การเปรียบเทียบวิธีการสกัดสารพันธุกรรมมนุษย์จากตัวอย่างปัสสาวะด้วย Wizard® SV Genomic Purification Kit และ Chelex® extraction สำหรับการระบุตัวบุคคลจากสารพันธุกรรม

Protocol comparison for human DNA extraction from urine sample using Wizard® SV Genomic Purification Kit and Chelex® extraction methods for DNA profiling

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บทคัดย่อ

สารพันธุกรรมมนุษย์สามารถนำมาใช้ประโยชน์ทั้งทางด้านการระบุตัวบุคคลและการตรวจวินิจฉัยโรคปัจจุบันมีชุดสกัดจากหลายบริษัทแต่ส่วนใหญ่มักมีสารเคมีหลายชนิดและการสกัดมีหลายขั้นตอนการสกัดสารพันธุกรรมด้วย Chelex® เป็นวิธีการสกัดที่ทำได้ง่าย ใช้สารเคมีในการสกัดเพียงสามชนิดและมีขั้นตอนไม่ยุ่งยากการศึกษานี้มีจุดมุ่งหมายเพื่อเปรียบเทียบปริมาณสารพันธุกรรมซึ่งสกัดด้วย Chelex® เรซิน ที่ใช้หลักการ ion exchange กับชุดสกัดที่ใช้หลักการของ solid-phase extractionโดยตัวอย่างปัสสาวะของอาสาสมัครชายจะถูกสกัดและวัดปริมาณสารพันธุกรรมด้วย Qubit™ 3.0 Fluorometer ที่วัดปริมาณ DNA จากปริมาณแสงฟลูออเรสเซ็นที่ปล่อยออกมาหลังจาก DNA จับกับสีฟลูออเรสเซ็นที่จำเพาะกับ DNA ก่อนที่จะไปทำPCRผลการทดลองพบว่าจากการสกัด 3 ครั้ง ค่าเฉลี่ยความเข้มข้นของ DNA ในตัวอย่างปัสสาวะ 1-5 ml ที่สกัดโดยใช้ Chelex® มีค่าเท่ากับ 63.5 –470.5 นาโนกรัม ในขณะที่ค่าเฉลี่ยความเข้มข้นของ DNA ในตัวอย่างปัสสาวะที่สกัดโดยชุดสกัด Wizard®SV Genomic purification มีค่าเท่ากับ 7.7 – 36.4 นาโนกรัม เมื่อนำสารพันธุกรรมที่สกัดได้มาทำ PCR พบว่าปริมาณปัสสาวะตั้งแต่ 4 มิลลิลิตรขึ้นไปที่สกัดด้วย Chelex® สามารถให้ผล PCR จาก primer ซึ่งเป็นหนึ่งในตำแหน่ง STR ที่ใช้ในการตรวจพิสูจน์DNA ในทางนิติวิทยาศาสตร์ได้ ในขณะที่การสกัดจากชุดสกัด Wizard® SV Genomic purification kitไม่สามรถให้ปริมาณที่เพียงพอในการทำ PCR ได้การศึกษานี้ชี้ให้เห็นว่าการสกัดโดยใช้เรซิน Chelex®เป็นวิธีที่คุ้มค่าและมีประสิทธิภาพในการสกัดสารพันธุกรรมมนุษย์จากตัวอย่างปัสสาวะ ผลที่ได้จากการศึกษานี้ทำให้สามารถประมาณปริมาตรของตัวอย่างปัสสาวะที่จะเก็บเพื่อนำมาวิเคราะห์สารพันธุกรรมในการระบุตัวบุคคลด้วยวิธี STR และยังสามารถบอกวิธีที่เหมาะสมในการสกัดสารพันธุกรรมมนุษย์จากตัวอย่างปัสสาวะหนึ่งในการประยุกต์ใช้ DNA profile คือนำไปใช้ยืนยันตัวบุคคลที่เป็นเจ้าของตัวอย่างปัสสาวะในการตรวจสารกระตุ้นในนักกีฬา

คำสำคัญ: ปัสสาวะ, การสกัดสารพันธุกรรม, ปริมาณสารพันธุกรรม, วิธี Chelex®, ชุดสกัดสารพันธุกรรม, solid-phase extraction และ ion exchange principle

Abstract

Human DNA can be used for individual identification and diagnostic purposes. Several commercial kits are available for DNA extraction, but many steps and reagents are required. Chelex® method is a simple DNA extraction method that requires only a few reagents and steps. This study aimed to compare the DNA yield obtained from Chelex® extraction method, which is based on ion exchange principle, with those from Wizard® SV Genomic purification kit, which is a commercial DNA extraction kit based on solid-phase extraction principle. One to five milliliters of urine samples obtained from a male volunteer were extracted using either Chelex® resin or Wizard® SV Genomic purification kit. Qubit™ 3.0 Fluorometer was used to quantify the DNA concentration based on the amount of fluorescent signal emitted when DNA specific fluorescent dye binds to DNA molecule. DNA profiling was performed after DNA quantification step. From three replicates, the results showed that, for Chelex® extraction, the average of total DNA concentration from 1-5 ml urine was 63.5 – 470.5 ng in total volume of 500 µl extract, while total DNA concentration extracted by Wizard® SV Genomic purification kit gave 7.7 – 36.4 ng in total volume of 70 µl. High yield of DNA could be obtained in 45 min for Chelex® extraction method while the DNA yield from Wizard® SV Genomic purification kit showed lower DNA concentration and the whole process takes approximately one hour.DNA extracted from Chelex® method can be amplified by PCR using THO1 primer, which is one of STR primer used for forensic DNA identification. Results also showed that DNA profiling can be performed from 4 ml of fresh urine sample extracted by Chelex® method while extracted DNA from Wizard® SV Genomic purification kit did not yield enough DNA to perform DNA analysis. From this study, Chelex® method is a more cost-effective and suitable for extracting human DNA from fresh urine sample. These results can be used to estimate the amount of urine collection for DNA analysis and suggest the suitable extraction method for urine sample. An example of the application for DNA profiling from urine is to prove the ownership of the urine sample for the athlete in case of doping agent test is in doubt.

Keywords: Urine, DNA extraction, DNA concentration, Chelex®, solid-phase extraction, ion exchange principle

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1. Introduction

Urine is a product from excretory system of human. The main objective of urination is to filter wastes from blood through nephrons of the kidney. Several chemicals are extracted with urine, including water, urea, sodium, chloride, sulfate, potassium, phosphate, creatinine, ammonia, uric acid, calcium and magnesium in different proportions (Chemical composition of urine, 2016). Urine samples can be used for different

purposes, such as drug and narcotic test and diagnostic, including pregnancy testing. Apart from these chemicals, urine also contains epithelial cells and white blood cells which are the source of DNA. Although everyone excrete the urine with some amount of DNA from epithelial cells, studies found that, urine from female contains higher DNA concentration (Bali et al., 2014 and Shan et al., 2012)(25.0-96.9 ng/ml) compared to male's urine (0.02-21.3 ng/ml) (Johnson et al., 2007). DNA extraction from urine sample has previously been studied for several purposes, such as for disease detection. The urine sample can be extracted and amplified with cancer-related primers (K-ras) in order to predict the chance of having cancer in the patient (Arcila et al., 2011). An example of another purpose is to determine the authenticity of athlete's urine in doping control using DNA analysis. In this case, the urine is used for detecting illegal substances including narcotics and doping agents. DNA profile can be used as evidence to prove the ownership of the urine sample (Devess et al., 2015).

Many DNA extraction methods have been developed and sold commercially, including those based on solid-phase extraction principle and ion exchange principle (Cortes and Griffiths, 2014). For Wizard® SV Genomic purification kit (Promega Biotechnology company, United states), which is a solid-phase extraction method, DNA and other components from sample is passed through a column and trapped inside. After this step, every component except DNA is then washed out by a washing solution that contain high concentration of chaotropic salt which has efficiency to remove the contaminant (Promega DNA purification, 2017). DNA which remains in the column is then eluted out after the washing step. The disadvantages of this technique are that the cost of extraction per sample is quite high (100 Baht per reaction) and there is the need to transfer the sample to different tubes during extraction, and therefore there is a higher chance of contamination occurring. Chelex®(Bio-Rad Laboratories, United States) extraction method is based on ion exchange principle. Chelex® resin has the ability to bind with cations such as Mg^{2+}, which is a cofactor of nuclease that can degrade DNA. Once Chelex® binds with those ions, nuclease enzyme will not function well without the cofactor thus the DNA will less likely to be destroyed. Boiling step is performed to destroy protein and also cause the denaturation of DNA (Elkins, 2013). After spinning the sample tube, proteins and cellular debris accumulate at the bottom of the tube while DNA is in the aqueous phase, which can then be transferred to a new tube. Chelex® method also has the disadvantage due to the resin itself is a PCR inhibitor, and therefore care must be taken to transfer DNA to a new tube to avoid resin carry over. The DNA extracted from this method is single stranded.

This study aimed to compare the quality and quantity of DNA obtaining from Chelex® extraction method which is based on ion exchange principle, and Wizard® SV Genomic purification kit based on solid phase extraction method, from urine samples. The quantity of DNA obtained was measured using fluorescence spectrometry, which employ the use of DNA-specific dye. The DNA was then amplified with a short tandem repeat (STR) primer to assess its quality.

2. Materials and methods

2.1 Sample preparation

2.1.1 Urine collection

Fifty milliliters of urine from male volunteer were collected into sterile 50 ml tube then put on ice immediately until use.

2.1.2 Cell collection

One milliliter of each sample was aliquot into the 1.5 ml tubes and centrifuged at 4,000 g for 30 min to allow the pellet to accumulate at the bottom of the tube, and the liquid was discarded. The number of times of sample adding and centrifugation for urine sample was performed depending on the volume of the urine sample in use, e.g. 2 times for 2 ml, and 3 times for 3 ml.

2.2 DNA extraction from fresh urine using Wizard® SV Genomic purification kit (Promega Biotechnology company, Medison, Wisconsin, United states)

After cell collection step, 400 µl of lysis buffer was added followed by 20µl of 20mg/ml of proteinase K and gently mix. The samples were incubated at 56°C for 30 min. The supernatant was transferred to the spin column assembly and centrifuged at 13,000 g for 1 min then the liquid was discarded. Six hundred and fifty microliters of washing solution were added into a column and centrifuged at 13,000 g for 1 min, and the liquid was discarded. This washing step was performed 3 times, and finally, the column was centrifuged at 13,000 g for 2 min to dry the membrane. The spin basket was transferred to a new tube and the DNA was eluted with 70 µl of warm TE, incubated at room temperature for 2 min, and centrifuged at 13,000 g for 1 min for 2 times (50 µl for the first elution and 20 µl for the second elution), then the spin basket was discarded, and stored the DNA at -20°C until use.

2.3 DNA extraction from fresh urine using Chelex®(Bio-Rad Laboratories, Hercules, California, United States)

Twenty microliters of 20mg/ml proteinase K and 500 µl of PBS (pH 7.4) were added into the pellet from cell collection step. The samples were incubated at 56°C for 30 min. One hundred and fifty microliters of 20% Chelex® suspension were added to the sample tubes. The sample was centrifuged at 13,000 g for 2 min and boiled for 10 min. The sample tubes were centrifuged again after boiling step for 2 min at 13,000 g. All supernatant was transferred to a new tube, and stored at -20°C until use.

2.4 DNA quantification by Qubit™ 3.0 Fluorometer

Quantification by Qubit™ required a mixture of Qubit reagent and Qubit buffer in a 1:200 ratio. The total volume of each sample-reagent mixture before measuring is 200 µl. The standards (std.1 and std.2) provided by the kit were measured first to calibrate the instrument. The measuring method was performed according to the manufacturer's protocol (Qubit®ds DNA HS Assay Kits For use with the Qubit®Fluorometer (all models), 2015). Then 2 - 10 µl of the samples were mixed with the reagent mixture to final volume of 200

µl. The tube was then inserted to the instrument and the DNA quantity was measured. Six nanograms was used in the PCR.

2.5 DNA amplification using THO1 primer pair

Amplification of THO1 was performed. THO1 primers were used as the representative for STR locus to estimate the chance of obtaining STR profile from the sample DNA. The components of PCR consist of 1xPCR buffer, 1.5m $MMgCl_2$, 0.2 mM dNTPs, 10 pmole of forward and reverse primer (THO1) and 1U of Taq DNA polymerase. Six nanograms of DNA from each sample were used as DNA template. Sterile distilled water was added so that the final volume of reaction was 25µl. Six nanograms of DNA from buccal cells extracted using Wizard® SV Genomic purification kit were used as positive control. Sterile distilled water was added instead of DNA template for negative control. The PCR reaction used the following thermal conditions: Initial denaturation at 95°C for 5 min, 34 cycles of denaturation 95°C for 30 sec, annealing 60°C for 30sec, extension 72°C for 1 min and final extension 72°C for 7 min. The PCR products were kept at -20°C to prevent DNA degradation. The primer sequences used in this study were obtained from Polymeropoulos M. H.,(1991).
Primer Sequences: Forward primer 5'–GCTTCCGAGTGCAGGTCACA-3'

Reverse primer5'-CAGCTGCCCTAGTCAGCAC -3'

2.6 PCR product detection using gel electrophoresis

One point five percent agarose/TBE gel with pre added ethidium bromide was used to separate the DNA fragment. Eight microliters of PCR product was mixed with 2 microliters of 10x bromophenol blue. Ten microliters of the mixture were loaded and DNA fragments were separated using 70 V for 90 min. The gel was visualized using SYNGENE G:BOX HR, gel documentation system. The expected product size from THO1 primer pair is about 160 – 200 bp.

2.7 PCR product detection using CE

Two microliters of PCR product was mixed with 9.5 microliters of Hi-di formamide and 0.5 microliters of internal size standard (500 LIZ™ dye size standard). The mixture was heated at 95°C for 5 min and put on ice immediately. The mixture was kept on ice at least 2 min before detection. The product was separated by capillary electrophoresis using ABI Prism® 310 Genetic Analyzer, (Applied Biosystems, Foster city, CA). GeneMapper Software (AppliedBiosystems, Foster city, CA) was used to analyze the data. An electropherogram for 1 locus was obtained. The analytical threshold of peak height for allele calling was set at 50 RFU (Butler, 2009). PCR product size and the peak height of each sample were observed and compared with the positive control. In this study, the male volunteer is homozygous for THO1 locus, thus there was only one peak expected.

3. Results and discussions

3.1 DNA concentration after extracted by Chelex® method Vs Wizard® SV Genomic purification kit

The concentration of DNA extracted from one to five milliliters of fresh urine by Chelex® resulted in 0.127–0.941 ng/µl of DNA concentration. The concentration of DNA extracted from one to five milliliters of fresh urine by Wizard® SV Genomic purification kit resulted in 0.110 – 0.520 ng/µl. (Fig. 1) The DNA extracted from Wizard® SV Genomic purification kit showed lower DNA concentration than those obtained from the Chelex® method(Fig. 1).In order to get an accurate estimation of DNA concentration, a method which employs fluorescent dye to specifically measure the amount of DNA in sample called Qubit™ 3.0 Fluorometer was used in this study (Qubit®ds DNA HS Assay Kits For use with the Qubit®Fluorometer (all models), 2015). The specificity of DNA quantification must be concerned. The advantages of quantifying by Qubit™ 3.0 Fluorometer are that the sensitivity is higher than UV- absorbance quantification. The effective range covers a sample concentration range of 10 pg/µl to 1 µg/µl of DNA for Qubit™ 3.0 Fluorometer, whereas the effective range for UV- absorbance quantification is 2 ng/µl to 15 µg/µl (Qubit Fluorometer versus competitors, 2017). Although quantification by Qubit™ 3.0Fluorometer is suitable for the sample with the low amount of DNA, but it cannot indicate the presence of contamination and the purity of DNA (Qubit Fluorometer versus competitors, 2017).

3.2 THO1 amplification and capillary electrophoresis

PCR was performed using THO1 primer pair, which is a short tandem repeat (STR) locus used in human DNA identification (Butler J. M., 2009). THO1 primer pair was used to estimate the chance of obtaining STR profile from the sample DNA. For Chelex® method, PCR product from 4 and 5 milliliters of urine could be detected, while the 1-3 ml samples could not. This was due to the fact that DNA concentration after extraction of 1-3 ml samples were very low compared to the 4 and 5 ml samples. The intensity of DNA band and peak height of the PCR products appeared and began to increase when 4 ml or more urine were used (1,805 and 3,982 RFU) Respectively due to the increase in concentration of DNA as higher amount of urine was used in extraction. (Table1and Fig. 2). DNA product size after separated by CE was approximately 164 bp which was in accordance with the results from gel electrophoresis (Fig. 2). For Wizard® SV Genomic purification kit, the results showed that no amount of urine sample yielded enough PCR products to be detected by both gel electrophoresis and capillary electrophoresis (Fig. 2 & Table 1). The whole process of DNA extraction by Wizard® SV Genomic purification kit took more than one hour while Chelex® method took 45 min. Another disadvantage of this type of commercial kit is that this method required multiple tubes transfer and it could be the source of contamination and error. Moreover, the cost of this commercial kit is more expensive than the Chelex® method. One reaction of the commercial kit costs about 100 Baht while one reaction of Chelex® is only 30 Baht. Therefore, Chelex® method is more a cost-effective comparing to Wizard® SV Genomic purification kit.

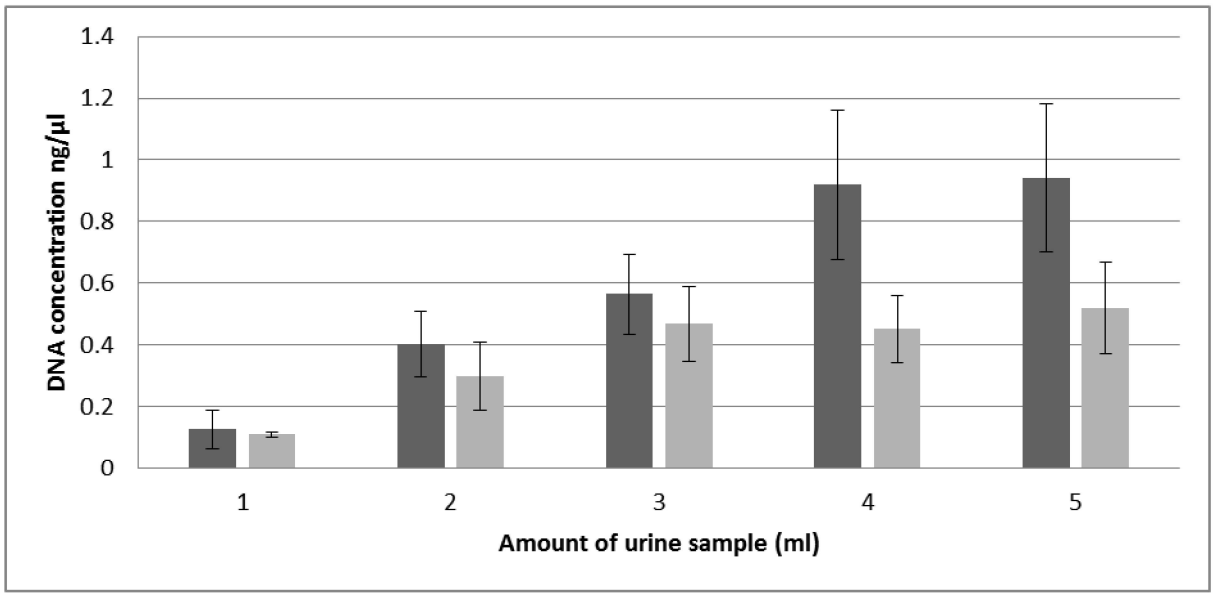

Fig 1.The quantity of urine sample Vs average DNA concentration obtained from Chelex® methodand Wizard®SV Genomic purification kit

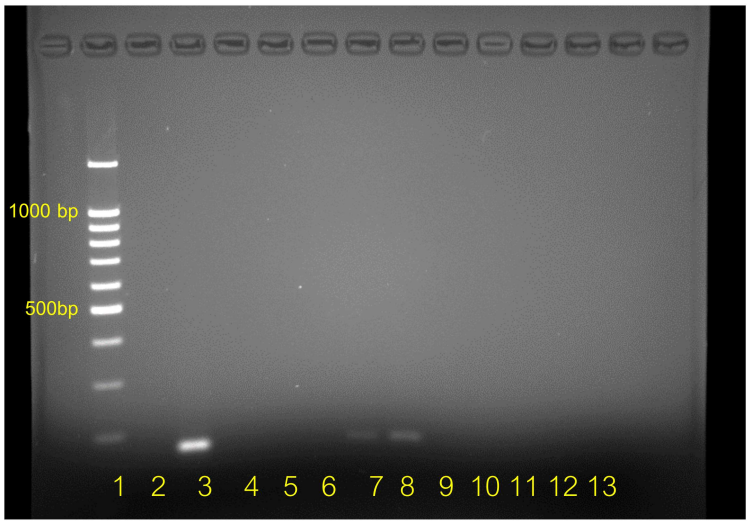

Fig.2 PCR products of extracted DNA from urine sample using Chelex® resin and Wizard® SV Genomic purification kit after separated by gel electrophoresis (1 = 100 bp DNA ladder, 2 = Negative control, 3 = Positive control, 4 = 1ml from Chelex® method, 5 = 2 ml from Chelex® method, 6 = 3 ml from Chelex® method, 7 = 4 ml from Chelex® method, 8 = 5 ml from Chelex® method, 9 = 1 ml from Wizard® kit, 10 = 2 ml from Wizard® kit, 11 = 3 ml from Wizard® kit, 12 = 4 ml from Wizard® kit, 13 = 5 ml from Wizard® kit)

Table 1. Peak heights separating PCR product by Capillary electrophoresis (Chelex® method and Wizard® SV Genomic purification kit)

Sample	Extraction method			
	Chelex® method		Wizard® SV Genomic purification kit	
	THO1	Height (RFU)	THO1	Height (RFU)
Negative	-	-	-	-
Positive	✓	6,058	✓	6,058
1 ml	-	-	-	-
2 ml	-	-	-	-
3 ml	-	-	-	-
4 ml	✓	1,805	-	-
5 ml	✓	3,982	-	-

*✓ indicate that there was a peak showed up at THO1 locus

Table2. Comparison between Chelex® and Wizard® SV Genomic purification methods

Extraction method	Advantages	Disadvantages
Chelex®method (Bio-Rad Laboratories, Hercules, California, United States)	- Quick (<1 hr.) - Only three reagents are required - Low cost - No multiple tubes transfer - The higher volume of extracted DNA obtained	- Generate single stranded DNA - Resin is PCR inhibitor
Wizard® SV Genomic purification kit (Promega Biotechnology company, Medison, Wisconsin, United states)	- All reagents is provided in a kit - Obtained doubled stranded DNA after extraction process	- Lower of DNA extract total volume - Expensive - Possible contamination issue due to multiple tubes transfer - Time-consuming - Several reagents and steps are required

Even though Chelex® extraction method generated single-stranded DNA, it can still be used as template in PCR analysis. Based on these results Chelex® resin seemed to be a more cost-effective and robust method of extraction. The results can be applied to establish a protocol for testing ownership of the urine for athletes when there is a dispute.

4. Conclusion

This study aimed to compare two extraction methods, Chelex® resin and Wizard® SV Genomic purification kit, in order to evaluate a suitable DNA extraction method from urine samples. From three replicates, the results showed that Chelex® method yield higher amounts of DNA. Moreover, this method did not take as much time comparing to the method using Wizard® SV Genomic purification kit. The concentration

of DNA extracted from both methods showed reasonable trend, the concentration increased as the amount of urine sample increased. The results suggested that DNA profiling can be performed from 4 ml of fresh urine sample extracted by Chelex® method, while extracted DNA from Wizard® SV Genomic purification kit required more than 5 ml of fresh urine sample. From this study, Chelex® method is a robust and cost-effective method for extracting human DNA from fresh urine.

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