TECHNICAL NOTE

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NaOH Treatment to Neutralize Inhibitors of Taq Polymerase

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ABSTRACT: The introduction of polymerase chain reaction (PCR) into the forensic field has greatly extended the ability to analyze DNA from small or degraded samples. However, one significant problem with PCR analysis is the sensitivity of Taq Polymerase to inhibitors found in many substrates commonly encountered with evidentiary materials. We hypothesize that the most problematic of these compounds intercalate into double stranded DNA (dsDNA) and have significantly less affinity for single stranded DNA (ssDNA). This study presents a comprehensive analysis of a novel method for the neutralization of Taq inhibitors by denaturation and washing with NaOH in Microcon-100 filtration units. The data show that DNA recovered following NaOH repurification routinely amplifies when other inhibitor neutralization techniques are unsuccessful. Genetic profiles have been obtained with both AmpliType PM + DQA1 and D1S80 systems. However, the NaOH protocol is not advised when the quantity of DNA is limited since the treatment results in significant loss of DNA.

KEYWORDS: forensic science, DNA typing, polymerase chain reaction, inhibitors, Taq polymerase, inhibitor removal

Two basic problems have hampered forensic PCR analysis. First, until recently, few systems had been implemented for routine casework, resulting in significantly lower discrimination than for RFLP methods. A second common problem encountered with forensic samples is the sensitivity of Taq polymerase to various inhibitors (1–5). Inhibitory substances (both environmental and textile dyes) are regularly found on substrates from which critical evidentiary material is obtained.

Significant effort has been directed to the development and implementation of additional PCR loci, resulting in greater discriminating power and more sensitive DNA typing systems for forensic casework. However, attempts to ameliorate inhibition have been less productive. Inhibitors from outside crime scenes such as soil, sand, wood, and leaf litter continue to plague PCR analysis. Similarly, wood, leather, and textile dyes present at interior crime scenes regularly contain Taq inhibitors. Proper collection and preservation of the evidentiary samples may minimize but not eliminate the presence of inhibitory compounds.

Techniques previously developed to overcome Taq inhibitors employed one of two strategies. The first strategy attempted to remove inhibitors from DNA. Repurification was accomplished by extensive TE washing in Microcon-100 filtration units (6), re-extracting the DNA, or chromatography (7,8). The second strategy was to inactivate or block the activity of inhibitors. Hot start and heat soak procedures (3), the addition of BSA (1), along with supplementing the reaction with greater quantities of Taq (9) have been routinely attempted. Other methodologies incorporated elements of both strategies to circumvent inhibitory substances (10).

In general, current methods have not proven sufficiently robust for routine forensic PCR typing. Since it is difficult to predict which inhibitor(s) might be in an evidentiary sample, any strategy should be rigorously tested on a wide variety of Taq inhibitors and optimized for significantly different DNA quantity and quality. This study represents a comprehensive analysis of a simple, inexpensive method for the neutralization of PCR inhibitors from DNA by denaturation and washing with NaOH. The efficacy of NaOH treatment is demonstrated as approximately fifty percent of case samples that failed to amplify using currently available techniques did so following repurification.

Materials and Methods

DNA Sample Preparation

Two hundred microliters of known blood were spotted onto substances which often inhibit Taq polymerase: wood, bark, rock, sand, soil, and leaf litter. Blood was deposited onto clean glass as a positive control. The blood was allowed to dry overnight at room temperature. Each sample was collected using care to minimize substrate removal. The DNA was purified using standard CSP Forensic Science Laboratory protocols (phenol/chloroform extraction followed by Microcon-100 purification and concentration (11). The quantity and quality of the DNA recovered were determined by electrophoresis in 1% agarose gels and by the Quantiblot procedure (PE-Biosystems).

PM and DQA1 and D1S80 Amplifications

Amplitype PM and DQA1 and D1S80 amplification were performed using 5–10 ng of input DNA in a Perkin Elmer 480 thermal

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cycler according to manufacturer's protocols (PE-Biosystems). Amplification products were visualized in 1% agarose gels containing ethidium bromide. Purified K562 DNA served as the NaOH/amplification control throughout the study. The genotype of the known blood was determined by repeated PCR typing (data not shown).

NaOH Treatment

DNA from each substrate that failed to amplify initially or following standard inhibitor neutralization strategies (heat soak, hot start, BSA, extra Taq, and extensive dilution) was subjected to NaOH treatment. Approximately 30–50 ng of DNA were placed into a Microcon-100 unit along with 200 μL of 0.4N NaOH. The volume was reduced to 5 μL by centrifugation at 500 × g and the eluate discarded. The chamber was refilled with 400 μL of 0.4N NaOH and centrifuged as described. This step was repeated once. (The number of NaOH washes was varied in Fig. 3.) The sample was neutralized by washing once with 400 μL of 10 mM Tris (7.5) and recovered in 15 μL of 10 mM Tris (7.5). The quantity and quality of DNA were determined by standard agarose gel electrophoresis and the Quantiblot assay.

Casework Samples: Inhibitors in Fabrics and Other Sources

DNA was purified from 28 case samples with blood deposited predominantly on common textile products (e.g., denim, cotton, leather, and carpet) or other substrates. All samples originally failed to amplify (AmpliType PM + DQA1) and were repurified by NaOH treatment. The samples were subsequently reamplified for AmpliType PM + DQA1 and D1S80 (data not shown) as described.

Results and Discussion

Many Taq inhibitors co-elute with DNA following standard extraction strategies. Since inhibition often cannot be overcome simply by dilution, these compounds are thought to bind DNA. If, in fact, these substances intercalate into dsDNA, denaturation could significantly reduce their affinity for DNA. Stains such as ethidium bromide and Hoechst 33258 intercalate into dsDNA with high affinity, yet have considerably lower affinity for ssDNA.

Figure 1A is a schematic of the NaOH protocol. NaOH was chosen as the denaturant since it is a simple, cost effective method for denaturing DNA and has long been used in capillary transfers without consequence to samples.

Human DNA which previously did not amplify after attempts with other inhibitor removal strategies (hot start, heat soak, BSA, additional Taq, and extensive dilution) were treated with NaOH (Fig. 1B). Lane 3 of Fig. 1B demonstrates successful amplification of 5–10 ng of DNA following NaOH treatment.

The results demonstrate the efficacy of NaOH treatment in neutralizing Taq inhibitors, thus permitting the amplification of many

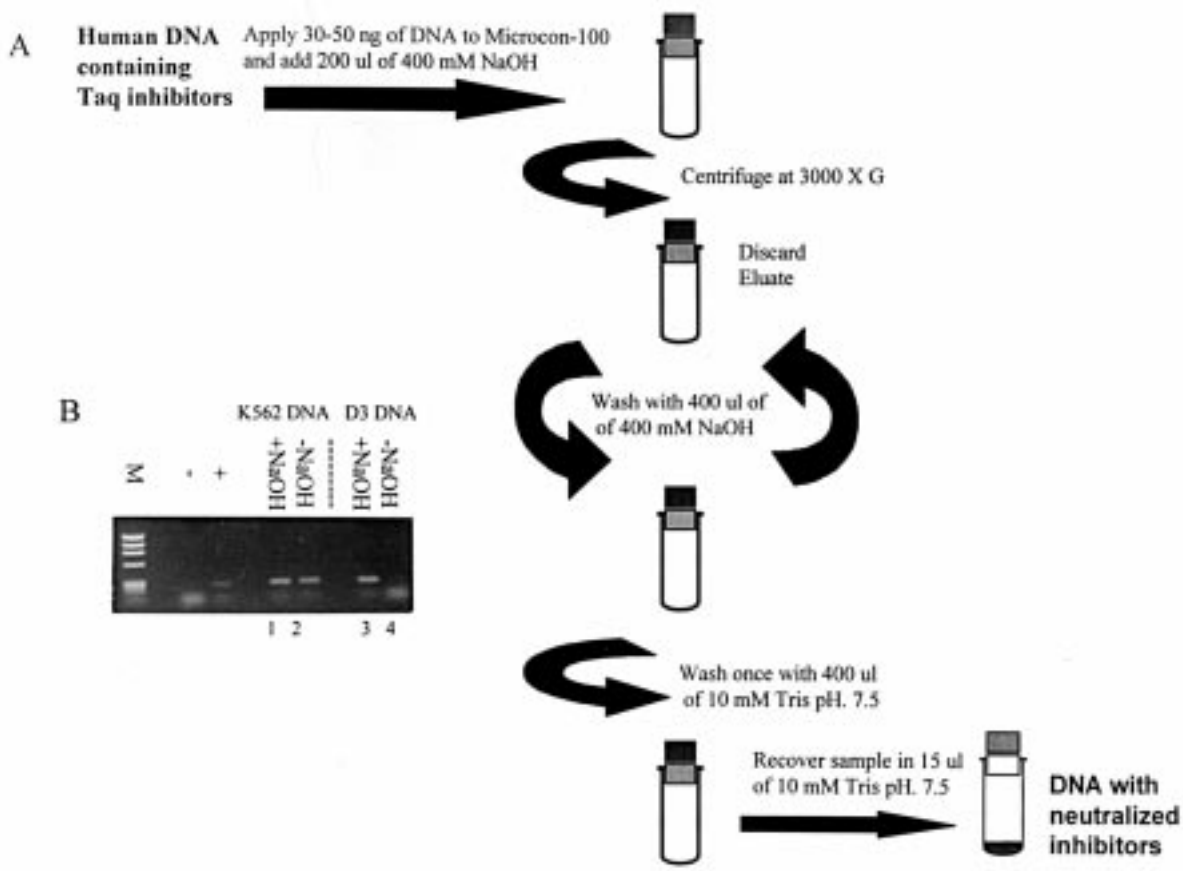

FIG. 1—*A: A schematic of the NaOH treatment procedure. B: Effect of NaOH treatment on an inhibited DNA sample. DQA1 amplification products from inhibited DNA (D3) with and without NaOH treatment; lanes 3 and 4, respectively. M: ϕ × 174 DNA digested with Hae III. K562 DNA is a positive control for the NaOH process. Lanes 1 and 2, K562 control DNA treated and untreated, respectively.*

difficult samples. Under alkali conditions DNA is single stranded, possibly reducing the affinity of the inhibitory agents for DNA and permitting their dilution/removal.

The capacity of NaOH treatment to overcome a variety of inhibitors was then evaluated. Two hundred μL of known blood was deposited onto seven different substrates thought to contain Taq polymerase inhibitors: sand, soil, bark, rock, leaf litter, lumber (pine), and soil stains on white cotton cloth. Known blood was deposited onto clean glass as a control. All test samples remained colored following the standard extraction procedure suggesting that an inhibitor is present. The quantity/quality of recovered DNA varied greatly among the different substrates, mimicking standard case results (Fig. 2A, lanes 2–8). Quantiblot tests indicated that the majority of DNA was human in all samples (data not shown). 5–10 ng of DNA from each sample were amplified for the DQA1 locus. The absence of amplification product confirms that each sample contains inhibitors (lanes 2–8 of Fig. 2B). Subsequently, each sample was treated with NaOH and the DNA recovery determined as described, Fig. 2C. NaOH treatment resulted in removal of pigment from five of the DNAs; only the bark and leaf litter samples retained color. The low recovery of DNA (approximately 50% of input) and the extent of degradation after treatment demonstrate a potential limitation of this protocol. The degradation of the recovered DNA is likely due to hydrostatic shearing of the ssDNA and imperfect renaturation of the DNA following neutralization. Hence, NaOH treatment may not be suitable for highly degraded or low yield DNA samples.

Five nanograms of each treated DNA were amplified for the DQA1 locus and the amplification products visualized as described (Fig. 2D). Amplification product was detected in five of the test samples (lanes 2,3,5,7,8). These results demonstrate that NaOH treatment can overcome inhibitory substances present in a wide variety of substrates. The two test samples that failed to amplify were the samples (tree bark and leaf litter) that remained colored following treatment. AmpliType PM and D1S80 profiles were determined for the five test samples that amplified. Genotypes detected were as expected (data not shown).

Our initial hypothesis was that denaturing conditions would release intercalated inhibitors and that denaturing washes would allow for their removal. However, it was theoretically possible that alkaline (or denaturing) conditions alone could inactivate the inhibitors, thus obviating the necessity for NaOH washes and potentially increasing the quantity/quality of DNA recovered.

To determine the mechanism of inhibitor neutralization/removal, DNA (D3) containing Taq inhibitors was denatured by three different methods: NaOH, formamide, or heating to 95°C (Table 1). Each denatured sample was split into three aliquots and washed under denaturing (NaOH or formamide) or native (TE) conditions. To determine if denaturing conditions alone would overcome the Taq inhibitors, tubes of D3 DNA (inhibited) were maintained denatured using either NaOH, formamide, or heat for the length of the experiment but not washed. DQA1 amplifications were then performed on 5–10 ng of DNA for all samples.

The results summarized in Table 1 clearly demonstrate that neutralization of inhibitors requires a combination of denaturation and inactivation by high pH. The only method that produced full amplification was NaOH denaturation combined with NaOH washes. Minimal amplification was observed with formamide denaturation coupled with NaOH washes as well as with the NaOH incubation

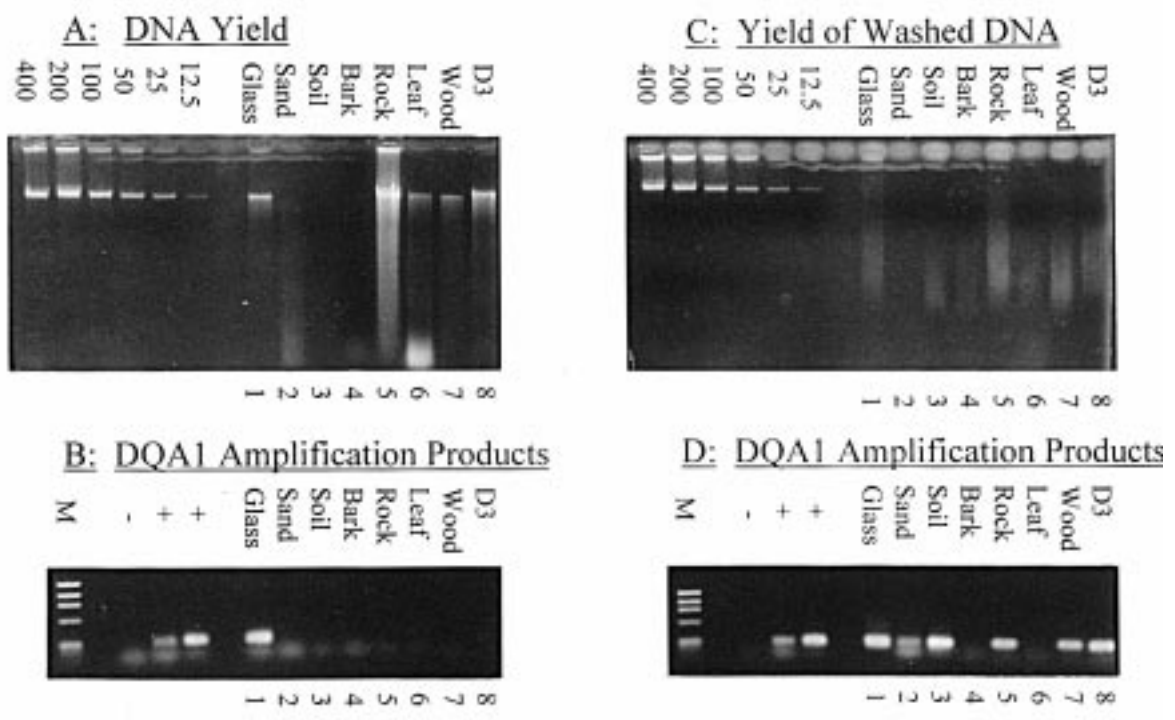

FIG. 2—*Effect of the NaOH process on inhibitors from seven different substrates: sand, soil, tree bark (bark), rock, leaf litter (leaf), lumber (wood), and soil on cotton cloth (D3). Glass was used as a positive control A: DNA yield following standard extraction procedure as compared to known quantities of K562 DNA in ng. B: DQA1 amplification products from the seven test DNAs. C: DNA yield following NaOH treatment process. D. DQA1 amplification products from NaOH-treated test DNAs.*

TABLE 1—*Mechanism of NaOH Action*

Denaturing Condition	Washes	DQA1 K562	Amplification Inhibited DNA (D3)
	NaOH	+	+
NaOH	TE	+	−
	Formamide	+	−
	NaOH	+	+/−
Formamide	TE	+	−
	Formamide	+	−
	NaOH	+	−
95°C	TE	+	−
	Formamide	+	−
Equal Time			
Incubation NaOH	None	+	+/−
Formamide	None	+	−
95°C	None	+	−

+ = Amplification
− = No amplification
+/− = Weak amplification

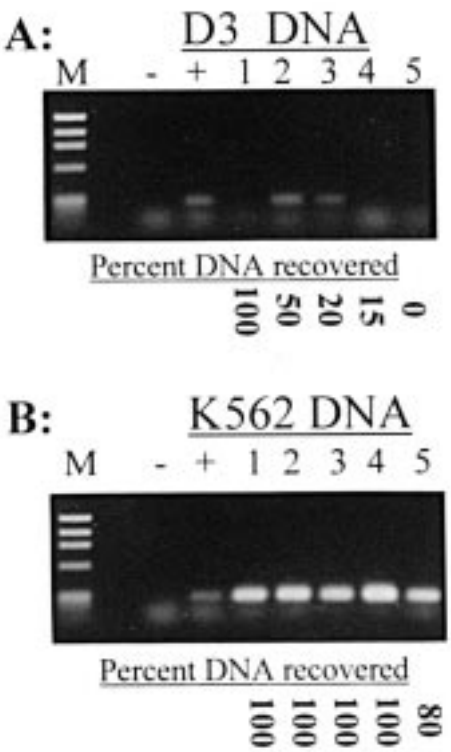

FIG. 3—*Recovery and DQA1 amplification of DNA following NaOH treatment. A: D3 (inhibited) and B: K562 DNA were washed in NaOH from one to five times. + = amplification positive control. 1–5 equals number of washes.*

alone. However, neither denaturation nor alkaline conditions individually can completely overcome inhibition.

Inhibitors were removed most successfully after NaOH denaturation and numerous NaOH washes. Unfortunately, extensive NaOH washes appear to significantly degrade the DNA in most field samples, therefore, the number of washes must be limited to maximize DNA recovery while still removing inhibition. The recovery of DNA with increasing number of NaOH washes was determined (Fig. 3). Twenty ng of K562 and D3 (inhibited) DNA were washed 1–5 times. Subsequently, 25% of each recovered DNA (5 ng assuming 100% recovery) was amplified for DQA1. The quantity of DNA was determined as described following each wash. Optimal amplification from the repurified D3 sample was achieved with two washes; the amount of recovered DNA diminished with each additional wash. A single NaOH wash yielded DNA that was still inhibited and three or more washes resulted in insufficient DNA recovery.

It is interesting to note that significant DNA loss did not occur with the K562 samples, suggesting that high molecular weight DNA (Fig. 2A, lane 8) from the D3 "field" sample, unlike the K562 control DNA, contained a significant level of single strand nicks. It is likely that the nicked condition of the D3 DNA, combined with hydrostatic shearing of ssDNA during NaOH treatment, resulted in the observed DNA loss. It may be possible to reduce DNA loss by using a smaller molecular weight cut off filtration unit (e.g., 50 kD) while still permitting the passage of inhibitors. Additional washes may be attempted with persistently inhibited samples containing large quantities of DNA if the loss of DNA could be tolerated. The goal with NaOH treatment is to balance inactivation of inhibitors with DNA loss, since success is dependent on both the quality and quantity of the DNA recovered.

The data suggest that the quality of the DNA is more important than the substrate or type of inhibitor. Amplification failure is most often correlated with significant sample degradation or low yield prior to the repurification step. However, there are many excep-

TABLE 2—*The efficacy of NaOH treatment for neutralizing Taq inhibitors from case samples.*

Substrate	DNA Yield	DNA Condition	Amplification
	APPAREL		
Blue denim jeans	125 ng	B†	No
	1000 ng	A	Yes
Dark blue cloth	No visible yield	Unknown	No
	No visible yield	Unknown	No
Blue jacket	100 ng	B	Yes
Blue denim jeans	50 ng	A	Yes
Green cotton shirt	250 ng	B	Yes
	50 ng	B	No
Blue cotton shirt	20 ng	B	No
Blue denim jeans	150 ng	B	No
Purple T-shirt	25 ng	B	No
Gray T-shirt	600 ng	B	Yes
	300 ng	B	Yes
Brown cloth	250 ng	C	No
	6000 ng	C	Yes
	6000 ng	C	Yes
Dark red cloth	50 ng	A	Yes
	50 ng	A	No
	LEATHER		
Sneaker,	200 ng	A	Yes
White leather	50 ng	A	No
Watch band,	No visible yield	Unknown	Yes
Black leather	No visible yield	Unknown	No
	CARPET		
Red color carpet	500 ng	B	Yes
Gray color carpet	35 ng	C	No
Rose color carpet	50 ng	A	No
Rust color carpet	500 ng	C	Yes
	ENVIRONMENTAL		
Rock	5000 ng	B	Yes
Toothpick	50 ng	A	Yes

A = Mostly high molecular weight DNA.
B = Some high molecular weight DNA with moderate degradation visible.
C = No high molecular weight DNA, highly degraded.

tions to this general rule. Several of the case samples that amplified post NaOH treatment contained a limited quantity or highly degraded DNA.

The efficacy of NaOH treatment for neutralizing Taq inhibitors from common evidentiary materials was assessed (Table 2). NaOH treatment was attempted on 28 case samples that failed to amplify under standard conditions. Following repurification, genotypes could be determined for 15 of the samples. These results demonstrate that NaOH treatment will effectively neutralize inhibitors from clothing dyes and various environmental sources.

Conclusion

NaOH treatment has proven to be a valuable tool for enhancing the utility of PCR in criminal investigations. This technique has permitted the analysis of numerous samples that otherwise would not have amplified. Although NaOH treatment is not always effective, it is a simple method for eliminating or neutralizing Taq DNA polymerase inhibitors found in many commonly encountered substrates.

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