr12 (p13.31)	556 bp
CYP3A4	Forward: AACAGGGGTGGAAACACAAT Reverse: CTTTCCTGCCCTGCACAG	chr7 (q21.1)	592 bp
GDF5	Forward: GGTGAGGTTGCAGGGAAT Reverse: CAGGGGAACTTGTGGATAA	chr20 (q11.2)	1106 bp

centrifugation or filtration should be avoided due to the co-removal of cells and their gDNA by these approaches. The report by Siddiqui et al. indicated that small-sized micro-beads appeared to yield more gDNA from urine samples than large-sized ones [25]. Hence, it is reasonable to speculate that the performance could be further improved using nano-sized particles as solid extraction phases.

Consequently, the present study was undertaken to develop a robust urine genomic DNA extraction method using nano-sized magnetic solid phases, applicable for both fresh and sediment-containing (previously frozen) urine specimens. We noted that urine sediment induced by freezing could be rapidly and efficiently dissolved with the addition of EDTA (and adjusting solution pH), thus allowing any sedimented cells to be re-suspended for binding by carboxyl-group modified magnetic nanoparticles (CMNPs). The quality of extracted DNA is assessed by its yield, molecular weight, and the ability to serve as a substrate for PCR amplification. Accordingly, gDNA extracted by CMNPs was of high quality as successful amplification was achieved with sample volumes as small as 50 μl of urine. This simple, rapid, yet sensitive and environmentally friendly gDNA extraction method is particularly suitable for routine laboratory use, and could form the basis of automated urine extraction systems for various diagnostic purposes.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and reagents (DNA isolation and analysis) were of analytical or molecular biology grade, respectively, and were obtained from commercial sources. Carboxyl-group modified magnetic nanoparticles were prepared according to our previously reported method [27]. $FeSO_4$ and $FeCl_3$ were used to prepare bare Fe_3O_4 nanoparticles prior to their coating with poly(methacrylic acid) through polymerization of monomer methacrylic acid in toluene. The coated CMNPs were washed successively with acetone, ethanol, and water prior to dispersal in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) at a concentration of 10 mg/ml. Particle size of the CMNPs, as determined by transmission electron microscopy (TEM), was around 10 nm, as illustrated by both TEM and scanning electron microscopy (SEM) images (Fig. 2A and B).

2.2. Urine sample collection

Morning void urine samples were collected in 100 ml sterile tubes from healthy adult male and female volunteers. The freshly collected urine was immediately mixed with EDTA to a final concentration of 10 mM, with a portion of the samples kept at 4 °C and processed within 4 h, while the remaining aliquots were stored at −20 °C. Whole blood (with 23 mM citric acid, 80 mM D-glucose, 45 mM sodium citrate, pH 5.5 as an anti-coagulant) was taken from healthy donors and served as a reference sample for human gDNA. Unless otherwise stated, the urine samples used for optimization of DNA extraction and PCR reactions were of female origin, stored at −20 °C, and thawed at room temperature before use.

2.3. Cell enrichment from urine

Frozen urine was thawed at room temperature with the sample pH adjusted to around 6.0–7.1 using 1 M NaOH. Any visual sediment in the urine quickly vanished after pipetting the solution 2–3 times. Urine samples were then transferred to a 1.5 ml tube containing CMNPs and 0.6 vol of binding buffer (30% PEG6000, 2 M NaCl). The volume of beads was 10% of the urine volume. The mixture was incubated at room temperature for 5 min to form cell-nanoparticle complexes, which were then immobilized on a Promega™ magnetic separation stand, after which the supernatant was aspirated and discarded. TEM was used to characterize the urine cells captured by CMNPs, with images obtained using a Hitachi H-600 microscope operating at 75 kV.

2.4. DNA isolation

The immobilized urine cells were lysed by adding 10 μl of lysis buffer (3 M NaI; 5 M urea; 40 g/l Triton X-100; 10 mM EDTA, 25 mM Tris–HCl, pH 6.5) prior to incubation at room temperature for 3 min. To facilitate binding of the released DNA to the nanoparticles, 20 μl of isopropanol was added to the suspension for a further 5 min at room temperature. After magnetic separation of the DNA–nanoparticle complexes, the supernatant was discarded, and the immobilized DNA was rinsed twice with 50 μl of cold 70% ethanol solution. After removal and evaporation of the ethanol, the DNA was eluted in 20 μl TE buffer (10 mM EDTA, 25 mM Tris–HCl, pH 8.0) at room temperature for 10 min. The MNPs were then immobilized with the supernatant transferred to a DNase/RNase free EP tube.

In a control study, gDNA was isolated from pelleted urine cells (1500 × *g*, 5 min) using a modified phenol/chloroform method [10]: pelleted cells were washed with TNE buffer (10 mM Tris, 100 mM EDTA and 100 mM NaCl, pH 8.0) and re-centrifuged followed by the addition of 600 μl of lysing solution (1% SDS in TNE buffer). The mixture was incubated for 1 h at 55 °C, followed by the addition of proteinase K to a final concentration of 100 μg/ml. After incubation for another 3 h at 55 °C, the lysate was extracted once with phenol, twice with phenol–chloroform–isoamyl alcohol (25:24:1), and finally once with chloroform. DNA was precipitated in a mixture of 0.1 vol 3 M sodium acetate (pH 5.2) and 0.6 vol isopropanol. After washing with 70% ethanol, the DNA was resuspended in 30 μl of TE buffer. Positive control DNA for PCR use was isolated from 100 μl of blood using the magnetic nanoparticle-based method reported by Xie [14]. Negative isolation control was performed using binding buffer only. The isolated gDNA was analyzed on a 0.8% agarose gel stained with GoldView (SBS Genetech, China).

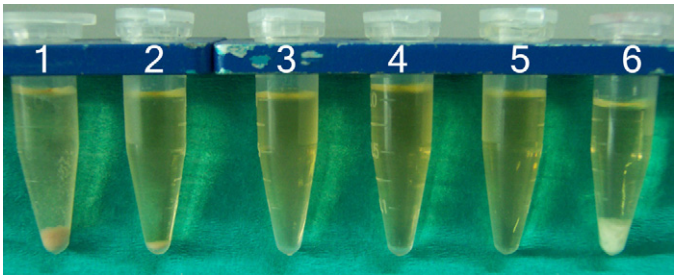

Fig. 1. Effect of EDTA addition and pH adjustment on the dissolution of urine sediments. 10 mM of EDTA was added to urine samples prior to freezing at −20 °C for 72 h. The pH (from left to right) was 5.6, 5.8, 6.0, 6.6, 7.1, and 8.0, respectively. Note that sediment color differs between acidic (tubes 1, 2) and alkaline (tube 6) environments.

2.5. PCR amplification

The quality of extracted gDNA from urine was assessed by PCR amplification of four different sized fragments of different genes (Table 1) using a Bio-Rad MyCycler Thermal Cycle (Bio-Rad Laboratories, Hercules, CA). Amplification reactions were carried out in a final volume of 50 μl containing 1× PCR buffer (50 mM Tris–HCl, 100 mM NaCl, pH 8.3), 10 μl of isolated DNA sample, 4 mM Mg^{2+}, 50 μM dNTP, 6 U Taq DNA polymerase and 20 pmol each of the PCR primers. Amplification profile for *K-ras* was: 94 °C for 5 min, followed by 30 cycles of 50 s at 94 °C, 45 s at 55 °C (the temperature for *GAPDH*, *CYP3A4* and *GDF5* were 52, 53 and 50 °C, respectively), 20 s (the duration for *GAPDH*, *CYP3A4* and *GDF5* were 40, 50 and 75 s, respectively) at 72 °C, and a final extension performed at 72 °C for 5 min [28–30]. The reactions were then held at 4 °C until analysis. Finally, amplified products (5 μl) were loaded onto a 3.0% agarose gel in TBE (90 mM Tris–borate pH 8.0, 1 mM EDTA) buffer for electrophoresis, with bands visualized under ultraviolet light by GoldView staining using the Gel Doc EQ System.

3. Results and discussion

The use of magnetic nanoparticles to isolate nucleated cells directly from urine is of significant advantage from both ease of use and purity perspectives. This facile approach concentrates and extracts gDNA from urine, and eliminates the influence of physiological metabolites present in the sediments of centrifuged or frozen urine samples that would otherwise inhibit enzyme activities. Despite considerable efforts to extract gDNA from urine sediments, traditional gDNA extraction methods are easily contaminated by various known enzyme inhibitors (phenol, urea and salts) originating from the urine and/or extraction process. Consequently, it would be necessary to re-dissolve the sediment prior to cell isolation, as adopted by protocols assaying other urine components (e.g., proteins) [26]. Despite reports that incubating urine samples at

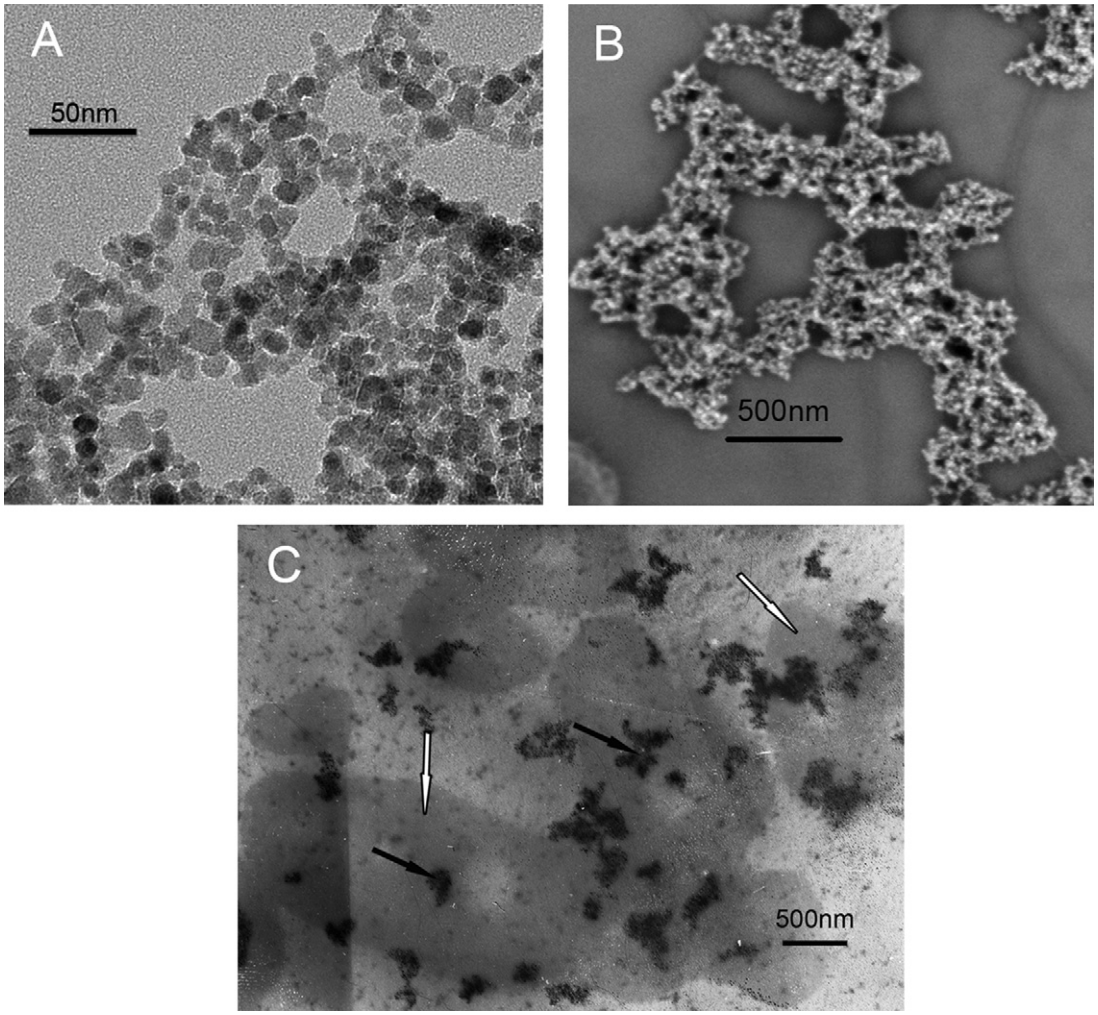

Fig. 2. TEM and SEM images. (A) TEM image of CMNPs; (B) SEM image of CMNPs; (C) urine cells captured by CMNPs. Note that a few aggregates of CMNPs (indicated by black arrows) are visible on the surface of cells (indicated by white arrows).

high temperatures [9] or room temperature with vigorous agitation [26] could re-dissolve the sediments, these approaches were unsatisfactory in our experience, particularly when the urine samples had a high sediment content. Regardless, the physical conditions employed by these approaches may disrupt the cell's integrity. Very recently, the ability of EDTA and pH to reduce the precipitation of urine sediment were examined by Saetun et al. [26]. Specifically, the addition of EDTA (5 mM) to urine samples which experienced freeze–thaw cycles reduced the formation of precipitate by 75%, while adjusting the urine pH between 5.8 and 7.4 prior to freezing slightly reduced urine sedimentation. However, the combined effects of EDTA and pH adjustment on urine sediment precipitation or dissolution were not evaluated. In the present work, urine sedimentation induced by freezing could be completely re-dissolved by the addition of EDTA and adjustment of pH, as shown in Fig. 1. Typically, at least 3 mM EDTA, commensurate with a urine pH adjustment to 6.0–7.1, was adequate to re-dissolve any urine sediments, with some variations in these manipulations required depending on the amount and composition of urine sediment. If the addition of EDTA and pH adjustments are both performed prior to freezing, urine sediment remains after thawing; but if EDTA is added prior to freezing, while the pH is adjusted after thawing, urine sediments can be efficiently dissolved. As an added benefit, the addition of EDTA prior to freezing increases the stability of gDNA during low temperature storage [31]. Neither EDTA nor pH adjustment (regardless of addition/adjustment prior to, or following freezing) alone could completely prevent (or dissolve) urine sedimentation; both manipulations must be employed to be effective.

Once a sediment-free urine solution was achieved, re-suspended cells were then available for binding by magnetic nanoparticles. Efficient binding between cells and the magnetic solid phase may be achieved by specific or non-specific interactions, the latter offering greater cost effectiveness. The non-specific binding of cells to a solid extracting matrix may be achieved by manipulating the chemical and physical nature (hydrophobicity or charge) of the surface of the solid support [13,32] and/or conditions of the cells milieu (e.g., pH or solution composition) [19,25]. For example, the non-antibody ligand-coated Bugs' n Beads, originally developed for non-specific bacterial capture from complex mixtures [23], was adapted for non-specific human cell capture from urine samples with the help of a binding buffer [25]. As for carboxylated magnetic nanoparticles, it has been reported that even without the assistance of a binding buffer, nanoparticles can form stable complexes with cells from a variety of sources such as whole blood, saliva, and bacterial culture [14,21,24]. In the present study, while stable cell-CMNP complexes could be formed in the absence of binding buffer, the extraction of urine cells was promoted by the addition of PEG/NaCl or phosphate buffer solution. Fig. 2C displays the TEM image of magnetically captured cells obtained after incubating CMNPs with a urine sample in the presence of binding buffer. We can see a few aggregates of CMNPs deposited on the surface of the urine cells, allowing the magnetically labeled nucleated cells in the urine samples to be easily recovered with the help of a permanent magnet. We noticed that the CMNPs were randomly deposited on the surfaces of the urine cells, rather than covering the entire cell surface in a specific pattern, indicating the non-specific nature of their interaction [12,33]. The high efficiency of cell enrichment and recovery displayed by the present method demonstrate its utility for urine cell-based genetic analysis and diagnostics.

During the extraction of intracellular biomacromolecules (e.g., DNA and proteins), the CMNPs attached to the cell surfaces do not need to be removed as the magnetic nanoparticle/cell complexes can be directly lysed. After cell lysis, the CMNPs can be further utilized to remove cellular impurities [19,34] or directly capture target biomolecules [14,23–25]. In the present study, the PCR template preparation procedure was simplified by subjecting the nanoparticle/cell complexes directly to lysis without prior removal of the magnetic nanoparticles. For binding of DNA to the same nanoparticles, isopropanol (or ethanol) were added to help form DNA/nanoparticle complexes [14,23]. As both the enrichment of urine cells and the adsorption of genomic DNA can be realized with the same nanoparticles, many residual impurities and enzyme inhibitors present in the urine or cell lysate can be readily removed. Further, the adsorbed DNA can be easily eluted using TE buffer. Electrophoretic analysis demonstrates that the DNA extracted from both fresh and frozen urine samples contained a continuous "smear" of various sized fragments ranging from about 100 bp to more than 23 kb (lanes 1 and 2, Fig. 3). DNA extracted by the phenol–chloroform method displayed similar electrophoretic patterns of DNA fragments, but with lower yields (lane 3, Fig. 3) while DNA extracted from whole blood appears as a single distinct band on the agarose gel (lane 4, Fig. 3).

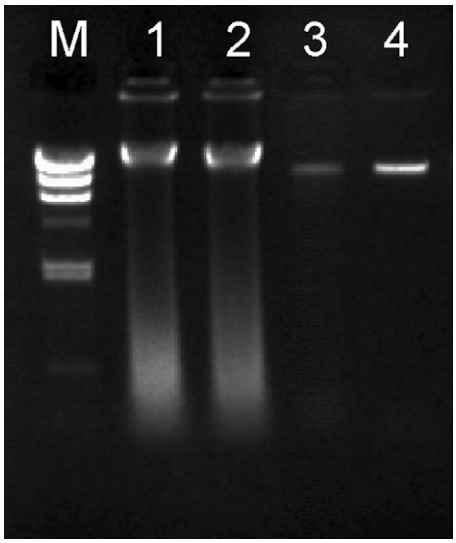

Fig. 3. Agarose gel electrophoresis of genomic DNA. Lane 1, DNA extracted from a fresh urine sample using CMNPs; lane 2, DNA extracted from a frozen-thawed urine sample using CMNPs; lane 3, DNA extracted from fresh urine using the phenol-chloroform method; lane 4, blood DNA extracted using CMNPs; lane M, λ-*Hind* III digest (Takara Bio, Shiga, Japan).

To investigate the sensitivity of the DNA preparation method, a 214-bp DNA sequence in exon 4B of the *K-ras* gene for extracted genomic DNA was amplified, with three different sample volumes (50, 100 and 200 μl) tested by PCR. As shown in Fig. 4, DNA bands of expected size (~214 bp) were observed in both urine (lanes 1–4) and blood samples (lane 5). Successful PCR amplification was detected from as small as 50 μl of sample (lane 1), while at least 200 μl urine was needed to generate enough PCR templates in a similar report [25]. This high extraction performance can be ascribed to the carboxylated surfaces of the nanoparticles, which bind mammalian cells with a high affinity [14,24,25,35,36], especially when used with the binding buffer employed in the present study. The higher surface area:volume ratio and good dispersal properties inherent to the nanoparticles employed in this study conferred an advantage over commonly used micro-sized particles, as evidenced by their superior binding capabilities with mammalian cells. As expected, the intensities of PCR bands became stronger with increased sample volume (from 50 to 200 μl); for consistency, a volume of 200 μl is recommended for DNA extraction.

The quality of extracted urine DNA was assessed by PCR amplification of four fragments of different sizes derived from different genes (Table 1). Specifically, PCR was performed on *K-ras*, *GAPDH*,

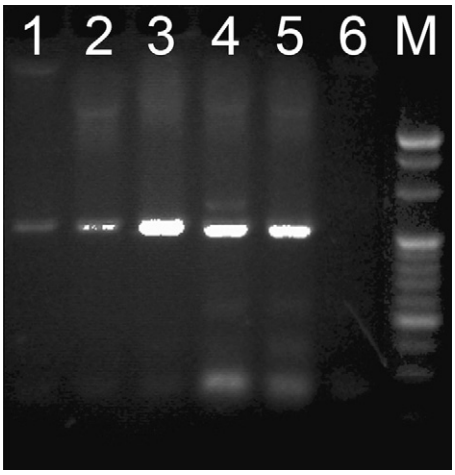

Fig. 4. Agarose gel electrophoresis of PCR products obtained after amplification of genomic DNA isolated from different volumes of urine sample. Lanes 1–3: DNA extracted using CMNPs using 50, 100 and 200 μl of urine sample, respectively; lane 4: DNA extracted from urine sample using the traditional phenol/chloroform extraction method; lane 5: positive control with DNA extracted from blood; lane 6: negative control without template DNA; lane M: 20 bp DNA ladder marker (Takara Bio, Shiga, Japan).

CYP3A4 and *GDF5* genes that require 0.21, 0.55, 0.59 and 1.1 kb DNA fragments, respectively, with results for the PCR products shown in Fig. 5. The PCR reactions were 100% successful for both urine DNA and control blood DNA, verifying the high DNA quality extracted by our method. One of the major advantages of the present magnetic nanoparticle-based method is its ability to isolate DNA from sediment-containing urine samples. When the supernatant of the sediment-containing urine sample was carefully removed for DNA extraction and PCR analysis, no bands were detected, indicating that most of the nucleated cells and DNA fragments were precipitated along with other urine components during freezing. Conversely, PCR analysis revealed no notable differences between DNA extracted from fresh (lanes 1, 4, 7 and 10, Fig. 5) and frozen urine samples when the sediments were re-dissolved as described (lanes 2, 5, 8 and 11, Fig. 5).

The four sets of primers also show successful amplification of DNA taken from a 600 μl male urine sample (lanes 1, 3, 5 and 7, Fig. 6), however, the band intensity observed on agarose was weaker than that produced by a 200 μl female urine sample. The gender differences in PCR sensitivity between male and female urine is due to female urine samples containing considerably more epithelial cells than that of males, thus providing more PCR templates [9,25]. The successful amplification of the human genomic DNA fragments from DNA isolated from either frozen or fresh

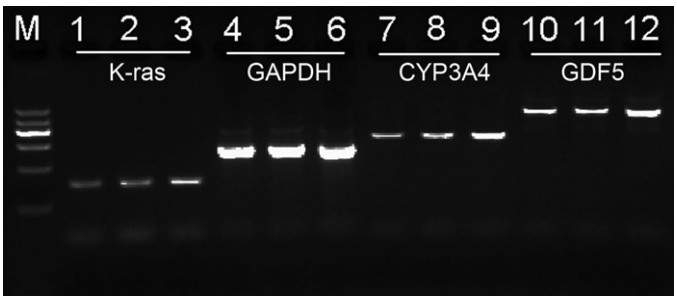

Fig. 5. Agarose gel electrophoresis of PCR products generated from urine genomic DNA. Lanes 1, 4, 7 and 10: DNA isolated from a fresh urine sample; lanes 2, 5, 8 and 11: DNA isolated from a frozen-thawed urine sample; lanes 3, 6, 9 and 12: DNA extracted from whole blood; lane M: DNA marker II (Tiangen Biotech, Beijing, China).

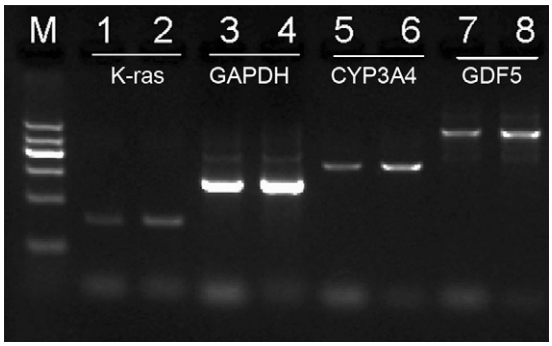

Fig. 6. Agarose gel electrophoresis of PCR products generated from urine genomic DNA. Lanes 1, 3, 5 and 7: DNA isolated from a frozen-thawed male urine sample; lanes 2, 4, 6 and 8: DNA isolated from a frozen-thawed female urine sample; lane M: DNA marker II (Tiangen Biotech, Beijing, China).

urine samples, regardless of whether it was obtained from male or female donors, indicates that the proposed DNA extraction method is widely applicable for a variety of urine samples, as has been confirmed by our repeated experiments.

As the recovery of cells and the purification of DNA were all accomplished with rapid magnetic separation rather than by centrifugal processes, the present procedure required less handling and could be carried out in a single microcentrifuge tube. Further, the methodology described requires no hazardous reagents (such as phenol and chloroform) during the entire process. Finally, the entire CMNP extraction process required less than 30 min, a significant time savings over the traditional phenol/chloroform method, which needed at least several hours to complete [9,10].

4. Conclusion

Carboxylated magnetic nanoparticles were used to develop a novel method for PCR-ready genomic DNA extraction from urine samples. CMNPs were used to both extract cells from urine samples, and adsorb genomic DNA from the lysates. Sedimentation associated with the freezing of urine samples could be re-dissolved with the addition of EDTA and a subsequent pH adjustment, allowing cells to be re-suspended for highly efficient binding by magnetic nanoparticles. The quality of extracted urine DNA was confirmed by PCR amplification of different gene fragments of different sizes. Relative to traditional methods, the present procedure required less handling, no hazardous reagents, and could be carried out in a single microcentrifuge tube within 30 min. These methods, while simple, rapid, sensitive and environmentally friendly, are suitable for routine laboratory use, but also hold promise for construction of automated urine extraction systems for various diagnostic purposes.

Acknowledgments

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