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ORIGINAL ARTICLE

Pressure cycling technology (PCT) reduces effects of inhibitors of the PCR

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Abstract A common problem in the analysis of forensic human DNA evidence, or for that matter any nucleic acid analysis, is the presence of contaminants or inhibitors. Contaminants may copurify with the DNA, inhibiting downstream PCR or they may present samples effectively as containing fewer templates than exist in the PCR, even when the actual amount of DNA is adequate. Typically, these challenged samples exhibit allele imbalance, allele dropout, and sequence-specific inhibition, leading to interpretational difficulties. Lessening the effects of inhibitors may increase the effective yield of challenged low template copy samples. High pressure may alter some inhibitors and render them less effective at reducing the yield of PCR products. In an attempt to enhance the amplicon yield of inhibited DNA samples, pressure cycling technology was applied to DNA exposed to various concentrations of hematin (0, 1.25, 2.5, 5, and 7 μM) and humic acid (0, 1.25, 2.5, 5, and 7 ng/μL). The effect of high pressure on the inhibitors, and subsequently the PCR process, was assessed by measuring DNA quantity by quantitative PCR and evaluating short tandem repeat typing results. The results support that pressure cycling technology reduces inhibitory effects and thus, in effect, enhances yield of contaminated amplified products of both hematin and humic acid contaminate samples. Based on the results obtained in this study, this method can improve the ability to type challenged or inhibited DNA samples.

Keywords PCR inhibition · Pressure cycling technology · Forensic DNA analysis · Hematin · Humic acid · Hi-Flow column

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Introduction

Samples that contain a suboptimal quantity and/or limited quality of DNA are commonly encountered in forensic DNA analyses. Several materials act as potent inhibitors of PCR, including collagen, calcium ions, melanin, hematin, and humic acids found in soil [1, 2]. Many inhibitory substances may copurify with the DNA and impact the PCR process. Inhibition has been shown to affect the accuracy of template quantification by quantitative PCR (qPCR) [3]. In addition, reduced PCR performance can result in a number of short tandem repeat (STR) typing problems, including heterozygote allele imbalance, allele dropout, and sequence-specific inhibition, all of which can impact negatively the interpretation of a DNA profile [4–9].

To effectively access available genetic material, PCR inhibitors must be efficiently removed or their effects mollified. Various approaches have been developed for the detection of PCR inhibitors and for their removal. A common approach, when the presence of inhibitors is suspected, is the dilution of the DNA sample to dilute the inhibitor concentration. However, for analysis of low copy number DNA samples, i.e., samples containing few template molecules for the PCR, this dilution approach may be undesirable as it concomitantly decreases the quantity of template DNA [8, 10–14]. Additionally, newer, more robust kits such as AmpFlSTR® Identifiler® Plus, PowerPlex® ESI and ESX

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systems, and AmpFlSTR® MiniFiler™ have been developed to reduce the effects of inhibition. While these alternative buffer systems have proven successful, this study explores an alternative strategy for improving amplification of inhibited samples, i.e., pressure cycling.

Pressure cycling technology (PCT; Pressure BioSciences Inc., South Easton, MA, USA) uses hydrostatic pressure alternating between ambient and ultrahigh levels to perturb molecular interactions. Pressure cycling has been shown to assist in extraction of nucleic acids, proteins, lipids, and small molecules from cells and tissues [15–17]. While pressure is a well-understood thermodynamic parameter orthogonal to temperature, its effects on enzyme activity and protein conformation are very complex and present rich opportunities for research. Indeed, high pressure has been shown to weaken hydrophobic interactions between aliphatic amino acid side chains, while electrostatic interactions are known to be enhanced under pressure [18, 19]. Moreover, main pressure effects on biological macromolecules are attributed to pressure perturbation of the interactions of said molecules with the solvent, leading to reversible partial denaturation of proteins, weakening of lipid bilayers, and dissociation of multimeric protein complexes [20]. Pressure acts synergistically with chaotropes and detergents leading to protein denaturation; however, pressure-perturbed proteins were shown to assume conformational forms drastically different from those resulting from thermal or chemical treatment [21].

During an investigation into the use of PCT to attempt to increase DNA yield in challenged samples, it was observed that PCT reduced the effects of inhibition on downstream DNA analyses. Possibly, the conditions of extreme pressure may alter the conformation of some inhibitors, thus improving the yield of PCR products. Pressure generally has no effects on covalent bonds. Therefore, natural compounds such as flavors, aromas, dyes, and pharmacologically active molecules are typically not altered by high-pressure treatment at room temperature [22–24]. Notable exceptions to this general phenomenon are Diels–Alder and several other types of cycloaddition reactions, involving conjugated double bonds and a substituted alkene, which have been shown to be associated with a significant volume reduction and, therefore, are enhanced under pressure [25]. This observation suggests that reactivity of aromatic compounds, including porphyrins and polycyclic aromatics, such as tannins, humic acid, phenolic compounds, and terpenes, could be somewhat enhanced under pressure, especially at elevated temperatures.

Formation of hydrogen bonds is associated with a small negative volume change and is, therefore, reinforced by pressure. Nucleic acids and polysaccharides appear to be pressure-resistant biological macromolecules because their secondary structure is predominantly held together by hydrogen bonds. Ionic interactions as well as hydrophobic interactions have been shown to be disrupted by pressure. Ionization of acids, bases, salts, and dissociation of water is promoted under pressure [26, 27].

Hydrophobic interactions, involved in the stability of proteins, micelles, and lipids, are differentially altered by pressure [28]. As an exception, pi-stacking has been shown to be increased under pressure, although very little is known to date about pressure effects on polycyclic aromatic compounds. The pressure may lead to aggregation of some polycyclic aromatic compounds via the pi-stacking mechanism. Considering possible Diels–Adler reactions in aggregated phenolics or porphyrins, pressure can be considered potentially as a selective way to remove such compounds from solution, leading to lower amount of PCR inhibitors present in the reaction mixture.

For this study, two potent PCR inhibitors, hematin and humic acid, were evaluated. Hematin is a metal chelating molecule found in red blood cells [29–31]. Hematin forms a stable complex with the DNA polymerase and may also cause a dissociation of the DNA–polymerase complex, thereby inhibiting polymerase activity [32–34]. Humic acid is a group of commonly found compounds in soil and often is encountered in samples that have been buried, such as skeletal remains. Proposed mechanisms of inhibition include the chelation of magnesium ions needed for DNA polymerase activity or that humic acid inhibits the PCR via sequence-specific binding to DNA, thus limiting the amount of available template [29, 35, 36]. The objective of this study was to determine if pressure cycling technology affects or modifies inhibitor compounds. The study was performed in both the absence and presence of DNA in order to evaluate the interaction of the DNA and the inhibitor. DNA quantity by qPCR and STR typing results were assessed to determine the efficacy of pressure on reducing the effects of the selected inhibitors.

Materials and methods

PULSE tubes and microtubes preparation

Specially designed *single use* pressure used to lyse samples for extraction (PULSE; Pressure BioSciences Inc., South Easton, MA, USA) tubes were used for this study. Two different types of PULSE tubes, the FT500-ND PULSE tubes and microtubes, were prepared for use as follows. The PULSE tubes and microtubes and their caps were cleaned prior to use in a 5 % bleach solution for 5 min with agitation. Tubes and caps then were washed three times with Nanopure water for 5 min with agitation. Tubes and caps then were washed in 70 % ethanol solution for 5 min with agitation and air-dried overnight. Following drying, tubes and caps were UV irradiated and assembled prior to use. After one use, the PULSE tubes were discarded.

DNA

Experiments were performed using the Quantifiler® Human DNA Quantification Kit (Life Technologies, Carlsbad, CA, USA) DNA Standard (Raji cell line; 200 ng/μL purified DNA). The standard was diluted to a final concentration of 1 ng/μL for each experimental sample. Experiments were performed in replicates of either three or five.

Preparation of inhibitors

Hematin

For experiments using Identifiler® amplification, porcine hematin (Sigma-Aldrich, St Louis, MO, USA) was dissolved in 0.1 N NaOH (Fisher Chemical, Fairlawn, NJ, USA) to a final stock concentration of 84.5 μM. Hematin was added to samples to final concentrations of 0, 1.25, 2.5, 5, or 7 μM. For experiments using Identifiler® Plus amplification, porcine hematin (Sigma-Aldrich, St Louis, MO, USA) was dissolved in 0.1 N NaOH (Fisher Chemical, Fairlawn, NJ, USA) to a final stock concentration of 100 mM. Three concentrations of hematin, i.e., 2.0, 2.4, and 2.8 mM, were tested, based on previous studies that showed that Identifiler® Plus amplifications were inhibited.

Humic acid

For experiments using Identifiler® amplification, technical grade humic acid (Sigma-Aldrich, St Louis, MO, USA) was dissolved using TE^{-4} buffer (10 mM Invitrogen UltraPure™ Tris–HCl, pH8.0; Invitrogen Corporation, Carlsbad, CA, USA, and 0.1 mM GIBCO UltraPure™ EDTA, pH8.0; GIBCO Products, Grand Island, NY, USA) to a final stock concentration of 500 ng/μL. Humic acid was added to samples to final concentrations of 0, 1.25, 2.5, 5, or 7 ng/μL.

For experiments using Identifiler® Plus amplification, humic acid was saturated at 10 mg/mL in molecular grade water. Humic acid was added to samples to final concentrations of 0, 0.01, and 0.02 mg/μL.

Pressure cycling technology

Samples undergoing PCT were placed in either single-use FT500-ND PULSE tubes or microtubes. Samples were transferred to the Barocycler® NEP3229 and subjected to 30 cycles of alternating pressures consisting of 35 kpsi for 20 s and ambient pressure for 10 s. Nonpressure-treated controls (NPC) also were prepared. NPC samples were placed in either PULSE tubes or microtubes, depending on the experiment, but not subjected to PCT.

Quantification and inhibitor effects

Quantity of DNA was determined using the Quantifiler® Human DNA Quantification Kit on the ABI 7500 Real-Time PCR System (Life Technologies). Quantification standard dilutions were prepared by performing a serial dilution of the 200 ng/μL stock solution from the kit to the following concentrations: 50, 16.7, 5.56, 1.85, 0.62, 0.21, 0.068, and 0.023 ng/μL in TE buffer (10 mM Tris–Cl pH8.0, 0.1 mM Na_2EDTA). The master mix was prepared by combining 4.2 μL of Primer Mix and 5 μL of Quantifiler® PCR Reaction Mix per reaction, multiplied by the number of reactions required. The master mix then was dispensed into an ABI PRISM™ 96-Well Optical Reaction Plate (Life Technologies) at 9.2 μL per reaction. Sample volume of 0.8 μL was added per reaction, with duplicate reactions of each quantification standard and single reactions of each analysis sample being run. The plate was placed in the 96-well sample block of an ABI 7500 Real-Time PCR System, and data analysis was performed by the SDS software to generate standard curve data for quantification standards, quantification results, and C_T values for the internal PCR Control (IPC).

Bone samples

Five human bones were obtained and prepared for DNA extraction. First, the outer surface of the bones was cleaned by immersing the bone fragment in 50 % commercial bleach (3 % NaOCl) for 5–15 min in a 50-ml conical tube. Next, the bones were repeatedly washed with nuclease-free water (4–5 washes). The bones then were immersed briefly in 95–100 % ethanol. The bones then were air dried overnight. The bones were crushed to powder using a 6750 Freezer/Mill (SPEX SamplePrep L.L.C., Metuchen, NJ, USA), filled with liquid nitrogen, using a protocol of a 10-min rechill followed by 5 min of grind time at 15 impacts per second. Approximately 0.2 g of bone powder was placed in a PULSE™ Tube. One milliliter of extraction buffer containing 0.5 M EDTA pH8.0 (Invitrogen Corporation, Carlsbad, CA, USA), 1 % sodium lauroyl sarcosinate (sarkosyl and *n*-lauroyl sarcosine; Sigma-Aldrich®), and 100 μg/mL Proteinase K (Invitrogen Corporation, Carlsbad, CA, USA), were added to each sample and vortexed. Both pressure-treated and NPC samples were placed into PULSE tubes. For three of the bones, the NPC sample contained 0.5 g bone powder (following Hi-Flow column protocol). The bones were incubated at 56 °C with constant agitation for either 2 h or overnight. Following incubation, samples were either subjected to PCT (30 cycles; 20 s at 35 kpsi and 10 s at ambient psi) or no pressure. Each sample was centrifuged at 2,545×*g* for 5 min, and the supernatant transferred to a 50-mL conical tube. Five volumes of buffer PB (QIAGEN Inc.,

Valencia, CA, USA) then was added, and the sample was vortexed. The entire sample then was added to a Hi-Flow column (Generon L.L.C., Maidenhead, UK) and centrifuged at 2,545×*g* for 10 min. The flow through buffer was discarded, and 5 mL of buffer PE (QIAGEN Inc., Valencia, CA, USA) were added to the column. Each sample then was centrifuged at 2,545×*g* for 5 min and the flow through buffer discarded. This step was repeated two more times. The "empty" column for each sample then was centrifuged at 2,545×*g* for 5 min to remove residual alcohol from the column. Each column then was transferred to a new 50-mL conical tube, and 100 μL of buffer EB (QIAGEN Inc., Valencia, CA, USA) were placed directly on the column membrane. Each tube was incubated at room temperature for 5 min and then centrifuged at 2,545×*g* for 1 min. Then, the eluate was collected from each tube. This step was repeated two more times for a total of three elutions per sample. The quantity of recovered DNA was determined for all elutions. Only the first elution of each sample was used for amplification. For all bones with the exception of bone 2, 10 μL of extract were used for amplification. For bone 2, the extract was normalized to 1 ng/μL.

Amplification and STR typing

Approximately one nanogram or ten microliters of each extract was amplified using the reagents contained in AmpFlSTR® Identifiler® PCR Amplification Kit (Life Technologies) according to manufacturer's recommendations. A subset of experiments was performed using the reagents contained in AmpFlSTR® Identifiler® Plus PCR Amplification Kit (Life Technologies) according to manufacturer's recommendations. PCR products were separated and detected on an AB 3130×l Genetic Analyzer (Life Technologies) following the manufacturer's recommendations. Samples were injected for 10 s at 3 kV and separated by electrophoresis in performance optimized polymer (POP-4™; Life Technologies) using the HIDFragmentAnalysis36_POP4 Module (Life Technologies) and a 1,500 s run time. Data were collected using the AB 3130×l Genetic Analyzer Data Collection Software 3.0. Electrophoresis results were analyzed with GeneMapper® ID software v3.2.1 (Life Technologies). The detection and interpretation thresholds were both set at 50 relative fluorescence units (RFU).

Results and discussion

PCT of inhibitor in absence of DNA

The effect of PCT was assessed on two inhibitors, hematin and humic acid. These initial experiments focused on the effects of pressure on inhibitors in the absence of DNA to determine if the inhibitor was affected directly by pressure. The ability to reduce the effect of PCR inhibitors on a sample using PCT was monitored by a shift in C_T values for the IPC in the Quantifiler® kit. If a DNA extract contains a PCR inhibitor, typically an increase in the C_T value is observed (i.e., more cycles required to reach a detection threshold) for the sample compared with the IPC C_T value for the control [5]. One milliliter of 84.5 μM hematin or 1 ml of 500 ng/μL humic acid was subjected to PCT in PULSE tubes. Various concentrations of hematin (0, 2.5, 5, and 7 μM) and humic acid (0, 2.5, 5, and 7 ng/μL) then were added immediately to the Quantifiler, i.e., qPCR, master mix in the absence of genomic template DNA and performance of the IPC monitored. In the presence of 2.5 and 5 μM hematin and 2.5 ng/μL humic acid, the C_T value of the IPC increased with concentration of inhibitor (Fig. 1a and b, respectively). However, following PCT, IPC C_T values were substantially lower for 5 μM hematin and 2.5 ng/μL humic acid. At 7 μM for hematin and 5 and 7 ng/μL of humic acid, the IPC was not amplified for either PCT or NPC samples.

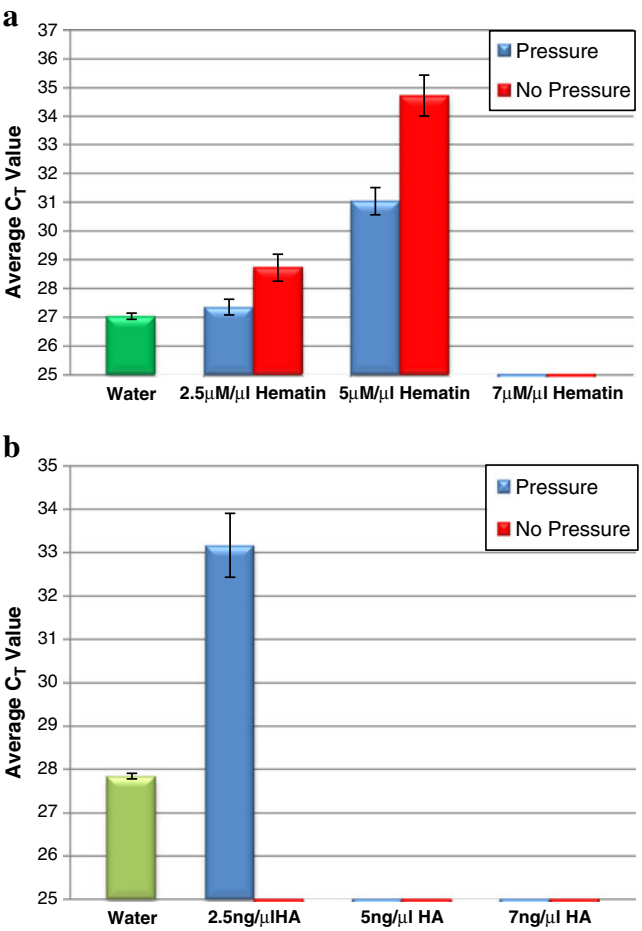

Fig. 1 Effect of PCT on IPC values of inhibitors hematin (**a**) and humic acid (**b**)

PCT of inhibitor prior to addition of DNA

PCT then was tested for its effects on inhibitors, which were immediately added to human DNA and the potential impact of pressure on inhibitors assessed on downstream analysis. One milliliter of 84.5 μM hematin or 1 ml of 500 ng/μL humic acid solutions was subjected to PCT. PCT-treated and NPC inhibitors then were added to a final concentration of 0, 2.5, 5, and 7 μM for hematin and 0, 2.5, and 5 ng/μL for humic acid to the qPCR master mix. DNA (0.8 μL of 1 ng/μL; Raji cell line) was added to each reaction and subjected to qPCR. An inhibitory effect was observed. Table 1 illustrates that as the concentration of inhibitor increased, the quantity of detectable DNA decreased. A difference was observed in PCT samples compared with NPC samples. Following PCT, IPC C_T values were lower for samples containing 5 μM hematin and 2.5 ng/μL humic acid. For NPC samples, the presence of 2.5 and 5 ng/μL of humic acid resulted in no detectable DNA, while DNA was not detected in pressure-treated samples, at a concentration of 5 ng/μL of humic acid. At 7 μM for hematin and 5 ng/μL of humic acid, the IPC was not amplified for either PCT or NPC samples.

The next step was to determine the effect of PCT on STR analysis. One milliliter of 84.5 μM hematin or 1 mL of 500 ng/μL humic acid was subjected to PCT. PCT and NPC inhibitors were added to a final concentration of either hematin (0, 2.5, 5, and 7 μM) or humic acid (0, 2.5, and 5 ng/μL) to a final concentration of 1 ng/μL DNA in a final volume of 100 μL. Subsequently, 1 μL of each sample was amplified using the AmpFlSTR® Identifiler® PCR Amplification Kit to generate STR profiles. While at concentrations of 2.5 μM hematin, dropout of larger amplicon loci occurred in both pressure and nonpressured samples, the negative effect of hematin was more pronounced in the nonpressured samples, illustrated by lower RFU values and increased allele and locus dropout. In the presence of 2.5 ng/μL of humic acid, full profiles were obtained for PCT samples but only partial profiles were observed for NPC samples (data not shown).

Pressure cycling treatment of inhibitor in the presence of DNA

To determine the effect PCT had on inhibitors in the presence of DNA, various concentrations of hematin (0, 1.5, 2.5, and 5 μM) and humic acid (0, 1.5, 2.5, and 5 ng/μL), (Fig. 2a and b, respectively) were added to 1 ng/μL of DNA in final volume of 100 μL, placed in microtubes and subjected to either PCT or not subjected to pressure. Subsequently, 1 μL of each sample was amplified and typed for STRs. Samples were run in five replicates and the outlier removed from each group. Average peak heights for all 16 loci for four replicates are shown (Fig. 2). Pressure-treated samples displayed higher RFU at 1.5 and 2.5 μM of hematin and 1.5, 2.5, and 5 ng/μL of humic acid compared with NPC samples.

Electropherograms shown in Figs. 3 and 4 are representative of STR typing results for hematin and humic acid treated samples, respectively. Increased RFU values for pressure-treated samples at concentrations of hematin up to 5 μM were observed compared with NPC samples (Fig. 3). Both pressure-treated and NPC samples failed to amplify at 7 μM hematin, while NPC samples failed to amplify at 5 μM hematin. PCT resulted in increased RFU values for samples with humic acid concentrations of 1.5 and 2.5 ng/μL (Fig. 4). At a concentration of 2.5 ng/μL humic acid, dropout of larger loci in the NPC samples occurred. No

Table 1 Effect of pressure treated inhibitors on amplification of DNA

Sample	Average quant (ng/μL)	StDev quant	Average IPC	StDev IPC
No Hem-PCT	0.96	0.12	27.50	0.08
No Hem-NPC	0.71	0.11	27.84	0.12
2.5 μM Hem-PCT	0.76	0.18	27.90	0.09
2.5 μM Hem-NPC	0.56	0.03	27.63	0.26
5 μM Hem-PCT	0.23	0.13	34.33	1.42
5 μM Hem-NPC	0.14	0.03	27.50	0
7 μM Hem-PCT	Undetermined	0	Undetermined	0
7 μM Hem-NPC	Undetermined	0	Undetermined	0
No HA-PCT	0.99	0.09	27.43	0.13
No HA-NPC	0.90	0.16	27.41	0.05
2.5 ng HA-PCT	0.75	0.03	33.21	0.12
2.5 ng HA-NPC	Undetermined	0	Undetermined	0
5 ng HA-PCT	Undetermined	0	Undetermined	0
5 ng HA-NPC	Undetermined	0	Undetermined	0

Quant quantifiler results, *StDev* standard deviation, *IPC* internal PCR control, *Hem* hematin, *HA* humic acid, *PCT* pressure cycling technology, *NPC* no pressure cycling

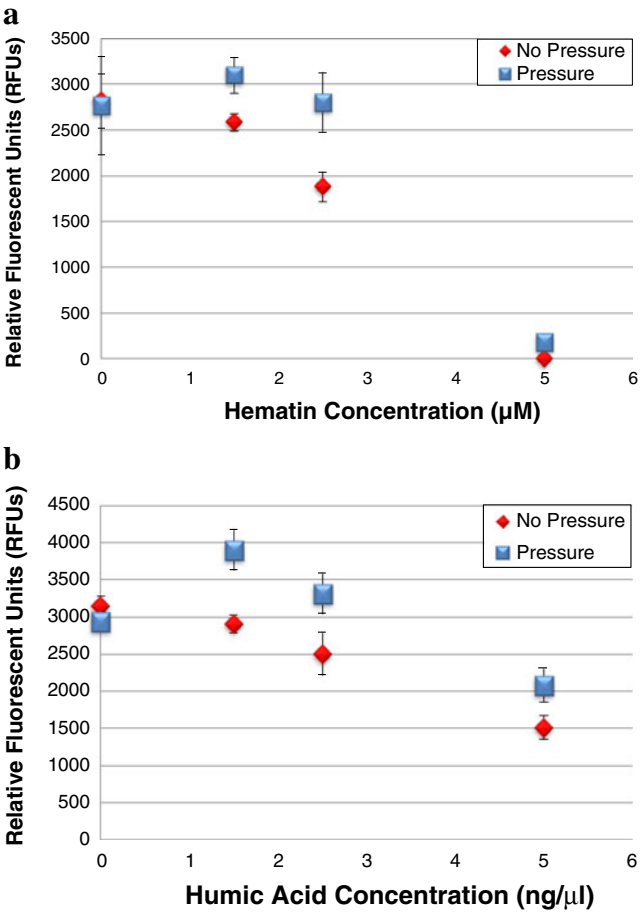

Fig. 2 Effect of PCT on average STR peak heights of inhibited samples. Hematin (**a**) and humic acid (**b**). Pressure-treated samples (*squares*) were compared with nonpressure-treated controls (*diamonds*)

differences were observed at 5 ng/μL humic acid between pressure and NPC samples.

The effect of PCT was determined when using a more robust amplification kit, i.e., the AmpFlSTR® Identifiler® Plus PCR Amplification Kit. Various concentrations of hematin (0, 2, 2.4, and 2.8 mM) and humic acid (0, 0.01, and 0.02 mg/μL) (Fig. 5a and b, respectively) were added to 1 ng/μL of DNA in a final volume of 100 μL, placed in microtubes and subjected to either PCT or not subjected to pressure. Subsequently, 1 μL of each sample was amplified and typed for STRs. Samples were run in five replicates. Average peak heights for all 16 loci for five replicates were obtained (Fig. 5). While not statistically significant, pressure-treated samples displayed higher RFUs at all concentrations of hematin. For 0.01 mg/μL of humic acid, pressure-treated samples displayed significantly higher RFU values compared with NPC samples.

Electropherograms are representative of STR typing results for no pressure-treated and pressure-treated humic acid-treated samples, shown in Fig. 6a and b, respectively. Significantly increased RFU values for pressure-treated samples (Fig. 6b) at concentrations of 0.01 mg/μL were observed compared with NPC samples (Fig. 6a). No significant differences were observed at 0.02 mg/μL humic acid, with both pressure and NPC samples failing to yield a profile (data not shown).

These results suggest that exposure to high pressure can reduce the inhibitory effects that some compounds can have on the PCR. For the IPC of the qPCR assay of human DNA and STR typing results, there was a general increase in performance observed based on yield of PCR product. The increase in yield effectively increased the available template DNA accessible in the PCR. For very limited samples that may contain inhibitory compounds, pressure treatment may be a more viable approach than sample dilution, one of the current methods of reducing inhibition.

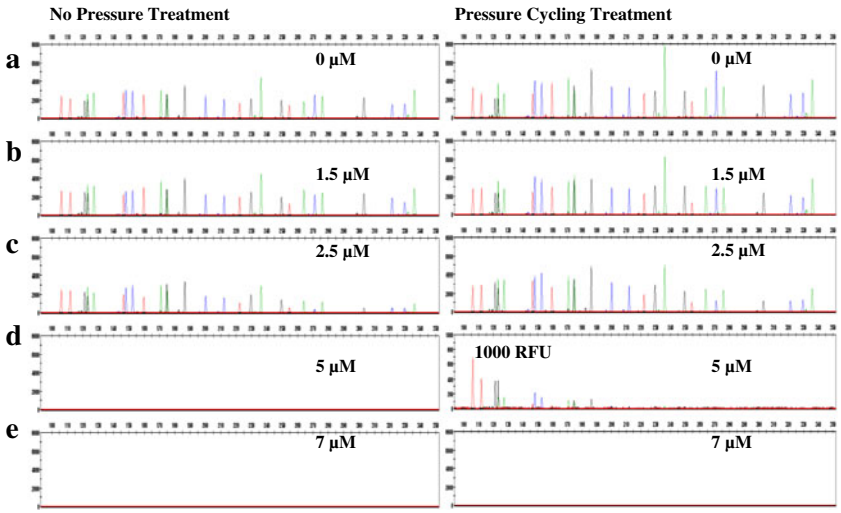

Fig. 3 Effect of PCT on hematin inhibition. Nonpressure-treated samples (*left panel*) and pressure-treated samples (*right panel*). Concentrations of hematin are 0 μM (**a**), 1.5 μM (**b**), 2.5 μM (**c**), 5 μM (**d**), and 7 μM (**e**)

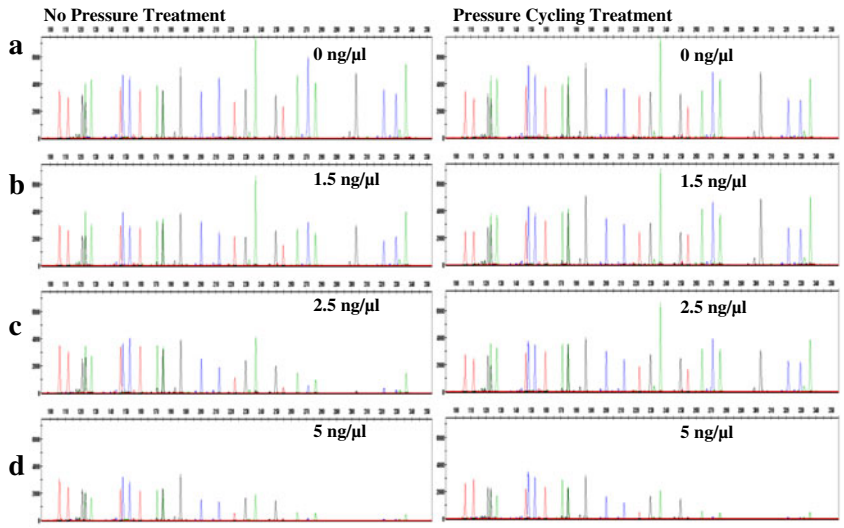

Fig. 4 Effect of PCT on humic acid inhibition. Nonpressure-treated samples (*left panel*) and pressure-treated samples (*right panel*). Concentrations of humic acid are 0 ng/µL (**a**), 1.5 ng/µL (**b**), 2.5 ng/µL (**c**), and 5 ng/µL (**d**)

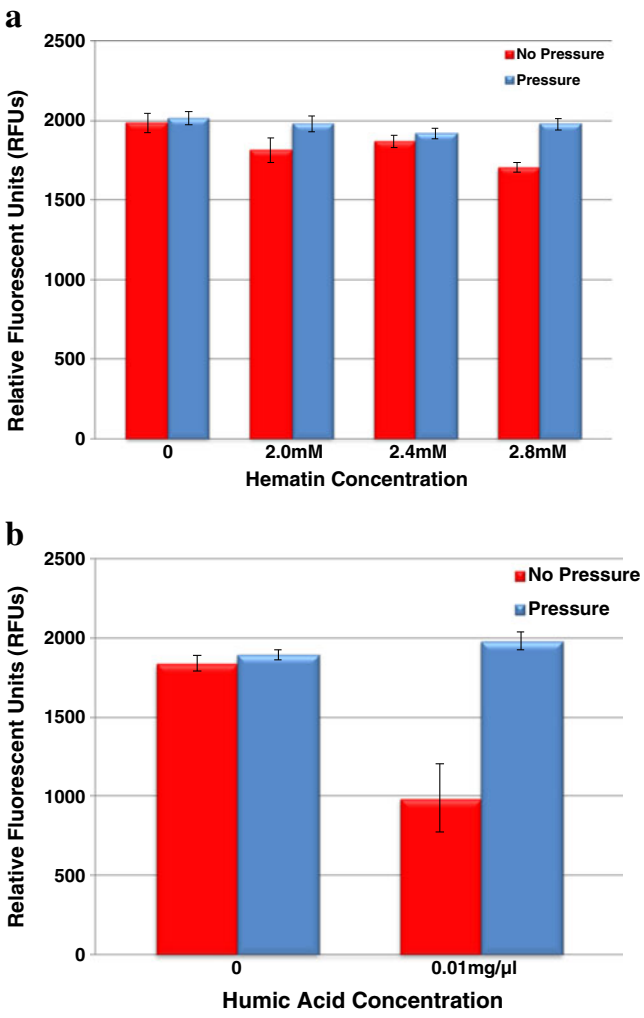

Fig. 5 Effect of PCT on average STR peak heights of inhibited samples. Hematin (**a**) and humic acid (**b**). Pressure-treated samples (*blue*) were compared with nonpressure-treated controls (*red*)

Elucidating a possible mechanism of increased DNA tolerance with pressure cycling

Considering possible Diels–Adler reactions in aggregated phenolics or porphyrins, we hypothesized that pressure acts in a selective way to remove these compounds from solution, resulting in a decrease in the amount of PCR inhibitors present in the reaction mixture. The effect of ethyl alcohol (EtOH) was tested by subjecting the inhibitors and DNA to pressure cycling in the presence or absence of a final concentration of 10 % EtOH. Two concentrations of hematin (0 and 2.5 µM) or humic acid (0 and 2.5 ng/µL) were added to 1 ng/µL of DNA in a final volume of 100 µL, placed in microtubes and subjected to PCT or no pressure. Samples containing 10 % EtOH or no EtOH were prepared for pressure and NPC samples. Subsequently, 1 µL of each sample was amplified using the AmpFlSTR® Identifiler® PCR Amplification Kit and then typed for STRs. Figures 7 and 8 show representative electropherograms of STR typing results for hematin and humic-acid-treated samples, respectively. For hematin-treated samples, the addition of alcohol reduced the ability to detect DNA for NPC samples, while pressure-treated samples showed slight increases in RFU values for larger amplicon loci (Fig. 7). For humic-acid-treated samples, the addition of alcohol greatly reduced DNA recovery for NPC samples; however, the addition of alcohol in pressure-treated samples yielded full DNA profiles compared with PCT alone (Fig. 8). These observations support the hypothesis, but more rigorous mechanistic studies are needed to elucidate the mechanism.

Challenged bone sample processing with pressure cycling technology

To demonstrate the potential of PCT on the forensic analysis of casework samples, three human bones were processed

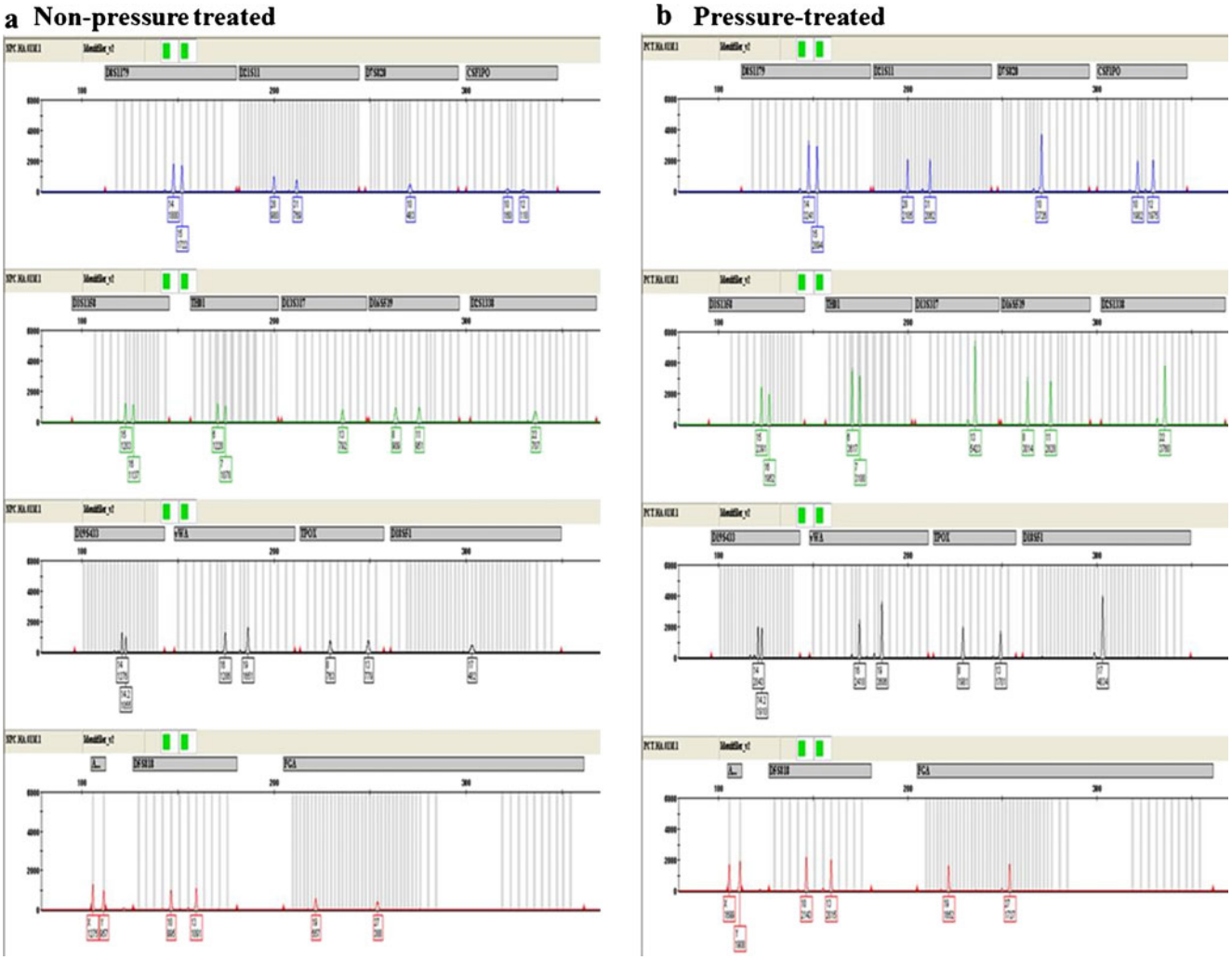

Fig. 6 Effect of PCT on Humic Acid Inhibition. Non-pressure treated sample (**a**) and pressure-treated sample (**b**). Concentration of humic acid is 0.01 mg/μL

with and without PCT and the results compared. Approximately 0.2 g of bone powder for each of the three bones was placed in separate PULSE tubes. Approximately 0.5 g of bone powder was used for the NPC samples, which were placed into PULSE tubes. The bones were incubated with extraction buffer at 56 °C with constant agitation for either 2 h or overnight. Following incubation, pressure-treated samples were subjected to PCT, and subsequently, DNA was extracted from all samples. Three elutions were collected for each bone sample. Each elution then was analyzed using qPCR. Table 2 shows the DNA quantity (ng/μL) for each elution and the elution volume for the bones incubated overnight. No shifts in IPC were observed for any of the bone samples either pressure-treated or NPC (data not shown). For samples with overnight incubation, two out of three PCT-treated samples yielded higher total DNA values (using two and a half times less bone powder). A 50 % increase in DNA yield was observed for bone 1 and a 33 % increase in bone 2 when subjected to PCT compared with NPC samples. Little difference was observed for bone 3.

Following qPCR, the first elution of each sample was amplified (for bones yielding <0.1 ng/μL, 10 μL was amplified; bone 2 elutions were normalized to 1 ng/μL) and typed for STRs using the AmpFlSTR® Identifiler® PCR Amplification Kit. Table 3 shows the total RFUs in each bone sample, for both 2 h and overnight incubation, and the number of alleles detected. Bone 2, which yielded high DNA quantities, also yielded full profiles for both pressure and NPC samples. For bones 1 and 3, pressure-treated samples yielded higher total RFU values as well as a greater number of alleles detected, for both 2 h and overnight incubation groups.

Shifts in the IPC for the three bone samples were not observed, which was somewhat in contrast with the Identifiler® STR results that indicated the presence of a PCR

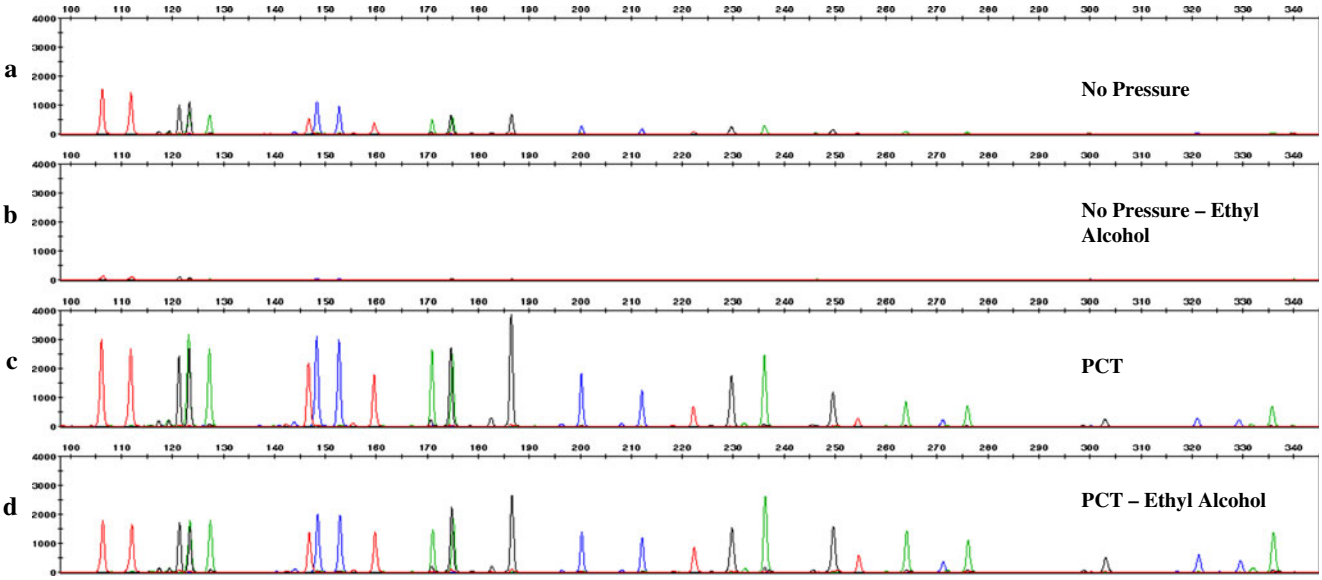

Fig. 7 Effect of ethyl alcohol addition on hematin inhibition. Nonpressure-treated samples containing 1 ng/μL control DNA and 2.5 μM hematin, without (**a**) and with (**b**) addition of 10 % ethyl alcohol. Pressure-treated samples (PCT) containing 1 ng/μL DNA and 2.5 μM hematin, without (**c**) and with (**d**) addition of 10 % ethyl alcohol

inhibitor. A recent study by Amory et al. [37] suggested that an inhibitor may be present in some bone samples but not be indicated by the IPC. To test whether or not inhibitors were present in the bone samples, 0.5 ng/μL of Raji cell line DNA was added to the first elution bone extract, pressure treated and compared with a NPC sample. The samples then were amplified for STRs. The STR profile of the known cell line DNA displayed peak heights that were lower in the pressure and NPC samples, which is indicative of the presence of an inhibitor. However, the inhibition was less pronounced in the samples, which were subjected to PCT, noted in the RFU values and decreased allele/locus dropout compared with NPC samples (data not shown).

As a final experiment, two additional human bones were processed with and without PCT and the results compared. Approximately 0.2 g of bone powder for each bone was placed in separate PULSE tubes. Two different quantities of bone powder were used for the

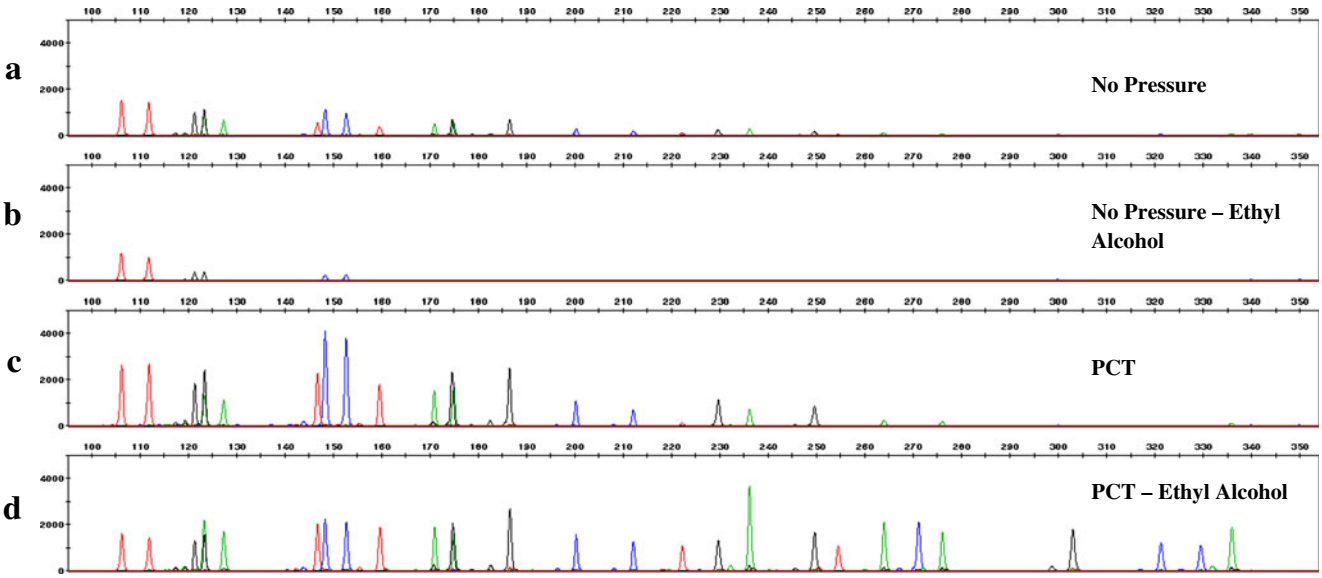

Fig. 8 Effect of ethyl alcohol addition on humic acid inhibition. Nonpressure-treated samples containing 1 ng/μL DNA and 2.5 ng/μL humic acid, without (**a**) and with (**b**) addition of 10 % ethyl alcohol. Pressure-treated samples containing 1 ng/μL DNA and 2.5 ng/μL humic acid exposed to PCT, without (**c**) and with (**d**) addition of 10 % ethyl alcohol

Table 2 DNA yield from bone processed with and without PCT

	Sample	Elution number	Quant value (ng/μL)	Elution volume (μL)	Total yield (ng)
Bone 1	Pressure	1	0.052	86	9
	Pressure	2	0.064	78	
	Pressure	3	0.000	96	
Bone 1	No pressure	1	0.057	73	6
	No pressure	2	0.021	78	
	No pressure	3	0.005	110	
Bone 2	Pressure	1	58.200	68	8702
	Pressure	2	37.150	107	
	Pressure	3	8.950	86	
Bone 2	No pressure	1	51.780	88	6084
	No pressure	2	15.220	80	
	No pressure	3	3.870	80	
Bone 3	Pressure	1	0.038	71	4
	Pressure	2	0.004	110	
	Pressure	3	0.005	96	
Bone 3	No pressure	1	0.021	81	3
	No pressure	2	0.010	96	
	No pressure	3	0.000	79	

NPC samples, 0.2 and 0.5 g, which then were placed into PULSE tubes. The bones were incubated with extraction buffer at 56 °C with constant agitation overnight. Following incubation, pressure-treated samples were subjected to PCT, and subsequently, DNA was extracted from all samples. Three elutions were collected for each bone sample. Each elution then was analyzed using qPCR. No shifts in IPC were observed for any of the bone samples either pressure-treated or NPC (data not shown).

Following qPCR, the first elution of each sample was amplified (all samples yielded <0.1 ng/μL, 10 μL were amplified) and typed for STRs using the AmpFlSTR® Identifiler® Plus PCR Amplification Kit. Electropherograms for bone 5 are shown in Fig. 9 for 0.5 g NPC (Fig. 9a), 0.2 g NPC (Fig. 9b), and 0.2 g PCT (Fig. 9c). For bone 5, 0.2 g of bone powder for the NPC sample failed to produce a profile. When 0.5 g of bone powder was used for the NPC sample as compared with 0.2 g for PCT, pressure-treated samples yielded significantly higher total RFU values as well as a greater number of alleles detected. Bone 4 failed to produce a profile for the NPC sample and yielded only four loci for the pressure-treated sample (data not shown).

Table 3 STR allele peak height from bone processed with and without PCT

	Sample name	Incubation time	Elution 1 DNA quantity (ng/μL)	Total RFUs in sample	Number of alleles detected
Bone 1	No pressure	Hours	2 h	2,516	12
	Pressure	Hours	2 h	2,734	21
	No pressure	Overnight	Overnight	2,460	15
	Pressure	Overnight	Overnight	3,693	26
Bone 2	No pressure	2 h	34.06	11,137	25
	Pressure	2 h	27.17	16,326	25
	No pressure	Overnight	51.8	7,005	25
	Pressure	Overnight	58.2	8,614	25
Bone 3	No pressure	2 h	0.045	736	8
	Pressure	2 h	0.048	1,917	21
	No pressure	Overnight	0.021		6
	Pressure	Overnight	0.038	612	8

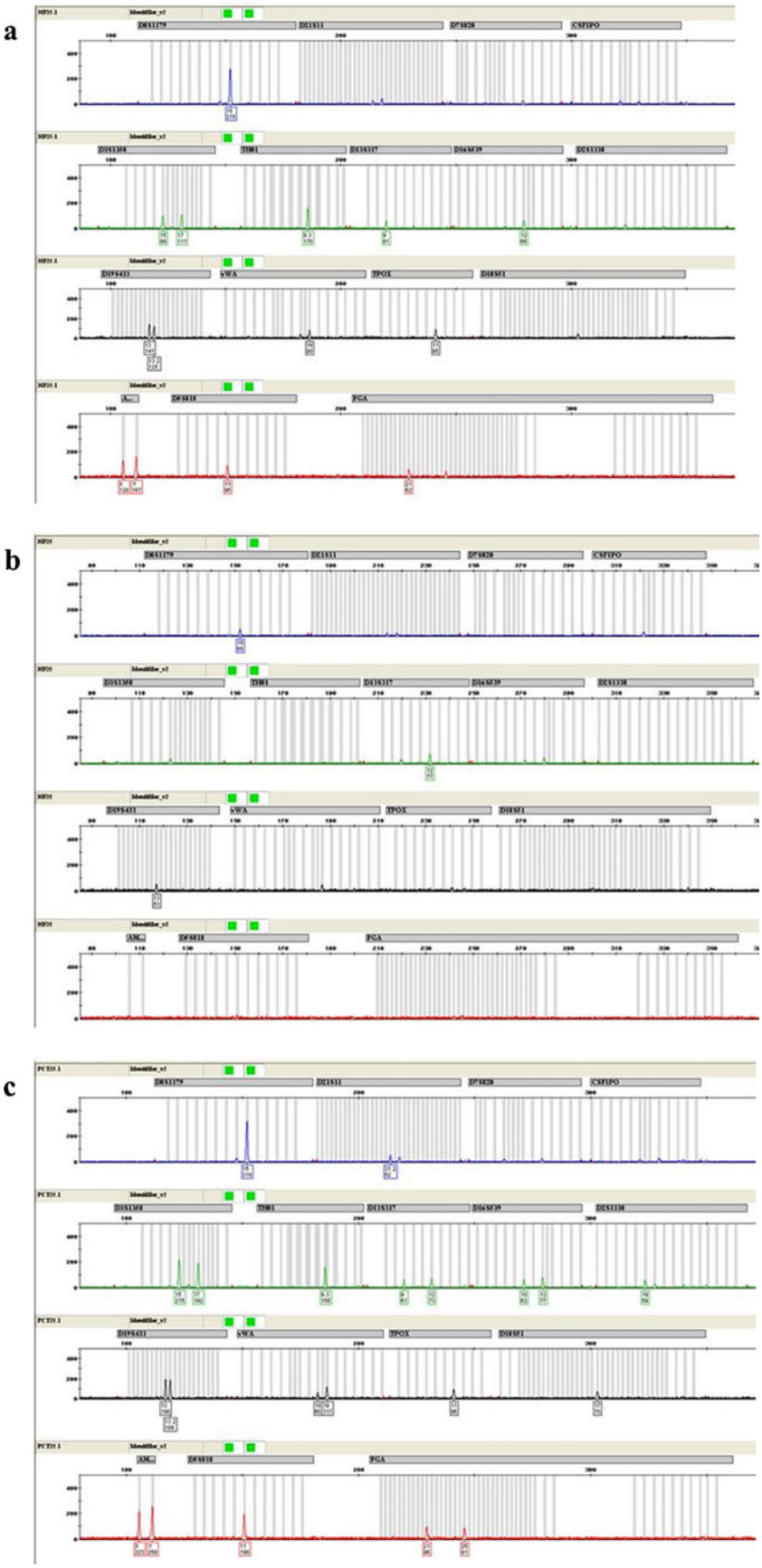

Fig. 9 Effect of PCT on human bone processing. Nonpressure-treated samples containing approximately **a** 0.5 g bone powder and **b** 0.2 g bone powder. Pressure-treated sample containing approximately **c** 0.2 g bone powder

Conclusions

This study shows potential enhanced PCR efficiency for samples containing an inhibitor when PCT treated compared with those samples not exposed to PCT. These results are a proof of concept that PCT may be a viable method to overcome the inhibitory effects on PCR of hematin and humic acid. This research study suggests that PCT potentially has applications for forensic DNA analysis of certain challenged forensic DNA samples by reducing the effects of inhibitors known to be present in some bone samples. Future research will focus on elucidating the mechanism(s) that overcomes the effect of inhibition.

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Conflict of interest The authors PLM, JLK, and BB declare that they have no conflict of interest. Authors NPL, AL, and VSG are employed by Pressure Biosciences Incorporated.

Ethical standards The work described above was performed in accordance with all laws (both Federal and State) that apply to research, researcher conduct, and the protection of human test subjects. We also operate under the guidance of and in accordance with the policies of the UNTHSC Institutional Review Board.

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