Amplification of Guthrie Card DNA: Effect of Guanidine Thiocyanate on Binding of Natural Whole Blood PCR Inhibitors

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Amplification of DNA from whole blood collected on Guthrie card filter paper presents considerable technical obstacles due to the presence of natural PCR inhibitors (protein, heavy metals, heme, and heme degradation products) and low copy number of genomic material. For this purpose we evaluated guanidine thiocyanate-impregnated filter paper (GT-903), a DNA collection device designed specifically to bind PCR inhibitors and preserve DNA in an aqueous extractable form. Compared to standard 903, which retains DNA and elutes inhibitors during aqueous extraction, we found GT-903 retained 90% of protein, hemoglobin, and iron. SDS-PAGE

analysis indicated that the majority of the protein released from standard 903 corresponded to albumin (70-) and globin (15-kDa); negligible levels of these proteins were eluted from GT-903. To evaluate PCR efficiency, we amplified the 491 bp region encoding the cystic fibrosis $\Delta F508$ mutation. Using comparable template, we found GT-903 amplification more efficient than standard 903 following qualitative (TBE-PAGE) and quantitative (anti-dsDNA EIA) determination. We conclude that GT-903 provides a good DNA collection device and addresses the complications associated with natural PCR inhibitors. *J. Clin. Lab. Anal.* 11:87–93. © 1997 Wiley-Liss, Inc.

Key words: metals; neonatal screening

INTRODUCTION

Whole blood collected on Guthrie card filter paper has provided an excellent means for screening neonatal disorders such as galactosemia, phenylketonuria, and homocystinuria by traditional methods of biochemical analysis (1). Recently, it has been proposed that Guthrie card specimens represent a vast, yet relatively underutilized repository of DNA for genetic analysis by polymerase chain reaction (PCR) (2–6). Amplification of Guthrie card DNA presents considerable technical problems due to the presence of high levels of natural whole blood PCR inhibitors (heavy metals, protein, heme, and heme degradation products) and low copy number of template (5–10). To overcome these limitations, investigators have resorted to a variety of DNA extraction methods using organic solvents (phenol/chloroform/isoamyl alcohol) (4, 10), chaotropic agents (cesium trifluoroacetate, guanidine-thiocyanate and -HCl) (11–13) and detergents (sodium dodecyl sulfate) (3, 4, 10). Unfortunately, these harsh extraction methods generally result in the simultaneous release of nucleic acid and PCR inhibitors. Thus treatment with proteinase K and/or metal-chelators (Chelex-100 resin, EDTA) is frequently required (4, 5, 8, 10). In some cases further DNA purification via ethanol precipitation may be necessary (4, 5, 10, 12).

In general these multistep labor intensive DNA extraction protocols are incompatible with screening methods. Thus we developed a water-based extraction protocol that selectively

eluted PCR inhibitors from Guthrie card blood spots and retained filter-bound DNA in amplifiable form (14, 15). Using this simple method, we demonstrated that PCR could be performed directly on as little as 1/16th of a 3 mm diameter bloodspot punch (0.2 μ l whole blood) (7, 15). We estimated that a typical Guthrie card collection filter contains sufficient genomic material for > 400 separate amplifications (7, 15).

Recently, Guthrie filter paper impregnated with guanidine thiocyanate (GT-903) was developed as an alternative whole blood DNA collection device (16). In contrast to traditional bloodspot collection filters, GT filter paper specifically binds PCR inhibitors and preserves DNA in an aqueous extractable form. Because of our interest in natural PCR inhibitors and the differences this device poses to our established method (7, 15), we sought to characterize the effect of guanidine thiocyanate on PCR inhibitors and subsequent amplification efficiency.

MATERIALS AND METHODS

Standard Guthrie card filter paper 903 (Std-903) and guanidine thiocyanate-impregnated filter paper 903 (GT-903) were

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from Schleicher & Schuell (Keene, NH). SDS-PAGE molecular weight standards were from Pharmacia-LKB (Piscataway, NJ). PCR reagents were from Perkin-Elmer (Norwalk, CT). Oligonucleotide primers were synthesized by automated phosphoramidite chemistry (Cyclone DNA Synthesizer, Milligen/Bioscience, Edison, NJ). Antidouble-stranded DNA (anti-dsDNA) EIA was from Sorin Biomedica, S.p.A. (Italy) distributed by Incstar Corp. (Stillwater, MN). Acrylamide and N,N'-methylene-bis-acrylamide were of electrophoretic grade. Unless specified, all other reagents were from Sigma Chemical Co. (St. Louis, MO).

DNA Preparation

Whole blood was collected on filter paper, air dried, and stored at room temperature (Std-903) or dried desiccated and stored desiccated at room temperature (GT-903) as recommended by the manufacturer. Template DNA was prepared from Std-903 as described previously (7, 15) and GT-903 as described by the manufacturer (Fig. 1). Briefly, bloodspots from Std-903 (3 mm diameter) were washed twice in 1 ml ultrapure water. The washed filter paper was cut to size (1/4 and 1/8) with a razor blade and amplified directly (see below). Bloodspots from GT-903 (3 mm) were rinsed in 0.5 ml ultrapure water (5 sec, pulse vortexed 3×) and transferred to 50 µl water. GT-903 DNA was eluted by heating at 95°C for 60 min (pulse vortexed 15× after 15 min and 60× after 30 min) (Fig. 1).

Fig. 1. DNA extraction methods for Std-903 (A) and GT-903 filter paper (B).

Quantitative Analysis

Protein was determined in the presence of bicinchoninic acid using bovine serum albumin as the standard in microtiter plate format (17). Hemoglobin concentration was determined spectrophotometrically (18). Iron was determined by ferrozine dye binding (Beckman Instruments, Brea, CA) (19). Data are representative of at least five independent determinations.

SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on Laemmli slab gels (20) as described (21). Prior to electrophoresis samples were concentrated 10-fold using a macrosolute concentrator (Amicon, Beverly, MA). Concentrated samples were prepared in 2X Laemmli sample buffer (20) and heat denatured (100°C, 5 min). Samples (30 µl) were electrophoresed (20 mA, constant current) on 10% polyacrylamide gels (R. Shadel, San Francisco, CA) at room temperature. Following electrophoresis, gels were stained with 0.2% (w/v) Coomassie brilliant blue (40% methanol, 10% acetic acid) and destained (40% methanol, 10% acetic acid) appropriately. Molecular weight standards were phosphorylase b (97-), bovine serum albumin (68-), ovalbumin (45-), carbonic anhydrase (29-), soybean trypsin inhibitor (20-), and lactalbumin (14.2-kDa).

PCR Amplification

PCR amplification was performed as described (7, 15). For direct amplification, 1/8 and 1/4 of a washed 3 mm Std-903 bloodspot punch were placed in 33 µl water and overlaid with 50 µl mineral oil. The tube was heated in a thermal cycler (Perkin-Elmer Corp., Norwalk, CT) at 95°C for 5 min and cooled to 25°C (7, 15). For GT-903 amplification, 6.25 and 12.5 µl DNA eluant was added to 27 and 20.5 µl water, respectively and overlaid with 50 µl mineral oil. PCR master mix (17 µl) was then added to all tubes to achieve final concentrations of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 200 µM deoxyribonucleotide triphosphates (dATP, dUTP, dCTP, and dGTP, each), 3 mM MgCl₂, 1.25 U Taq polymerase, 0.5 U uracil N-glycosylase, 0.01% (w/v) gelatin and 100 ng (each) oligonucleotide primers (sense, 5'-GCAGAGTACCTGAA-ACAGGC-3'; antisense, 5'-CATTCACAGTAGCTTACCCA-3') for the 491 bp region encoding the ΔF508 mutation (22, 23). Carryover prevention was performed (37°C, 10 min) and uracil N-glycosylase was inactivated at increased temperature (94°C, 10 min). PCR amplification consisted of denaturation (94°C, 1 min), annealing (55°C, 10 min), and elongation (72°C, 2 min) for 20–35 cycles. Amplification product was analyzed by TBE-PAGE and anti-dsDNA EIA (see below).

TBE-PAGE

Tris-borate-EDTA/polyacrylamide gel electrophoresis (TBE-PAGE) was performed as described (15, 24). Briefly,

PCR samples (20 μ l) were mixed with 6X sample buffer containing bromophenol blue and Ficoll-type 400 (24). Samples (15 μ l) were electrophoresed on 4% mini-polyacrylamide gels (0.75 mm thickness) (Bio-Rad Laboratories, Hercules, CA) in 1X TBE at 75 V (constant voltage) for 1 h at 25°C (15). Gels were stained with ethidium bromide (0.5 μ g/ml, 10 min) and photographed with background UV illumination. Electrophoretic migration was compared relative to 123 bp DNA molecular weight ladders (GIBCO-BRL, Grand Island, NY).

Anti-dsDNA EIA

The anti-dsDNA enzyme immunoassay (EIA) was performed essentially as described (25, 26). All reagents were allowed to come to room temperature prior to use. Briefly, PCR products (obtained from a normal individual, Δ F508 negative) were denatured to single-stranded molecules in a boiling water bath (15 min) and immediately placed in a ice water bath for 15 min to prevent annealing. Denatured samples (20 μ l) were mixed with 100 μ l hybridization buffer containing 1X SCC (150 mM NaCl, 15 mM trisodium citrate), 2X Denhardt's solution (0.4 g bovine serum albumin, 0.2 g Ficoll, and 0.2 g polyvinylpyrrolidone), 10 mM Tris-Cl, pH 7.5, and 1 mM EDTA. Samples were added to microtiter plate wells coated with normal (N) or mutant (M) single-stranded cDNA probes for the Δ F508 mutation. Hybridization was performed for 1 h at 45°C in a thermostatically controlled water bath. The wells were washed three times with phosphate-buffered saline (PBS, pH 7.5) containing 0.05% Tween-20 (wash solution). Anti-dsDNA solution (100 μ l, PBS, 10% fetal calf serum) was added and the incubation continued (1 h, 25°C). The wells were washed and enzyme tracer solution (horse-radish peroxidase-conjugated anti-mouse IgG, 100 μ l, PBS, 10% fetal calf serum) was added (1 h, 25°C). The wells were washed and 100 μ l chromogen/substrate solution containing tetramethylbenzidine (100 mM) and 0.01% H₂O₂ was added (30 min, 25°C). Following addition of stop solution (1 N H₂SO₄, 200 μ l), the microtiter plates were scanned at 450 nm and corrected for background (650 nm). A ratio of N/M > 2.0 is indicative of normal genotype (confidence interval, 98%).

RESULTS

Filter Paper Characterization

Extraction of standard filter paper (Std-903) and GT-impregnated filter paper (GT-903) by established protocols (Fig. 1) resulted in a substantial difference in bloodspot appearance (Fig. 2). As can be seen, Std-903 was almost completely cleared of whole blood contaminants following two-step water elution (Fig. 2A, C). In contrast, GT-903 retained most darkly stained material (Fig. 2B, D). Eluants obtained from Std-903 and GT-903 were further characterized.

Fig. 2. Whole blood collection on Std-903 (A) and GT-903 filter paper (B) and 3 mm diameter punch of Std-903 (C) and GT-903 (D). Pre- and postindicate pre and post DNA extraction as described in Figure 1. Note: different size of sample collection area (A vs. B) and staining intensity of bloodspot post extraction (C vs. D). Bar, 5 mm.

Quantitative Analysis of Released Material from Filter Paper

Elution 1 resulted in release of high levels of protein, hemoglobin, and iron from Std-903 (Fig. 3A–C). Lower release occurred in elution 2. During direct PCR, the washed filter paper is subjected to repeated heating and cooling, a process that may result in additional release of whole blood components. To evaluate this potential complication, the post-PCR material was evaluated. As can be seen, thermal cycling of washed Std-903 was ineffective in releasing additional material (post-PCR). In contrast to Std-903, only minor amounts of protein, hemoglobin, and iron were released from GT-903 (Rinse, DNA elution fractions) (Fig. 3A–C). Protein, hemoglobin, and iron released from Std-903 were 609.4 μ g, 1.975 au, and 459 ng, respectively. In comparison, only 3.2% of protein (19.2 μ g), 2.3% of hemoglobin (0.052 au), and 9.8% of iron (45 ng) were released from GT-903.

SDS-PAGE Analysis

Characterization of protein release from Std-903 and GT-903 by SDS-PAGE confirmed quantitative results (Fig. 4A). As can be seen, two intensely stained protein bands (a and c) were evident in elution 1 from Std-903. Comparison of electrophoretic migration with known molecular weight standards (Fig. 4B) indicated relative molecular weights of ~70- and 15-kDa for these two proteins (a and c) and likely correspond to albumin and globin, respectively. The abundance of these two proteins decreased substantially in elution 2 from Std-903 and was absent in the post-PCR fraction. In good agreement with quantitative results (see above), only a small amount of stainable protein was observed in either fraction obtained from GT-903 (Rinse, DNA elution) (Fig. 4A). Three minor proteins (a, b, and c) were, however, evident in the DNA elution fraction. Two of these (a and c) demonstrated electro-

Fig. 3. PCR inhibitors released from Std-903 and GT-903 filter paper: **A**, protein, **B**, hemoglobin, and **C**, iron. For fraction description, see Figure 1.

Fig. 4. SDS-PAGE analysis (**A**) and molecular weight determination (**B**) of protein released from Std-903 and GT-903 filter paper. Lanes: 1, elution 1; 2, elution 2; 3, post-PCR (Std-903) and 4, rinse; 5, DNA elution (GT-903). For fraction description, see Figure 1. Migration positions of proteins a, b, and c, are shown. r , correlation coefficient ($r = 1.000$, perfect correlation).

phoretic mobility similar to albumin and globin, respectively. The third protein (b) at 27-kDa was unidentified.

Filter Paper DNA Amplification

Amplification efficiency of Std-903 and GT-903 DNA was evaluated using a comparable amount of template and vary-

ing cycle number ($n = 20-35$). PCR was performed for the 491 bp region encoding the most common mutation in cystic fibrosis ($\Delta F508$) (22, 23). As can be seen, the 491 bp amplicon was detected at cycle number 25 using GT-903, whereas no product was observed with Std-903 (Fig. 5A). Doubling the template concentration did not alter amplification efficiency (Fig. 5B). No amplification product was detected at 20 cycles for either Std-903 or GT-903. Similar results were obtained using primers for a shorter amplification product (98 bp, data not shown). Quantitative analysis of the 491 bp PCR product by anti-dsDNA EIA confirmed electrophoretic results. EIA detection of amplification product from GT-903 resulted in

N/M values of 1.3, 2.7, 8.2, and 8.4 for cycle number 20, 25, 30, and 35, respectively (cutoff > 2.0). In contrast, N/M values for Std-903 were 0.8, 0.8, 2.8, and 7.1 for cycle number 20, 25, 30, and 35, respectively.

DISCUSSION

Impregnation of Guthrie card filter paper with guanidine thiocyanate (GT-903) resulted in substantial differences in the filter-binding properties of whole blood components that act as natural PCR inhibitors (5-10). Protein, hemoglobin, and iron bound to GT-903 with high avidity ($> 90\%$), whereas DNA was readily dissociable under aqueous conditions. Conversely, the majority of these components were solubilized from Std-903. The role of guanidine thiocyanate in the differential binding of DNA and PCR inhibitors to the filter paper matrix is unclear. Guanidine thiocyanate has been a widely used chaotrope for DNA and RNA purification (11, 12, 24). Although GT has been reported to enhance the affinity of DNA for silica and glass particles (11, 12), it appears to prevent or at least limit DNA interaction with the filter paper matrix while promoting inhibitor binding. The lack of elution of both high (protein) and low molecular (iron, heme) components from GT-903 would appear to suggest that these components have become irreversibly bound or fixed in the presence of guanidine thiocyanate. Thus both methods, proceeding by independent mechanisms, were effective in the separation of nucleic acids from whole blood PCR inhibitors. However, it is reasonable to assume that DNA stability would be better enhanced in the presence of guanidine thiocyanate (12, 13).

Amplification of DNA eluted from GT-903 was more efficient than direct amplification of Std-903. This observation may result from better preservation and/or increased yield of template DNA in the presence of GT (11, 12, 24), GT-mediated inhibition of endogenous nucleases (12, 13), or some combination thereof. Although comparable amounts of Std-903 and GT-903 were used for amplification, it was impossible to quantitate the DNA that was tightly bound to Std-903. In independent experiments, we found that the majority of DNA bound to Std-903 was nondissociable under a variety of conditions including extraction with sodium dodecyl sulfate and urea (unpub. ob.). The high affinity of DNA for Std-903 may account in part for low DNA yield achieved by traditional nucleic acid extraction procedures and our ability to amplify Std-903 directly (7, 14, 15). The mechanism of interaction between DNA and Std-903 is unclear; however, DNA integrity on Std-903 appears relatively stable. Previously, we demonstrated that amplification could be successfully performed on Std-903 blood spots stored at room temperature for up to 2 years (14) (see Table 3). In fact, one report cites amplification of a 330 bp region from a 17-year-old Guthrie card blood spot (4). Experiments are underway to evaluate relative stability of DNA (long-range PCR, see below) on Std-903 and GT-903.

Fig. 5. Qualitative evaluation of PCR amplification of DNA from Std-903 and GT-903 filter paper at two template concentrations and varying cycle number: 4% TBE-PAGE with ethidium bromide staining was performed as described (see Materials and Methods). Template concentration: in **A**: 1, 1/8 punch Std-903; 2, 6.25 μ l GT-903; in **B**: 1, 1/4 punch Std-903; 2, 12.5 μ l GT-903. PCR cycle number 20, 25, 30, and 35 is shown. Mw, 123 bp ladder.

Although the number of sample application sites is identical on Std-903 and GT-903 ($n = 4$), the larger size of the Std-903 sample collection area (Fig. 2, Table 1) should correlate to increased number of amplifications per filter paper (Table 2). However, differences in relative amplification efficiency (see above) and actual template required (6.25 μ l vs 10 μ l template recommended) should decrease this number under working conditions. The presence of guanidine thiocyanate on GT-903 should inhibit endogenous nucleases and thus better preserve integrity of filter-bound DNA. In fact, researchers have successfully amplified a 22,000 bp region for the β -globin gene from DNA eluted from GT-903 (16). Such long-range experiments are necessary to determine the integrity of DNA collected on Std-903.

We found that length of time and complexity of preparation of template DNA from Std-903 and GT-903 were comparable (Fig. 1). Std-903 has minimal storage requirements and is stable at room temperature pre- and post-sample collection. Due to the hygroscopic properties of guanidine thiocyanate (12, 13, 24), GT-903 must be stored desiccated prior to and following sample application (Table 2). Finally, GT-903 is incompatible for neonatal screening by traditional biochemical methods, thereby increasing cost at least two-fold (Table 2).

The use of GT-903 would facilitate method standardization for DNA extraction and amplification. Current Guthrie card microextraction methods utilize a variety of organic solvents, detergents, proteinases, chelating resins, precipitants, or some combination thereof (3, 4, 10–13). These “in-house” methods lack standardization and thus result in variable yield of template DNA (i.e., up to 8-fold in several studies) (5, 10, 27, 28) and level of contamination (8). Although decreased amplification efficiency at low copy number may be overcome by increasing cycle number, this practice also increases the likelihood of amplifying contaminating nucleic acid thereby increasing the false positive rate (29). Variable template yield consumes limited sample at different rates; thus the number of potential amplifications could vary widely between laboratories. Reliable yield of GT-903 template DNA would result in consistent PCR product generation, thereby ensuring a minimal level of sensitivity. Finally, if Guthrie cards

TABLE 1. Filter Paper Comparison

Parameter	Std-903	GT-903
Sample collection sites (n)	4	4
Collection site radius (mm)	7.5	5.0
Collection site area (mm ²)	176.6	78.5
Total collection area (mm ²)	707	314
3 mm punch per collection site (n)	8–10 ^a	5–6 ^a
Total punches (n)	32–40	20–24
PCR per punch (n)	4 ^b	5 ^c

^aTypical yield.

^bDirect PCR typically performed on 1/4 punch (7).

^cAs recommended by the manufacturer.

TABLE 2. Filter Paper Evaluation

Parameter	Std-903	GT-903
Biochemical analysis (PKU)	yes	no
PCR amplification	yes ^a	yes ^b
Amplification per filter (n)	128–160 ^c	100–120 ^d
DNA stability (mo)	30	na
Storage conditions (25°C)	none	desiccated
Cost	x	2x

^aUp to 500 bp (7).

^b22,000 bp reported (16).

^cTheoretical, based on established direct protocol (7).

^dManufacturer's recommendations.

are to be utilized as DNA data base, storage conditions for this repository also require standardization. In fact, a recent survey of 53 newborn screening laboratories in the United States found large disparity in Guthrie card storage conditions (i.e., room temperature, refrigerated, frozen, desiccated) (2). Given the fact that DNA yield decreases with storage time (5), GT-903 provides a simple means to collect and store stabilized genomic material. Furthermore, GT-903 is particularly effective in the elimination whole blood PCR inhibitors and should thus facilitate standardization.

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