

Recovery of Genomic DNA from Residual Frozen Archival Blood Clots Suitable for Amplification and Use in Genotyping Assays

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ABSTRACT

A greatly neglected source of DNA potentially useful for genetic or forensic studies is the clot remaining from blood samples collected for serum chemistry measurements. We have investigated the utility of residual clots remaining from venipunctures collected for California's Expanded Maternal Serum Alpha-Fetoprotein Screening Program. We report a protocol based on the salting out method for the extraction of DNA from samples which have been archived and frozen for up to 2.5 years. As much as 57 μg of high-quality DNA can be obtained from a 2-ml clot as determined by PicoGreen[®] (Molecular Probes, Inc., Eugene, OR) fluorescence measurements. Quality of the purified DNA was evaluated by its ability to serve as template in polymerase chain reaction (PCR) amplifications, using primers that flank the polymorphic regions of six genes of pharmacogenetic interest distributed throughout the human genome. Sizes of the gene regions successfully amplified range from 215 bp to 2064 bp, using as little as 10 ng of template DNA. Because many genotyping protocols routinely recommend the design of amplicons in the 100–200 bp range, and 10–50 ng of template, we conclude that the clot remaining after serum has been removed from blood collected for serum chemistry measurements can serve as a reliable source of DNA for genotyping studies.

INTRODUCTION

DNA EXTRACTION METHODS have greatly improved and diversified in recent years, allowing the efficient recovery of genetic material from a wide variety of sources. DNA purification protocols for clinical or forensic evaluations now include such varied origins as hair (Suenaga and Nakamura 2005), archival dried blood spots (Iovannisci 2000), laundered garments (Eminovic et al. 2005), and mummified (Aufderheide et al. 2004) or burned human remains (von Wurmb-Schwark et al. 2005). However, a significantly overlooked source of genetic material that might be useful for diagnostic or forensic studies is the blood clot that remains after the serum component has been removed for serum chemistry measurements. Laboratories have neglected to research the resource that blood clots might provide, assuming that the only valuable portion of a centrifuged vial of clotted blood is the serum. The presumption has been that any genetic material remaining in these samples would be too degraded to be of much value. However, there

are some noteworthy advantages to using such materials if their utility can be adequately demonstrated. Multiple use of a single blood draw can reduce the number of venipunctures experienced by patients, decreasing patient anxiety and discomfort. Fewer blood draws would also reduce the volume of biohazardous materials handled within the laboratory, lowering the risk of exposure incidents, as well as decreasing material use and biohazardous waste disposal costs. Because approximately 75% of California pregnant women participate in the Expanded Maternal Serum Alpha-Fetoprotein Screening Program (XAFP) between 15 and 20 weeks of gestation, we wondered if these samples might double as a reliable source of DNA for genetic screening applications. We have developed a specimen bank that contains thousands of such samples in which both serum and residual clots have been stored frozen, offering a potentially rich source of archival material for retrospective studies of pregnancy-related events, if their utility can be adequately demonstrated. However, because blood draws intended for XAFP are typically collected by primary care centers or blood

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collection facilities where they are often held for extended periods at ambient temperatures before processing, it is important to evaluate samples that have undergone similar handling. We therefore have evaluated frozen clotted samples left over from XAFP screening where the history of collection, transport and extended storage is known, for the ability to serve as a useful source of DNA for polymerase chain reaction (PCR) amplifications and genetic screening assays.

MATERIALS AND METHODS

Description of samples

Frozen archival blood clots were obtained through XAFP. An average time of 12 days passed from the time the bloods were drawn to the time they were frozen. All samples were collected and handled using universal precautions employed to prevent worker and sample contamination. Typically, samples were drawn in a clinic or physician's office (BD[®] Vacutainer SST[™] [Becton Dickinson, Franklin Lakes, NJ] with polymer gel for serum separation, 4-ml draw) and immediately spun to separate serum from the clot. Approximately 5 days elapsed while the bloods were transported at ambient temperature to a screening laboratory for processing and XAFP screening. Samples were held for an additional 7 days at 4°C until it could be confirmed that no additional serum aliquots would be needed for XAFP or other serum-based testing. After complete removal of the remaining serum, tubes containing blood clots and polymer separation gels were frozen at -20°C. The amount of time the samples remained frozen ranged from 2–2.5 years. Each residual clot was estimated to be 2 ml. A total of 20 samples was evaluated.

Extraction protocol

The MasterPure[™] DNA Purification Kit (Epicentre Technologies, Madison WI, Cat. No. MCD85201) based on the salting out method (Miller et al. 1988; Iovannisci 2000) was used for these studies. The detailed procedures of the extraction method are described in Table 1. Cell lysis was initiated by physical crushing of the blood clot and then vortexing in the presence of 2X Tissue and Cell Lysis Solution containing detergents. DNases, RNases and other proteins were inactivated with the addition of proteinase K and incubation in a high-temperature water bath; protein removal was completed by treatment with salt. After removing the impurities by centrifugation, the DNA was recovered by isopropanol precipitation.

DNA quantitation by picogreen[®]

The resulting DNA concentrations were determined using the PicoGreen[®] dsDNA Quantitation Kit (Molecular Probes, Eugene OR, Cat. No. P-11496) on a fluorescence plate reader (PerkinElmer, Inc., Wellesley, MA) according to the manufacturer's instructions. DNAs were subsequently diluted to a working concentration of 10 ng/μl for use as PCR templates.

PCR evaluation of purified DNA

Quality of the DNAs was evaluated by PCR amplification. The ability to amplify the following gene sequences of common interest for pharmacogenetic genotyping studies was examined: glutathione-S-transferase T1 (GSTT1, deletion region), glutathione-S-transferase M1 (GSTM1, deletion region), albumin (Intron 6), cytochrome P450-2C19 (CYP2C19, *6 SNP containing region), N-acetyltransferase 2 (NAT2, full-length genomic sequence), and cytochrome P450-2A6 (CYP2A6, *12

TABLE 1. PROTOCOL FOR DNA EXTRACTION FROM FROZEN ARCHIVED BLOOD CLOTS

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- I. Cell Lysis:
1. Remove the serum separation polymer plug from each tube using the cotton tipped end of a 6-inch cotton tip swab (cotton at one end only) and transfer each blood clot into a separate 50 ml conical tube.
 2. Break up clot by crushing it with the wooden end of the same or another cotton tip swab.
 3. Add an equal volume (2 ml) of 2X Tissue and Cell Lysis Solution to each conical tube.
 4. Vortex each tube for 10 sec to further break up blood clot.
 5. In a separate tube, combine 50 μl of 50 μg/μl Proteinase K with 4 ml of 2X Tissue and Cell Lysis Solution for each sample. Gently mix by swirling. Add 4 ml of the proteinase K/lysis mix to each conical tube containing a blood clot.
 6. Vortex each tube slightly. Do not overvortex.
 7. Incubate 60 min at 65°C, vortexing again after 30 and 60 min.
- II. Removal of Protein:
8. Add 4 ml of MPC Protein Precipitation Reagent to the total volume (8 ml) of the lysed sample and vortex vigorously for 10–15 sec. Chill on ice for ≥ 1 hour.
 9. Pellet the debris by centrifugation for at least 30 min at ≥ 1800g in a centrifuge. Faster centrifugation speeds will improve the quality of the preparation.
 10. Transfer the supernatant to a new 50-ml conical tube and discard the pellet. Approximately 10–12 ml supernatant should be recovered. Repeat centrifugation and transfer to a clean tube if supernatant contains unprecipitated debris.
- III. Precipitation of Nucleic Acids:
11. Add 25 ml (at least 2 volumes) of isopropanol to the recovered supernatant. Gently invert the tube several times. Freeze at -20°C, overnight-indefinitely.
 12. Pellet the DNA by centrifugation for 30 min at ≥ 1800g in a centrifuge.
 13. Carefully pour off the isopropanol without dislodging DNA pellet, and wash each with 10 ml of 70% ethanol.
 14. Centrifuge each tube for 10 min at ≥ 1800g. Pour off the ethanol and invert tubes over paper towels to dry.
 15. Resuspend dried pellet in 300 μl of TE buffer.
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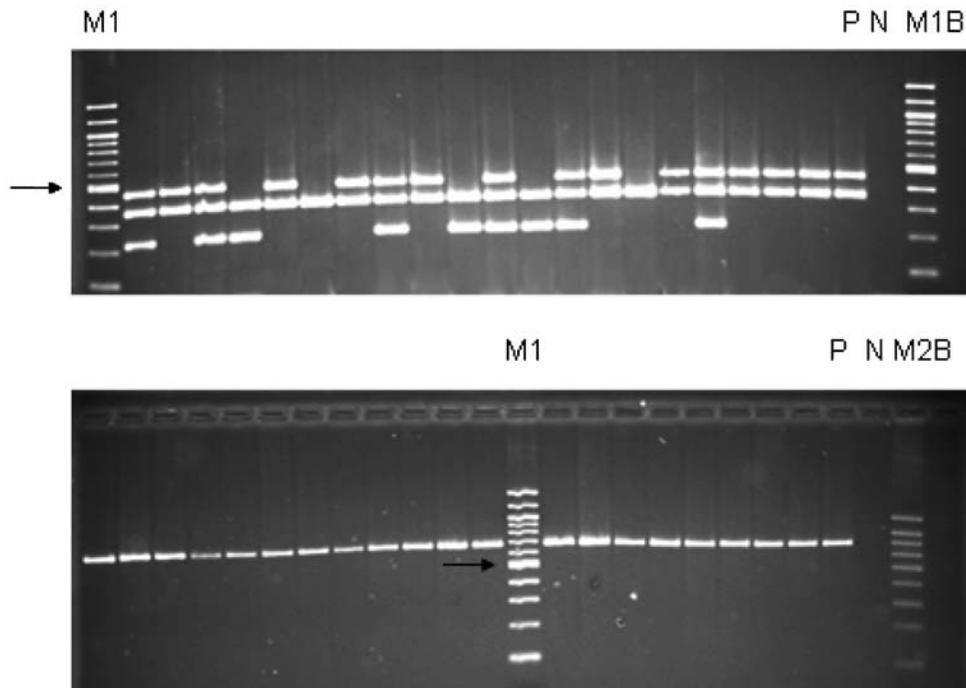


FIG. 1. Polymerase chain reaction (PCR) amplifications of four target sequences in the 200–700 bp size range from archival blood clot purified DNAs. All blood clot samples for each gel are displayed in the order 1 → 20. **Upper gel:** multiplexed coamplification of glutathione-S-transferase T1 (GSTT1; 480-bp band), albumin (350-bp band), and glutathione-S-transferase M1 (GSTM1; 215-bp band) sequences. Samples for which either the GSTT1 or GSTM1 bands did not amplify represent homozygous NULL genotypes for these deletion regions being analyzed; note homozygous NULL GSTM1 genotype for positive control lane. Arrows indicate 500/517 bp doublet band (M1, New England Biolabs, Ipswich, MA; 100-bp ladder). **Lower gel:** amplification of CYP2C19 sequences (675-bp band). P, positive control using DNA extracted from fresh whole blood; N, no template PCR-negative control; B, blank lane.

gene conversion region). PCR designs including the forward and reverse primers used to amplify each gene sequence as well as amplicon sizes and the chromosomal locations of the target genes are presented in Table 2. PCR amplifications were performed on an MJ PTC-200 thermal cycler as previously described (Doll et al. 1995; Arand et al. 1996; Ibeanu et al. 1998; Schoedel et al. 2004) in 10- μ l reaction volumes. Amplified DNA was examined by gel electrophoresis and ethidium bromide staining followed by visualization and recording with an Alpha Imager (Alpha Innotech, San Leandro, CA).

RESULTS

We successfully purified genomic DNA from frozen archived residual blood clots, employing the salting out method commonly used for DNA extractions. Variations of this final protocol were evaluated by us and included a red cell lysis step, eliminating the protease digest, extended protease digests at lower temperatures (55°C) and protein precipitation with higher salt concentrations, all with minimal success. Thus we have optimized a protocol using reagents that are commercially available and protocols that are similar to those frequently employed for the purification of DNA from other sources. The quantity of DNA obtained from each sample was determined by PicoGreen[®] fluorescence measurements. A range of 16 μ g to

57 μ g total DNA was recovered per sample (average, 36 μ g; data not shown) from clot volumes estimated to be approximately 2 ml.

Purified DNAs were also examined for their ability to serve as template in PCR amplifications (Figs. 1 and 2). Ten nanograms of each sample was evaluated in each of six amplification reactions ranging in amplicon size from 215 bp to 2064 bp using protocols currently in use within our laboratory, without modification. In each case all samples amplified for all PCRs attempted, with one exception. A single sample did not amplify for the largest (2064 bp) amplicon (Fig. 2, lower gel). Because most amplification protocols today recommend the design of amplicons less than 200 bp, particularly for automated methodologies (e.g., TaqMan[®], 100–150 bp; MALDI-TOF mass spectrometry, 100 bp; GoldenGate[®] assay, less than 100 bp) (Heid et al. 1996; Bray et al. 2001; Fan et al. 2003), frozen archival blood clots could clearly serve as a valuable source of material for genetic studies. However, because many genes of interest are members of multigene families, it is often difficult to identify PCR primer sequences that uniquely identify the desired gene family member and still adhere to such short amplicon design parameters. Therefore it is frequently necessary to preamplify longer chromosomal regions where unique priming sites can be identified to generate preamplified material that can subsequently serve as template in secondary, nested PCRs for final genetic analysis. For this reason we have included in

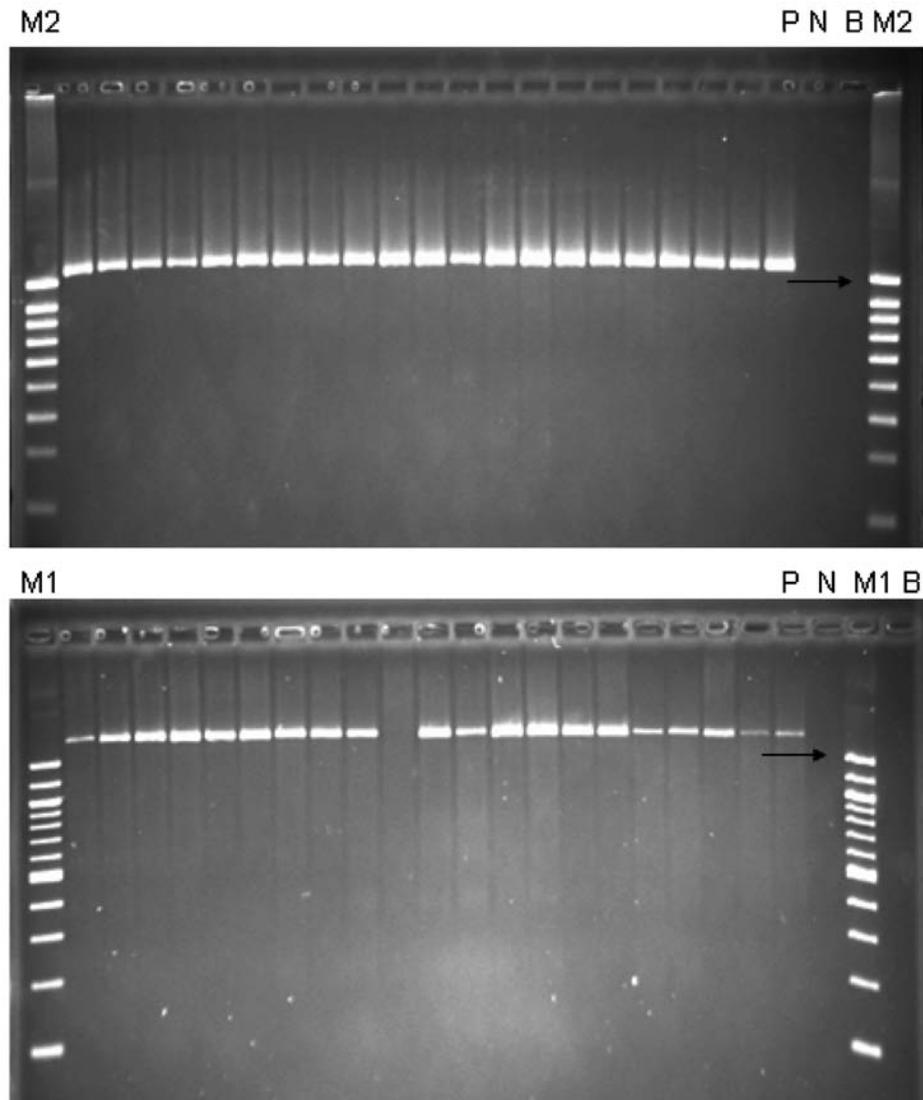


FIG. 2. Polymerase chain reaction (PCR) amplifications of two target sequences in the 1000–2100 bp size range from archival blood clot purified DNAs. All blood clot samples for each gel are displayed in the order 1 → 20. **Upper gel:** amplification of NAT2, entire gene, 1092 bp. **Lower gel:** amplification of CYP2A6 sequences, 2064 bp. Arrows indicate 1517-bp band (M1) and 1000-bp band (M2). M1, New England Biolabs, Ipswich, MA; 100-bp ladder; M2, Bioline Hyperladder IV (Bioline, Boston, MA); P, positive control using DNA extracted from fresh whole blood; N, no template PCR-negative control; B, blank lane.

our study the evaluation of three genes which are members of multigene families that exemplify such PCR design problems (NAT2, CYP2C19, and CYP2A6), and we demonstrate the ability to amplify the longer sequences required to uniquely select for the intended member of each multigene family. Even for these longer more demanding amplification reactions, the genomic DNA we have obtained is of a sufficiently high molecular weight that 10 ng is adequate for amplifications up to 2064 bp for most samples. Almost without exception, the amplification achievable with clot derived DNA was as good as or better than the same reaction using identical starting template concentrations of DNA prepared from fresh whole blood (Figs. 1 and 2; compare band intensities for all samples to their respective positive control, P).

Furthermore, we demonstrate that these DNAs can serve as targets for multiplexed amplifications (GSTT1, GSTM1, albumin; Fig. 1, upper gel) and that the amplified DNA serves as a suitable substrate for downstream genotyping applications. We have successfully used the following genotyping protocols on our extracted blood clot samples: end-point PCR analysis, restriction fragment length polymorphism (RFLP) analysis, matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry analysis, and DNA sequencing. End-point PCR analysis can be conducted on the gel presented in Figure 1 (upper gel). Genotypes for both GSTM1 and GSTT1 can be directly interpreted by the presence or absence of an amplified band corresponding to the presence or absence of the deletion alleles associated with these two genes, when

TABLE 2. PCR PRIMERS

Primer designation	Sequence (5' → 3')	Chromosome	Amplicon size (bp)
GSTM1-F	GAACTCCCTGAAAAGCTAAAGC		
GSTM1-R	GTTGGGCTCAAATATACGGTGG	1	215
Albumin-F	GCCCTCTGCTAACAAGTCCTAC		
Albumin-R	GCCCTAAAAAGAAAATCGCCAATC	4	350
GSTT1-F	TTCCTTACTGGTCCTCACATCTC		
GSTT1-R	TCACCGGATCATGGCCAGCA	22	480
CYP2C19*6-F	ATACAATGTAATATGAATCTAAG		
CYP2C19*6-R	CAGGACTCCAAATAAAAGATC	10	675
NAT2-F	GGAACAAAATTGGACTTGG		
NAT2-R	TCTAGCATGAATCACTCTGC	8	1092
CYP2A6*12-F	GCACCCCTCCTGAGGTACCAC		
CYP2A6*12-R	GTCCCCTGCTCACCGCCA	19	2064

the coamplification of another gene such as the albumin locus is also included as a PCR control (Arand et al. 1996). RFLP analysis was performed on NAT2 amplified sequences for the following three polymorphic sites, as previously described (Lammer et al. 2004): 857G>A (*Bam*HI), 481C>T (*Kpn*I), and 590G>A (*Taq*I). Both MALDI-TOF mass spectrometry genotyping as well as DNA sequencing of the same samples at these NAT2 polymorphic sites resulted in the same genotyping calls for each of the samples examined. Thus all three genotyping methodologies were concordant when blood clot derived genomic DNA was used as template for NAT2 genotyping protocols (data not shown).

While not exhaustive, each of the 6 target genes we have amplified is located on a different chromosome (Table 2) suggesting that chromosomal bias is unlikely in the DNA we have recovered from these archival samples. Because such large amounts of DNA can be obtained through the use of residual clots (36 μ g, average), it is evident that thousands of amplifications are possible from a sample which clinical laboratories currently discard.

DISCUSSION

Our laboratory has previously exploited the recovery of small amounts of genetic material that can be purified from residual blood remaining on newborn screen cards. This has allowed us to evaluate birth defect occurrence risks associated with an infant's genotype for a number of xenobiotic metabolizing activities when combined with information addressing *in utero* exposure to certain toxic substances such as cigarette smoke. We have observed substantially increased risks for the development of cleft lip and cleft palate when the infant possesses the variant xenobiotic metabolizing gene and the mother smokes during pregnancy (Lammer et al. 2004, 2005; Shaw et al. 2005). Because metabolism of toxins may also occur through the mother's metabolic activities, thus modulating fetal exposure, a more complete picture of risk may be possible to formulate if the mother's DNA were also available for study. In addition, this would provide an opportunity to identify variance from random inheritance through transmission disequilibrium analysis

(Weinberg 1999). However, obtaining the mother's DNA for such large population-based retrospective studies is logistically difficult, costly, time-consuming, and often compromised by poor participation. Because approximately 75% of California pregnant women participate in the XAFP between 15 and 20 weeks of gestation, we wondered if these samples might serve as a reliable source of maternal DNA. We therefore examined the utility of the residual material remaining after whole blood has been allowed to clot and serum has been removed, to serve as a genomic template for genotyping studies. Because these samples were not originally collected for genotyping, there may be some concern regarding contamination issues. However, samples collected for XAFP are handled in accordance with universal precautions used to prevent both worker exposure and cross contamination of samples. Furthermore, after the serum has been removed for analysis, the polymer gel plug remains in place during storage, serving as a physical barrier to contamination. Lastly, the lack of any visible contamination in our PCR gels combined with our observed concordance for three different genotyping chemistries suggests that sample contamination does not appear to be a significant issue.

We have developed a protocol for the extraction of DNA from archival samples that have been collected, transported and processed in the same way in which they are being handled for XAFP evaluation, and which have remained frozen for up to 2.5 years. Our work demonstrates the high quality and quantity of the recovered DNA and that it can serve as a reliable PCR template even for genes requiring preamplification of larger genomic regions (up to 2064 bp) in order to ensure the selective amplification of a specific member within a multigene family. Thus residual blood clot samples are expected to become an invaluable resource for retrospective genotyping studies within a very large California cohort.

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