



ORIGINAL ARTICLE

Rapid DNA Extraction from Dried Blood Spots on Filter Paper: Potential Applications in Biobanking

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Received: August 8,
2014
Revised: September
14, 2014
Accepted: September
29, 2014

KEYWORDS:

biobank,
dried blood spot,
filter paper

Abstract

Objectives: Dried blood spot (DBS) technology is a microsampling alternative to traditional plasma or serum sampling for pharmaco- or toxicokinetic evaluation. DBS technology has been applied to diagnostic screening in drug discovery, nonclinical, and clinical settings. We have developed an improved elution protocol involving boiling of blood spots dried on Whatman filter paper.

Methods: The purpose of this study was to compare the quality, purity, and quantity of DNA isolated from frozen blood samples and DBSs. We optimized a method for extraction and estimation of DNA from blood spots dried on filter paper (3-mm FTA card). A single DBS containing 40 μ L blood was used.

Results: DNA was efficiently extracted in phosphate-buffered saline (PBS) or Tris-EDTA (TE) buffer by incubation at 37°C overnight. DNA was stable in DBSs that were stored at room temperature or frozen. The housekeeping genes GAPDH and beta-actin were used as positive standards for polymerase chain reaction (PCR) validation of general diagnostic screening.

Conclusion: Our simple and convenient DBS storage and extraction methods are suitable for diagnostic screening by using very small volumes of blood collected on filter paper, and can be used in biobanks for blood sample storage.

1. Introduction

Biological samples collected in biobanks constitute a resource with significant research potential, but may also be subject to various ethical and deontological conflicts. Moreover, many samples now stored in biobanks were collected many years earlier, under nonstandardized conditions and in contexts where awareness of the need

to meticulously handle information and obtain consent was not yet widespread. The problems this presents for the use of samples has been noted worldwide and has been addressed in different ways [1].

In 2010, the Italian Joint Group CNB-CNBBSV (National Committee of Bioethics–National Committee for Biosecurity Biotechnologies and Life Sciences) published recommendations for the storage and use of

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dried blood spots (DBSs), and the development of a national network of regional newborn screening repositories for the collection of residual DBSs [2].

Collection of blood samples on filter paper was first performed >50 years earlier [1]. This simple collection and storage method has broadly been adopted in diagnostic screening [3], drug monitoring [4], and genetic analysis [5], and is particularly suitable for molecular epidemiologic studies in remote tropical areas, where transport and storage conditions are often suboptimal.

The DBSs used in this study were obtained by finger or heel pricks, and were spotted onto filter paper. The analysis of human blood dates back to 1963, when Dr Robert Guthrie used DBS specimens for phenylalanine analysis to identify newborns with phenylketonuria. This novel approach to blood collection facilitated population screening of newborns and other clinical testing applications [6].

DBSs can easily be collected by a heel or finger prick without the need for venipuncture; they can be stored at room temperature and easily transported to the testing laboratory with little biohazard risk. The use of stored DBS samples combined with clinical information from medical registries constitutes an ideal resource for large population studies of patients under a given age without selection bias. In addition, DBSs offer several advantages in areas that lack appropriate infrastructure for processing blood and a cold chain for the transportation and storage of blood products [7].

Generally, the use of neonatal DBSs is based on the removal of blood spots using punches, typically 3–6 mm in diameter, followed by physical or biochemical analysis of serum analytes released from the punched-out material by soaking it in alcohol or water [8]. More recently, DBSs have been used to screen for heritable traits at the DNA level; typically, they have been used for traits such as cystic fibrosis, thalassemia, and other traits that are readily assessed by polymerase chain reaction (PCR).

The use of DBSs has expanded from their modest beginning in the diagnostic arena for neonatal screening [3] to drug development, including discovery research [9,10], nonclinical and clinical toxico- and pharmacokinetic evaluations, and therapeutic drug monitoring [11–13]. This is due to the multifold advantages of DBS with respect to scientific, practical, ethical, and cost-effective aspects in nonclinical and clinical settings [14–17].

DBSs represent a valuable and comprehensive genetic repository because genomic DNA can be isolated from blood samples processed on these filter papers for use in PCR [18]. In addition, direct PCR amplification from DBSs that have been punched out has been reported [19].

We analyzed whole blood and buffy coats in comparison with DBSs, in terms of storage temperature, buffer stability, and DNA yield. We have presented a storage and extraction method for DBSs on filter paper,

with PCR verification. We have developed a reliable system for good blood storage practice, blood sample preparation and processing, conditions, and acceptance criteria for DBS applications.

2. Materials and methods

2.1. Collection and storage of blood specimens

Blood samples were collected in EDTA tubes from unaffected individuals tested at the Eulji University Hospital, Daejeon, Korea. All blood samples were obtained from young adults (20–40 years) as whole blood in EDTA and as DBS samples.

DBS samples (40 μ L each) were spotted on Whatman FTA cards (Whatman Inc., Brentford, UK) and were dried for 24 hours at room temperature. They were stored at room temperature or at -80°C until analysis, and then kept in separate clean zipper bags for 2, 4 weeks and 3 months. Liquid, EDTA-treated blood samples were also stored at 80°C for 2, 4 weeks and 3 months.

2.2. DNA extraction from DBSs

For single-stranded DNA (ssDNA) extraction, DBSs on the cards were cut into small pieces with scissors and transferred into 1.5-mL microtubes to which 500 μ L dH_2O had been added; the tubes were kept at room temperature for 5 minutes. The dH_2O was discarded, and the procedure was repeated twice. The filter papers were soaked in 500 μ L extraction buffer [phosphate-buffered saline (PBS) or 10 mM Tris-EDTA (TE)].

For lysis, red blood cell lysis buffer (100 μ L) was added to the filter papers, which were then incubated with 10 μ L (1 mg/mL) proteinase K at 37°C overnight. After blood cell lysis, the filter papers were pressed against the bottom of the tube several times with a clean pipette tip [20]. For increased extraction efficiency, cell lysates were incubated at 95°C for 15 minutes. Filter papers were removed after brief centrifugation (2–3 seconds), and the DNA-containing supernatant was stored at -80°C until analysis.

For double-stranded DNA (dsDNA) extraction, each filter paper sample that had been punched out was soaked in 40 μ L Tris-EDTA buffer or PBS, and incubated at 37°C overnight. Genomic DNA was isolated from blood using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, USA) as per the manufacturer's instructions.

2.3. DNA concentration

DNA concentrations were determined by measuring absorbance at 260 nm (A_{260}), and assuming that an A_{260} of 1.0 is equal to 50 ng/ μ L pure dsDNA or 35 ng/ μ L pure ssDNA. Concentrations of extracted DNA were measured using a SmartSpec plus spectrophotometer (Bio-Rad, Hercules, CA, USA).

2.4. Polymerase chain reaction

The levels of the GAPDH and β -actin transcripts were used to verify the quality of the extracted DNA. For GAPDH PCR, the PCR reaction mixture contained 3 μ L extracted DNA, 10 pmol of each primer, and Bioneer PCR premix in a final volume of 20 μ L. Thermal cycling was conducted in a CFX96 Touch system (Bio-Rad), with the following PCR program for GAPDH: initial denaturation of 8 minutes at 94°C; followed by 40 cycles of 94°C for 1 minute, 65°C for 1 minute, and 72°C for 1 minute; concluding with 7 minutes at 72°C and 1 minute at 4°C. A similar PCR program was used for β -actin PCR, except that the annealing temperature was 64°C. The primer sequences for GAPDH and β -actin are shown in Table 1 [21].

The PCR products were analyzed by electrophoresis on 2% RedSafe (Koma)-stained agarose gels in Tris-acetate-EDTA (TAE) buffer. After electrophoresis, the digital images were analyzed with a ChemiDoc MP imaging system (Bio-Rad).

3. Results

3.1. Quantitation of DNA from filter paper and frozen-liquid samples

The recovery ratio of dsDNA was measured as described in the “Materials and Methods” section. The dsDNA extracted from DBSs and liquid samples was stored at room temperature or -80°C for 3 months (Figure 1).

As shown in Figure 2, the recovery ratios of frozen-liquid and filter paper samples were compared. The dsDNA was reduced by approximately 68% in the whole blood and 40% in the buffy coat. The amount of DNA extracted from the frozen whole blood was approximately 39% higher than that extracted from the frozen buffy coat. The amount of DNA extracted from whole blood kept on filter paper at room temperature was approximately 11% higher than the yield from the buffy coat. The stability of DNA in samples collected on filter paper was higher than that for frozen-liquid samples. Furthermore, in a comparison of samples stored in the same manner, the quantity of DNA extracted from whole-blood samples was approximately 39% higher than the yield from buffy-coat samples stored frozen and approximately 11% higher than that from samples stored on filter paper at room temperature.

The ssDNA yields were measured following the procedure described in Figure 1. We determined that two storage methods were significantly higher extraction method of ssDNA quantity. The amount of ssDNA extracted from samples stored for 2 weeks was similar to that from samples stored for 4 weeks (Figure 3).

The amount of DNA extracted from DBSs was >62% and 60% of that obtained from frozen-liquid samples (whole blood and buffy coats, respectively). The amounts of DNA extracted from frozen-liquid samples and filter paper samples at 2 weeks did not differ much from those extracted at 4 weeks; however, the yield from frozen-liquid samples was approximately 62% lower than that from filter paper samples after 2 weeks and approximately 60% lower after 4 weeks. The stability and yield of frozen-liquid samples were associated with storage temperature.

DNA from DBSs stored at room temperature was of lower purity than that obtained from frozen-liquid samples because of impurities in the paper; however, the improved yield of the extracted ssDNA suggested greater stability than that obtained in the case of frozen-liquid samples.

3.2. Quantitation of ssDNA from DBS

To measure the DNA yields from samples stored under different conditions, samples were separated into whole-blood and buffy-coat samples, spotted on filter papers, and stored at room temperature; subsequently, the extracted ssDNA yields were compared (Figure 4). The amount of ssDNA extracted from buffy-coat samples was approximately 65% higher than that extracted from whole-blood samples after 2 weeks and approximately 44% higher after 4 weeks. Moreover, the purity of ssDNA extracted from buffy-coat samples was higher than that extracted from whole-blood samples (data not shown).

Buffy-coat sample yields were approximately three times higher than whole-blood sample yields after 2 weeks and approximately 2.4 times higher after 4 weeks; however, the difference decreased as the storage period increased. In the case of whole blood, there was little difference in ssDNA yield over time. However, the extraction yield from buffy coats decreased by approximately 20% after 2–4 weeks.

3.3. Yield of ssDNA from DBS at different storage temperatures and times

As the filter paper storage method provides higher ssDNA yields than the frozen-liquid method at room temperature, we investigated how the storage

Table 1. PCR primers (synthesized by GeneWorks, Adelaide, SA, Australia).

Gene	Primers (5'–3')		Product size (bp)
	Forward	Reverse	
<i>GAPDH</i>	TGA AGG TCG GAG TCA ACG GAT TTG GT	CAT GTG GGC CAT GAG GTC CAC CAC	983
<i>β-actin</i>	GCA CCA CAC CTT CTA CAA TG	TGC TTG CTG ATC CAC ATC TG	838

PCR = polymerase chain reaction.

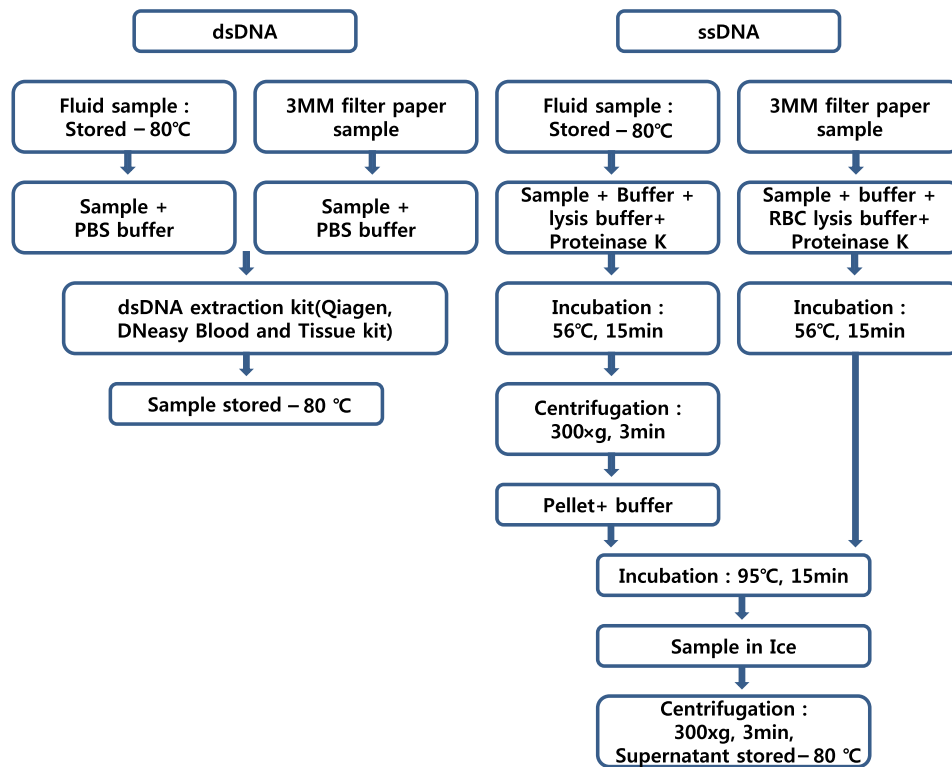


Figure 1. Workflow diagram. dsDNA = double-stranded DNA; RBC = red blood cell; ssDNA = single-stranded DNA.

temperature affected yields. As shown in Figure 5, ssDNA yields were compared between filter paper storage temperatures and times; there was little difference between the amounts of ssDNA extracted. However, there was a difference of 8% in the ssDNA yields between 2- and 4-week storage, while there was a 1% difference in ssDNA yields from samples stored at room temperature for the same period. Therefore, storage at room temperature was best for samples on filter paper.

3.4. Quantitation of ssDNA extracted from DBSs with different buffers

Extraction yields with PBS and TE buffers were compared (Figure 6). Neither buffer produced much difference in yields for different sample storage times; however, the ssDNA yield in TE buffer was approximately 5% higher than that in PBS buffer. Therefore, TE buffer was more suitable than PBS for optimal ssDNA yield.

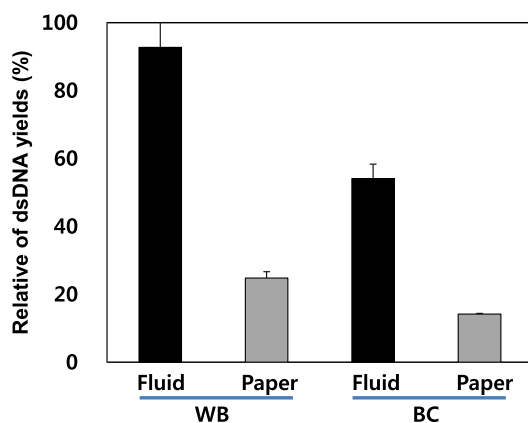


Figure 2. DNA recovery from whole blood and Whatman FTA cards. BC = buffy coat in dried blood spots stored at room temperature; dsDNA = double-stranded DNA; WB = whole blood in dried blood spots stored at room temperature.

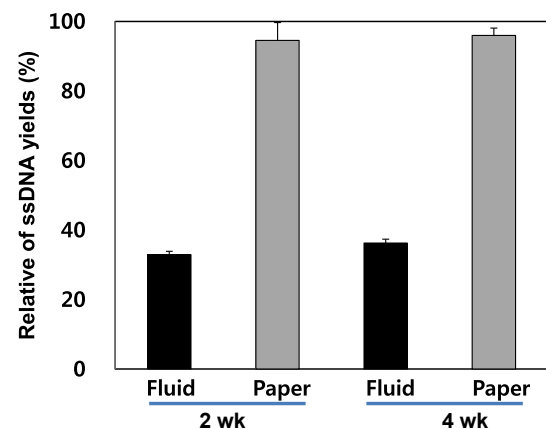


Figure 3. Average DNA yield over time. In the figure, fluid represents whole blood stored at -80°C and paper represents Whatman FTA cards stored at room temperature. ssDNA = single-stranded DNA.

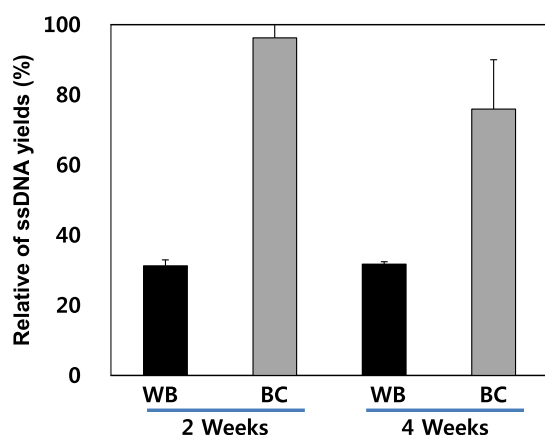


Figure 4. DNA extracted by boiling dried blood spots on filter paper after incubation at 56°C. BC = buffy coat in dried blood spots stored at room temperature; ssDNA = single-stranded DNA; WB = whole blood in dried blood spots stored at room temperature.

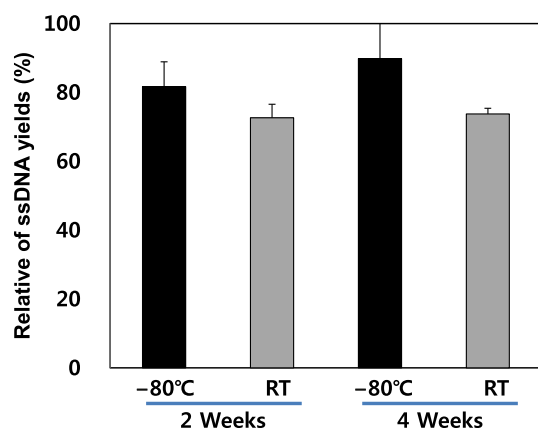


Figure 5. Yield of ssDNA from dried blood spots stored at -80°C and room temperature. ssDNA = single-stranded DNA.

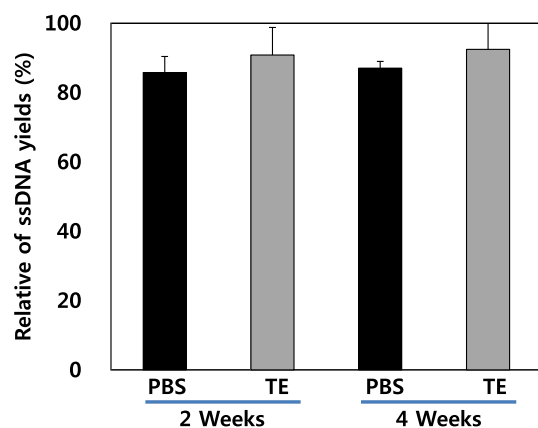


Figure 6. Efficiency of ssDNA extraction in PBS and TE. PBS = phosphate-buffered saline; ssDNA = single-stranded DNA.

3.5. Validation by PCR

To examine whether the extracted ssDNA could be used for PCR in medical and scientific research, we amplified GAPDH and β -actin from dsDNA or ssDNA extracted from frozen-liquid and filter paper samples.

Nonquantitative PCR amplification of GAPDH and β -actin DNA was performed with (1) ssDNA from frozen-liquid samples, (2) dsDNA from filter paper, and (3) ssDNA from filter paper (Figure 7). The results showed that the DNA obtained from DBS extraction was of sufficient quality to be used for PCR analysis and that the yield was better than that obtained from frozen samples.

4. Discussion

Compared with conventional plasma sampling, DBS requires much smaller blood volumes and simpler storage and transportation conditions, which makes it a very promising alternative sample-collection procedure. DBS sample preparation requires extraction of dried blood samples into a solution. This is a step unique to DBSs for large-molecule sample analysis and is crucial to the success of the method. For a given analyte, poor extraction recovery or poor correlation between the serum/plasma and DBS samples may indicate that further development of the method is necessary or that the analyte may not be a good candidate for the DBS method [22]. Therefore, sample-extraction conditions and recovery should be evaluated during method development and confirmed during method validation.

DBS sampling is most commonly used in clinical laboratories to assess small-molecule analytes during screening of newborns for metabolic disorders [2].

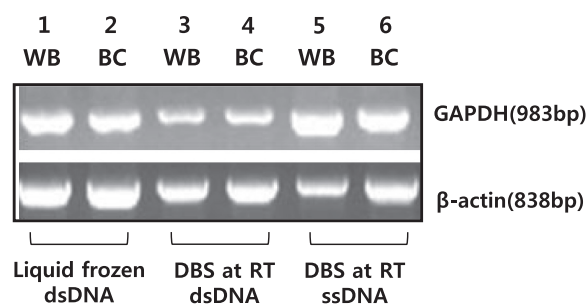


Figure 7. PCR amplification of GAPDH and β -actin DNA. DNA was extracted from frozen-liquid samples and dried blood spots. Lanes 1–2 show dsDNA isolated from frozen whole blood and the buffy coat; lanes 3–4, dsDNA isolated from DBSs, with whole blood stored at room temperature; and lanes 5–6, ssDNA isolated from DBSs, with whole blood and the buffy coat stored at room temperature and extracted with the boiling method. BC = buffy coat in dried blood spots stored at room temperature; DBS = dried blood spot; dsDNA = double-stranded DNA; PCR = polymerase chain reaction; ssDNA = single-stranded DNA; WB = whole blood in dried blood spots stored at room temperature.

Samples are prepared by spotting a small drop of blood directly onto the DBS collection card; only a portion of the DBS is removed for analysis. Ideally, the volume of blood transferred for the assay is defined by the diameter of the punch and, therefore, does not require precise measurement of the volume of blood that is spotted. However, there are several sources of variation unique to bloodspot sampling. For example, proper placement of the whole-blood sample on the filter paper is critical, and errors can be introduced if blood is blotted or smeared rather than drawn onto the filter paper by capillary action [16]. We minimized this source of error by rigorous training of the personnel for the procedure and punching out of the entire blood spot. We focused on increasing the DNA extraction yield from blood specimens and maintaining high structural stability of DNA to retain the most information, with the intent of securing high-quality human specimens for future medical and scientific research. We established a method to obtain high-quality DNA for research by analyzing dsDNA and ssDNA yields after varying the storage method, temperature, and elution buffer, all of which influence DNA extraction.

The importance of DNA recovery from Guthrie cards has been discussed at length in a recent comparative study by Sjöholm and colleagues [24]. They compared a number of commercially available kits and procedures for DNA recovery from Guthrie cards and demonstrated that only approximately 15–25% of the total DNA complement can be recovered. They measured DNA recovery from DBSs stored for up to 26 years and showed that standard 3-mm punches provide DNA yields (with the best available technology) of only approximately 30 ng per punch. However, they demonstrated that, in spite of the relatively low yields, the small amount of DNA obtained remains an excellent substrate for whole genome amplification and relatively complex multiplex single nucleotide polymorphism (SNP) analysis [24].

In the current study, a comparison of frozen-liquid storage and DBSs showed that the quantity of dsDNA extracted using DNA preparation kits was higher after storage as frozen liquid. The mean dsDNA concentration from frozen-liquid samples was 87 ng/ μ L, with a standard deviation of 9.6 ng/ μ L. However, the mean dsDNA concentration obtained from the DBS samples was 23 ng/ μ L, with a standard deviation of 1.7 ng/ μ L.

When economics are considered, the frozen-liquid method is limited by manpower and capital requirements. In this regard, DBSs stored at room temperature provide many economic benefits such as reduced cost and space requirements. However, genome-scale microarray analysis continues to require an input DNA concentration (250 ng) that is approximately 100 times greater than that required for simple PCR; the DNA must be double stranded and must

contain intact molecules approximately five times longer than those required for most PCR reactions.

Therefore, we tried to extract a large amount of DNA by optimization of an ssDNA extraction method for a customized DNA resource that can be used for PCR, which is widely used in DNA research. Extraction was performed after converting dsDNA to ssDNA by boiling the filter paper sample at 95°C, thereby releasing a large amount of DNA into the buffer. The mean ssDNA concentration obtained from the DBS samples was 24.5 μ g/ μ L, with a standard deviation of 0.54 μ g/ μ L.

Similar results were obtained by Mas et al [23] in a study on DBSs stored on treated filter paper matrices such as Whatman FTA or IsoCode, wherein extraction was performed according to the manufacturer's instructions. In that study, approximately 25% recovery was obtained in a single extraction, yielding up to 150 ng of ssDNA in 200 μ L solution, per 40 μ L adult human blood input.

The general recommendation is DBS sample storage at -20°C to prevent DNA degradation [24]. We compared DBS storage at room temperature versus that at -80°C . As shown in Figure 5, the concentration of ssDNA from DBS stored at -80°C was similar to the yield from DBS stored at room temperature, suggesting that our modified extraction method can be used for extraction of ssDNA from DBSs stored at room temperature.

The ssDNA products were compared with dsDNA products for use in common PCR experiments (Figure 7). GAPDH and β -actin, human housekeeping genes, were collinearly expressed in the DBSs and frozen samples. Thus, DBSs may not be used in microarray technology, but may be used to analyze the quantity and quality of ssDNA.

In this study, we analyzed the concentration of DNA extracted from DBS samples and frozen-liquid samples from the same healthy adults using a modified DNA extraction procedure and a commercial kit. The comparison revealed a slight advantage of the frozen-liquid samples over DBS samples with respect to dsDNA quantity, but the concentration of ssDNA was significantly greater in the DBS samples.

The advantages of sampling and storing blood on filter paper for genetic analyses of human pathogens highlight the need for reliable, sensitive, and cost-effective DNA extraction methods. The denaturation-based DNA extraction method described in this report has shown superior results in comparison to standard methods and commercial kits for the extraction of archived blood samples. Our method is rapid, simple, and inexpensive, and confers a reduced risk of cross-contamination due to minimal sample manipulation. Using our DNA extraction method, archival DBS samples can provide sufficient DNA for clinical studies and biobanking.

Conflicts of interest

Authors declare no conflicts of interest.

Acknowledgments

This work was supported by the Bio-Meditech Regional Innovation Center at Eulji University, under the Regional Innovation Center Program (Grant No. 2012-02-05) of the Ministry of Commerce, Industry, and Energy.

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