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Pressure Cycling Technology (PCT) Reduces Effects of Inhibitors of the PCR

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ORIGINAL ARTICLE

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

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Pressure cycling technology (PCT)

- Rapid cycles of hydrostatic pressure between ambient and ultra-high levels controls bio-molecular interactions
- During exposures to multiple cycles of pressure, biomolecules such as nucleic acids, proteins, lipids, and small molecules can be extracted into a lysis buffer from cells and tissues

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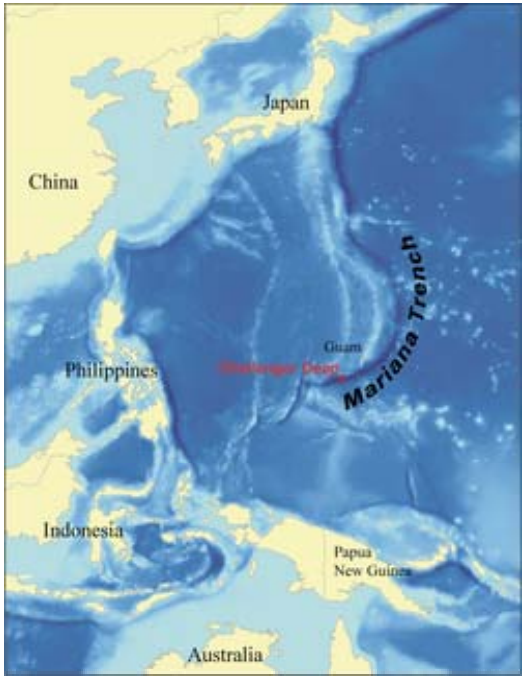
Mariana Trench



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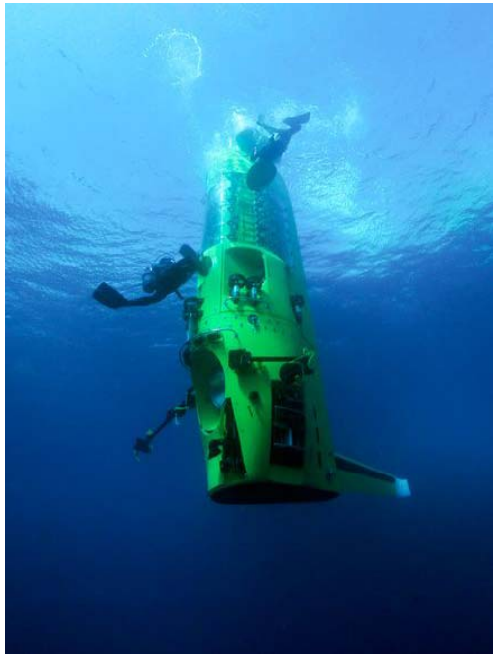
- Cinematographer and Explorer James Cameron piloted a custom designed submersible to the deepest part of the ocean, the bottom of the Mariana Trench.
- At 35,576 feet deep and 8 tons per square inch in pressure, that's about 1/3 of the pressure generated by the PBI Barocycler.



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PCT

- While investigating the use of PCT to attempt to increase DNA yield in challenged samples, we observed that PCT reduced the effects of inhibition on downstream DNA analyses.
- I hypothesized that conditions of extreme pressure alter the conformation of some inhibitors, rendering them less effective at reducing the yield of PCR products in forensic DNA analysis.

Pressure Cycling Equipment and Consumables

Barocycler® NEP3229

- Bench top instrument
- Capable of processing up to three samples simultaneously using PULSE Tubes or up to 48 samples using MicroTubes

Disc-less PULSE Tubes

- Pressure Used to Lyse Samples for Extraction hold up to 1.2 mL
- During the PCT process, the Ram compresses the sample and processing buffer against the inside of the Cap, resulting in pressure being transmitted to the sample

PCT MicroTubes with Cartridge Adapter

- Specially designed single use tubes are available in three sizes (50, 100, and 150µl)

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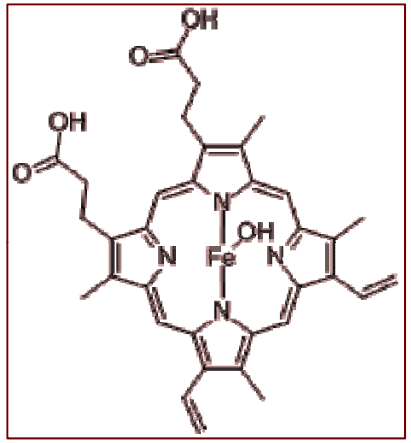
DNA INHIBITORS

Two potent PCR inhibitors, hematin and humic acid, were evaluated

Hematin

- Metal chelating molecule found in RBCs
- Forms a stable complex with the DNA polymerase
- May also cause a dissociation of the DNA-polymerase complex, thereby inhibiting polymerase activity



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Humic acid

- Component in soil
- Often encountered in samples that have been buried, such as skeletal remains
- Proposed mechanisms: chelates with magnesium ions needed for DNA polymerase or inhibits the PCR via sequence specific binding to DNA, thus limiting the amount of available template

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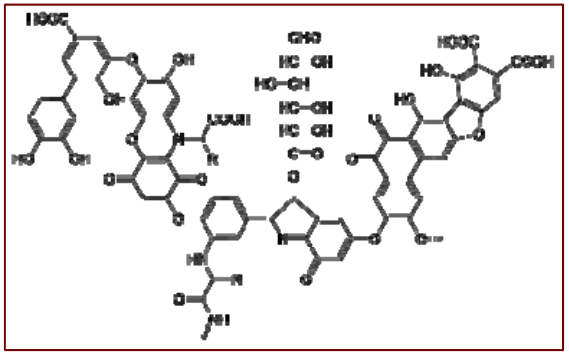

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Experimental design

Absence of DNA

- PCT was applied to stock solutions of hematin and humic acid and IPC values were compared among pressured and non-pressured samples

Presence of DNA

- PCT was applied to stock solutions of hematin and humic acid and added to 1ng/µl of DNA in concentrations of 0, 1.25, 2.5, 5, and 7 µM for hematin and 0, 1.25, 2.5, 5, and 7 ng/µl for humic acid
- PCT was applied to *mixtures* of 1ng/µl of DNA and several 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)

To assess the effects of these inhibitors on the PCR process, DNA quantity by qPCR and STR typing results of samples subjected to pressure cycling were compared with no pressure controls.

Materials & Methods: DNA

- All experiments were performed using the Quantifiler® Human DNA Quantification Kit (Life Technologies, Carlsbad, CA) Raji DNA Standard (200ng/µL purified DNA standard).
- The standard was diluted to a final concentration of 1ng/µl for each experimental sample.
- Experiments were performed in replicates of either three, five, or twenty, depending on the experiment.

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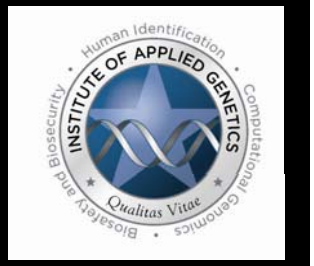
Materials & Methods: Inhibitor Preparation



Hematin

- Porcine hematin (Sigma-Aldrich) was dissolved in 0.1 N NaOH (FisherChemical) to a final stock concentration of 84.5µM
- Hematin was added to samples to final concentrations of: 0, 1.25, 2.5, 5, or 7µM

Humic Acid

- Technical grade humic acid (Sigma-Aldrich) was dissolved using TE^{-4} buffer (10 mM Invitrogen UltraPure™ Tris-HCL, pH 8.0 and 0.1 mM GIBCO UltraPure™ EDTA, pH 8.0) to a final stock concentration of 500ng/µl
- Humic acid was added to samples for final concentrations of: 0, 1.25, 2.5, 5 or 7ng/µl

PCT Parameters

- Samples undergoing PCT were placed in either FT500-ND PULSE tubes or MicroTubes, depending on the experiment.
- Samples were transferred to the Barocycler® NEP3229 and subjected to 30 cycles of alternating pressures consisting of 35 kpsi for 20 seconds and ambient pressure for 10 seconds.
- No Pressure Cycle (NPC) controls also were prepared. NPC samples were placed in either PULSE or MicroTubes, depending on the experiment.

Quantification, Amplification & STR Typing

Quantification: Quantifiler® Human DNA Quantification Kit on the ABI 7500 Real-Time PCR System

Amplification: 1 µl of each extract was amplified using AmpFISTR® Identifiler® PCR Amplification Kit

STR Typing

- Separation and detection performed on AB 3130xl Genetic Analyzer (10 s at 3 kV; POP-4)
- AB 3130xl Genetic Analyzer Data Collection Software 3.0
- GeneMapper® ID software v3.2.1
- The detection and interpretation thresholds were both set at 50 relative fluorescence units (RFU)

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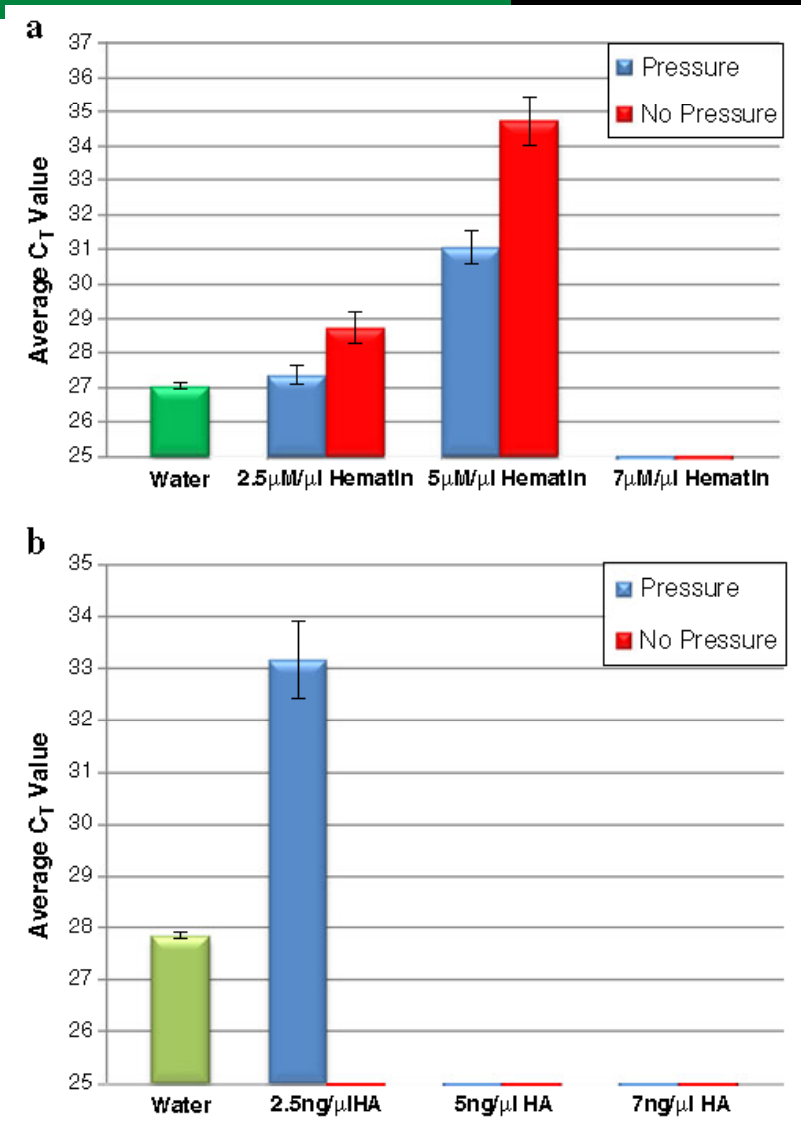
Effect of PCT on IPC values of Inhibitors

1mL of hematin (84.5µM stock) or humic acid (500ng stock) were subjected to barocycling. Various concentrations of hematin and humic acid were then added to the Quantifiler master mix and quantified using ABI 7500 Real Time PCR System. Nuclease free molecular biology grade water was also used as a control. The IPC values for each concentration are shown. Pressure samples were compared with non-pressured controls in replicates of 20.



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Effect of PCT 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

1mL of hematin or humic acid was subjected to barocycling in PULSE tubes. Various concentrations of hematin and humic acid were then added to 50 µl DNA (1 ng/µl). Samples then were analyzed by qPCR. Samples were run in replicates of five. The average DNA recovery and average IPC C_T values for each concentration are shown.

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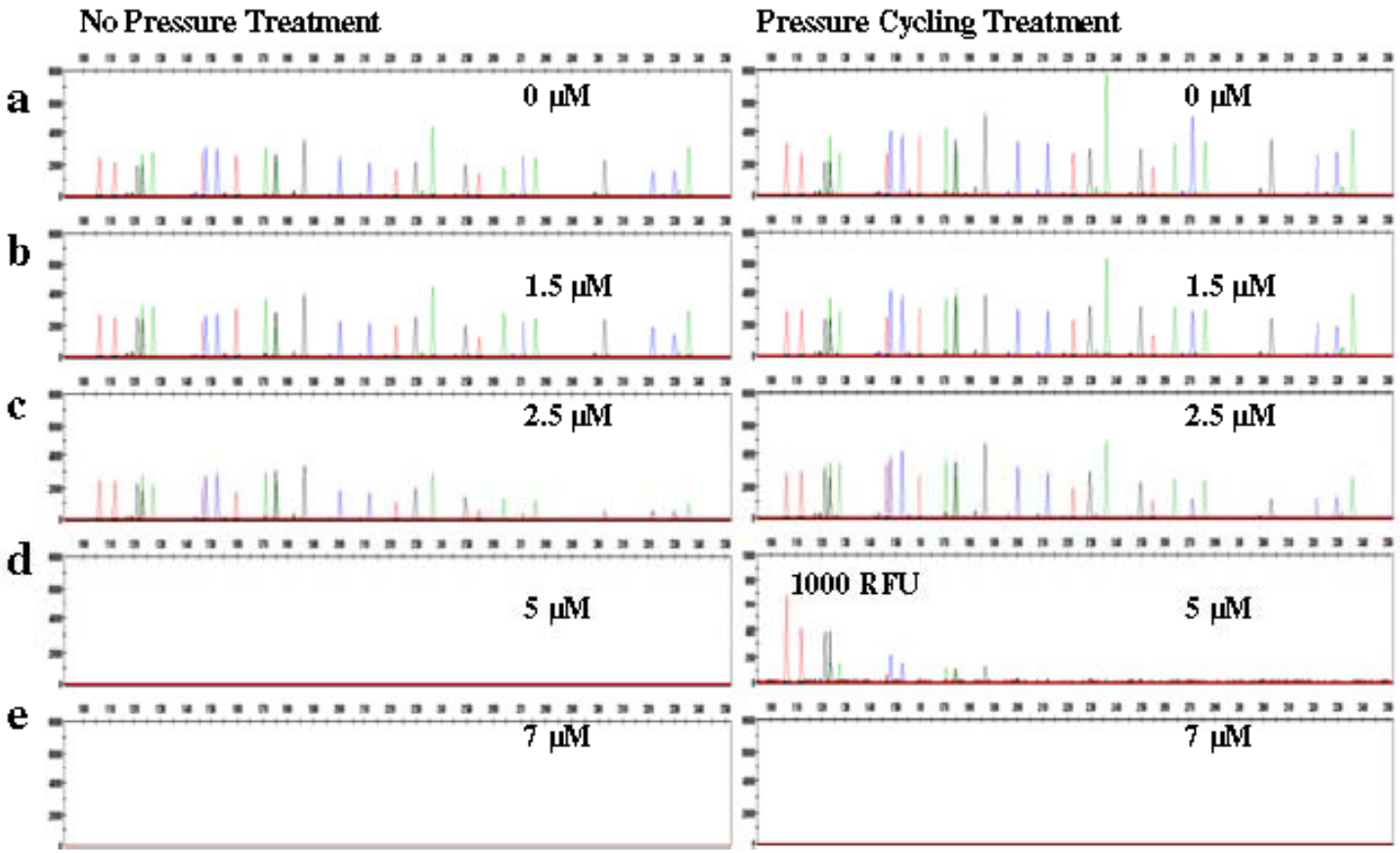
Effect of PCT on Hematin Inhibition

Hematin was added to 100µl DNA (1ng/µl), placed in MicroTubes and subjected to either barocycling or no pressure. 1µl of each sample was subsequently amplified and typed for STRs. Samples were run in replicates of five. Pressure samples were compared with non-pressured controls.



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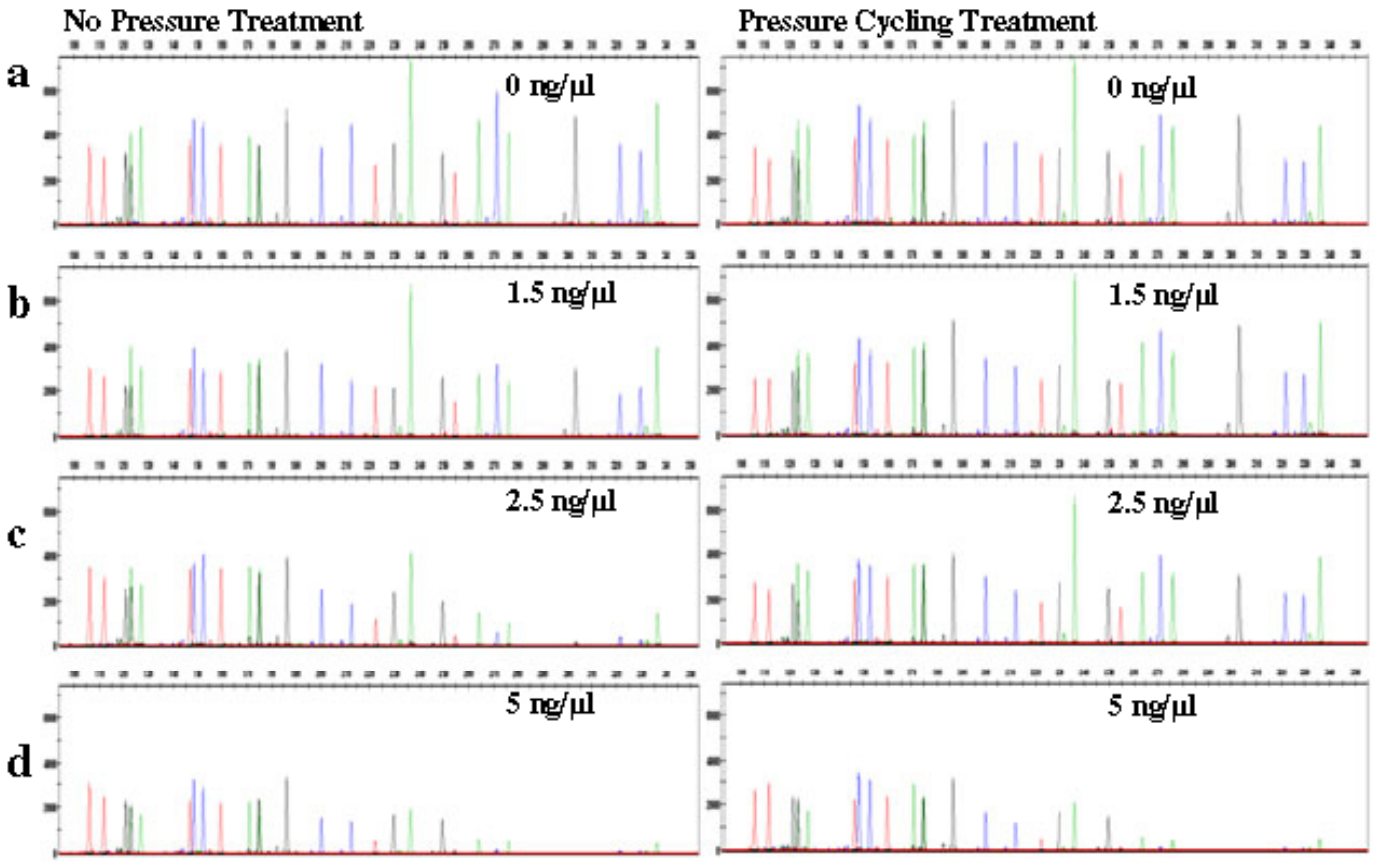
Effect of PCT on Humic Acid Inhibition

Humic acid was added to 100µl DNA (1ng/µl), placed in MicroTubes and were subjected to either barocycling or no pressure. 1µl of each sample was subsequently amplified and typed for STRs. Samples were run in replicates of five. Pressure samples were compared with non-pressured controls.



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Effect of PCT on average STR peak heights of inhibited samples

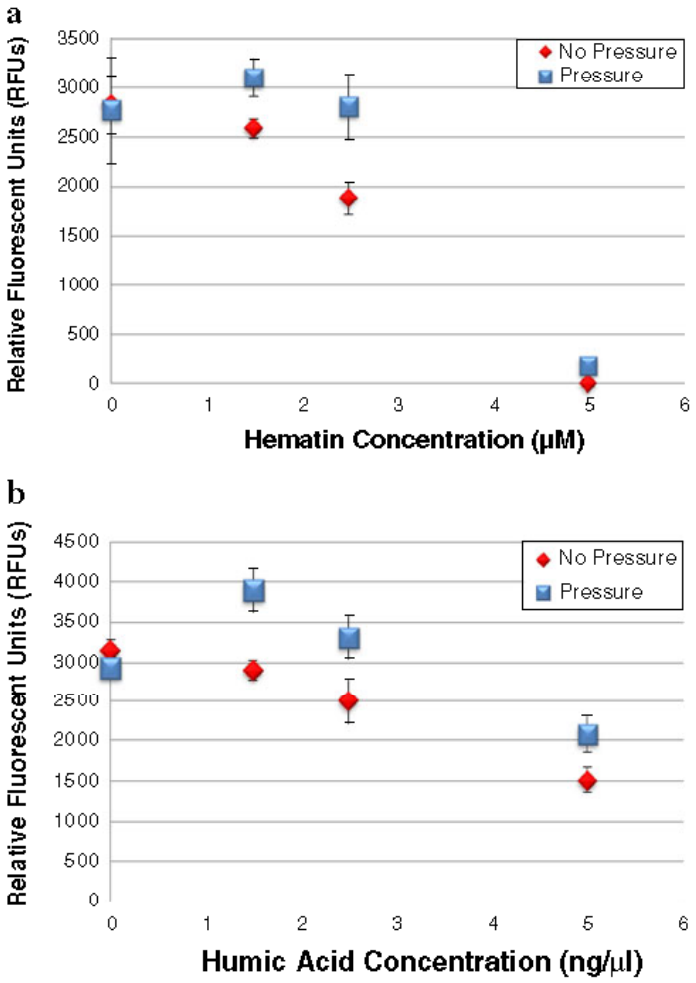

Fig. 2 Effect of PCT on average STR peak heights of inhibited samples. Hematin (**a**) and humic acid (**b**). Pressure-treated samples (*squares*) were compared with nonpressure-treated controls (*diamonds*)

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Elucidating a potential mechanism for PCT inhibitor tolerance

- Designed experiment to ascertain not only potential mechanisms of inhibitors but also the mechanism of how PCT is reducing effect of inhibitors:
  - Addition of 10% Ethyl Alcohol to Reaction
    - If Hematin and HA interact with DNA, then that interaction will be further disrupted by the addition of alcohol (reduced hydrophobicity and more potential for PCT to get to the inhibitor)
    - PCT may also impact the structure of the inhibitors themselves (ie by changing structure of hematin, PCT possibly reduces ability to chelate $Mg^{2}+$)

Effect of EtOH Addition on Hematin Inhibition

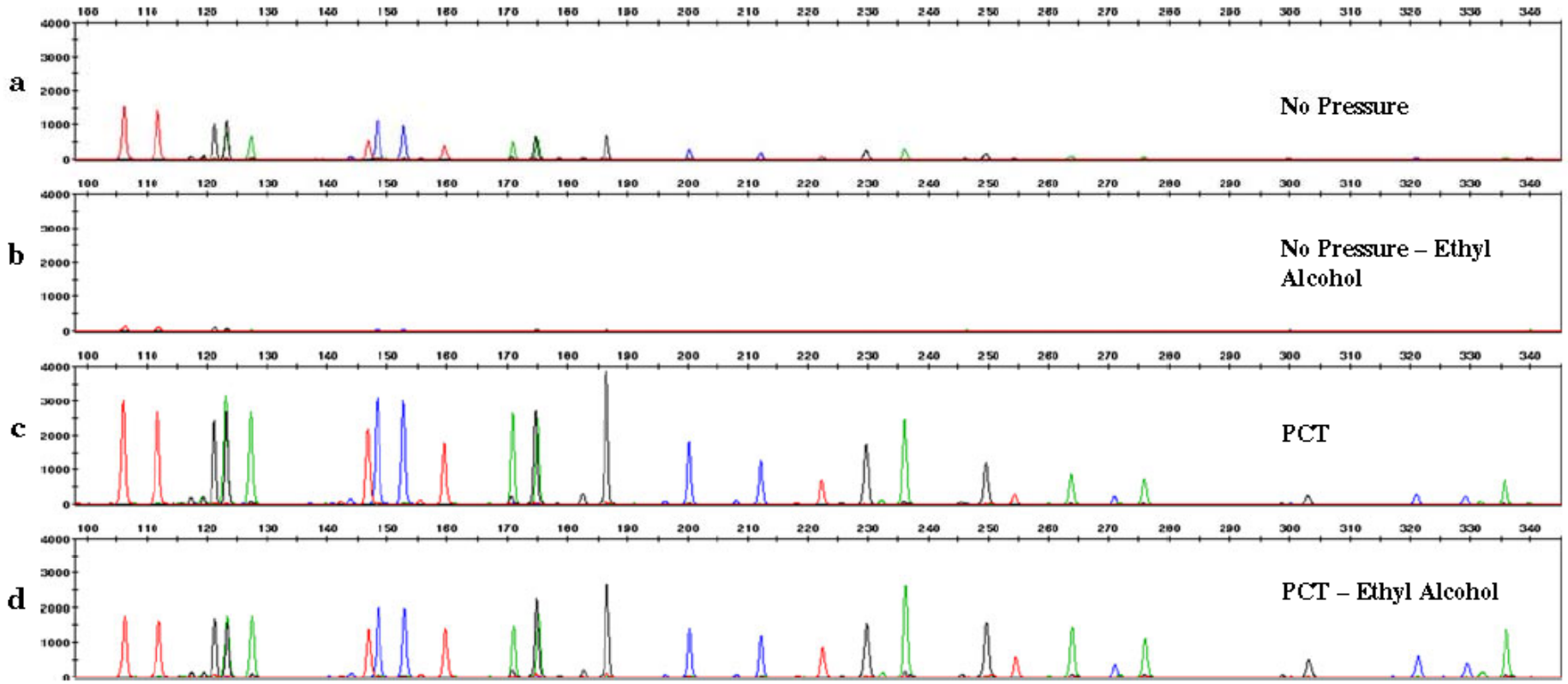

Fig. 7 Effect of ethyl alcohol addition on hematin inhibition. Nonpressure-treated samples containing 1 ng/μL control DNA and 2.5 μM hematin, without (**a**) and with (**b**) addition of 10 % ethyl alcohol. Pressure-treated samples (PCT) containing 1 ng/μL DNA and 2.5 μM hematin, without (**c**) and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

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Effect of EtOH Addition on Humic Acid Inhibition

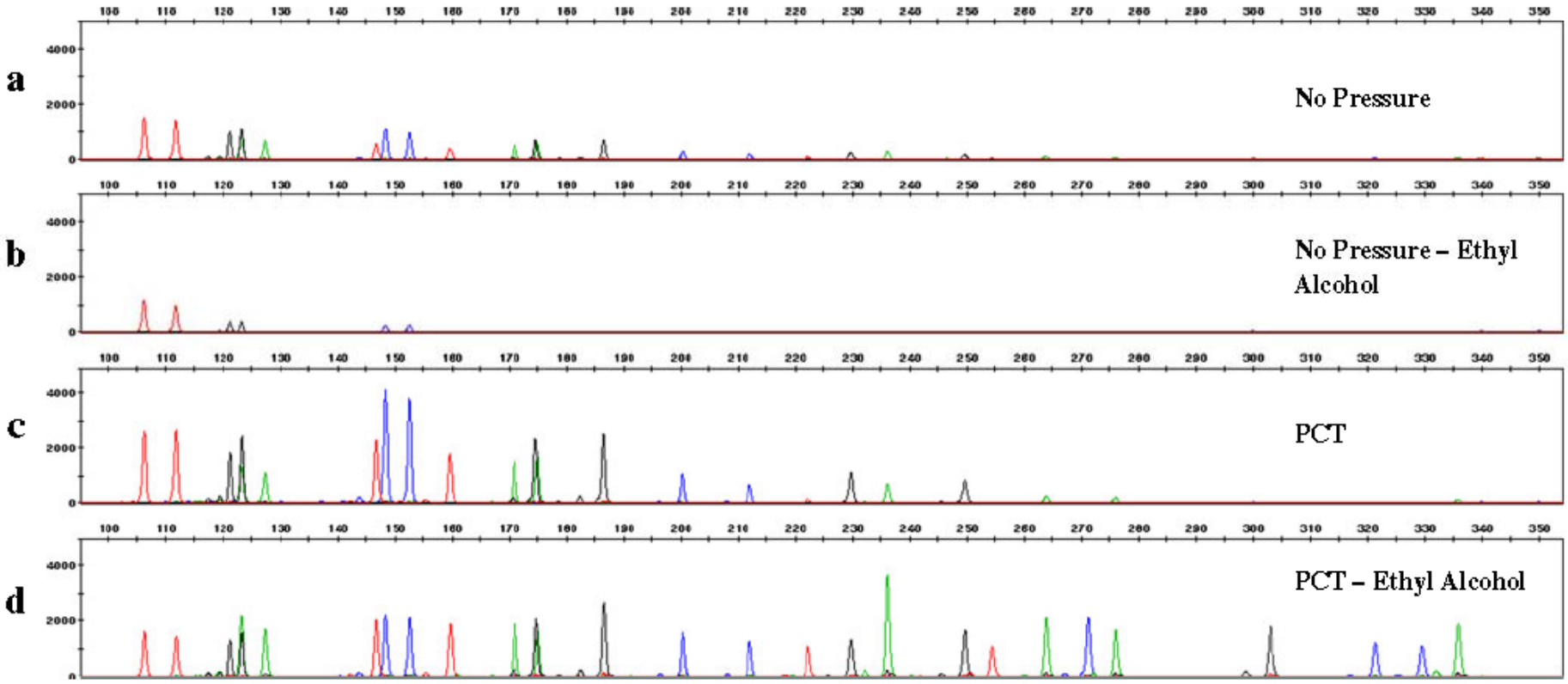

Fig. 8 Effect of ethyl alcohol addition on humic acid inhibition. Nonpressure-treated samples containing 1 ng/μL DNA and 2.5 ng/μL humic acid, without (**a**) and with (**b**) addition of 10 % ethyl alcohol. Pressure-treated samples containing 1 ng/μL DNA and 2.5 ng/μL humic acid exposed to PCT, without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

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Mock casework samples: Human Bones

- Originally, 3 human bones were selected
- 0.2g bone powder was used for PCT and NPC compared to original protocol using 0.5g bone powder
- 1ml EDTA/1% SLS + ProK
- Overnight (~16h) incubation at 56 degrees
  - For overnight samples, samples were pressured after overnight incubation and then extracted
- Following incubation, all samples were extracted using Hi-Flow Column Protocol
- Three elutions were collected; Following quantification, Elution 1 was used for Amp/CE
- Final study examined two additional bones with comparable results

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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	

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NPC vs PCT: Number of Alleles Detected

	Sample Name	Total RFUs in Sample	Average PH w/n Sample	Standard Deviation PH w/n Sample	# of Alleles Detected
Bone 1	NPC.2.001.1	2516	209.6666667	129.3208366	12
	PCT.2.001.1	2734	130.1904762	51.20216699	21
	NPC.24.001.1	2460	164	144.5426284	15
	PCT.24.001.1	3693	142.0384615	75.80843265	26
Bone 2	NPC.2.002.1	11137	445.48	221.8948925	25
	PCT.2.002.1	16326	653.04	335.1239174	25
	NPC.24.002.1	7005	280.2	147.6211367	25
	PCT.24.002.1	8614	344.56	179.605373	25
Bone 3	NPC.2.003.1	736	92	58.81690719	8
	PCT.2.003.1	1917	91.28571429	38.28987184	21
	NPC.24.003.1				
	PCT.24.003.1	612	102	76.57153518	6

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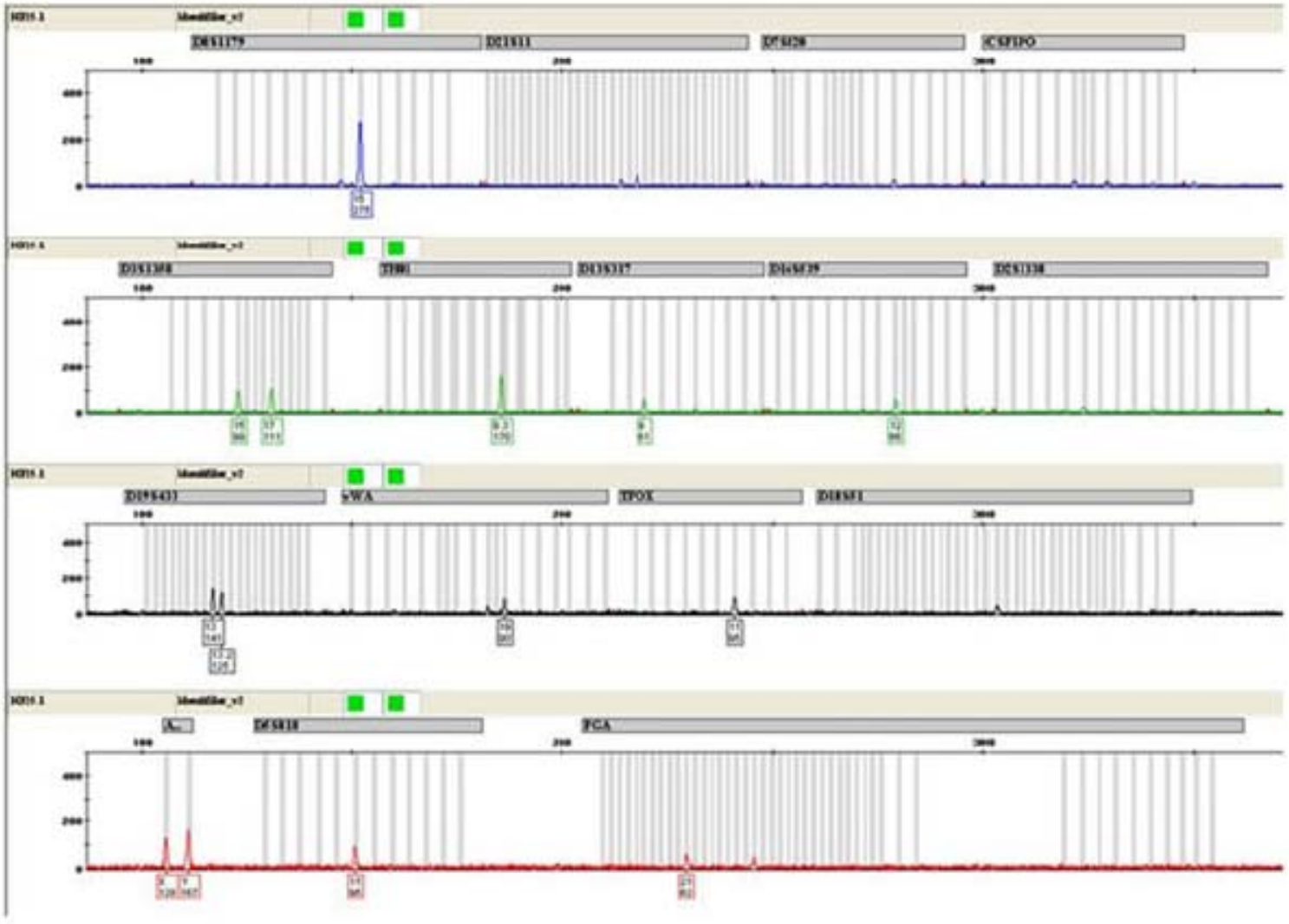
Effect of PCT on Human Bone Processing

Non Pressure Treated Sample Containing 0.5g Bone Powder



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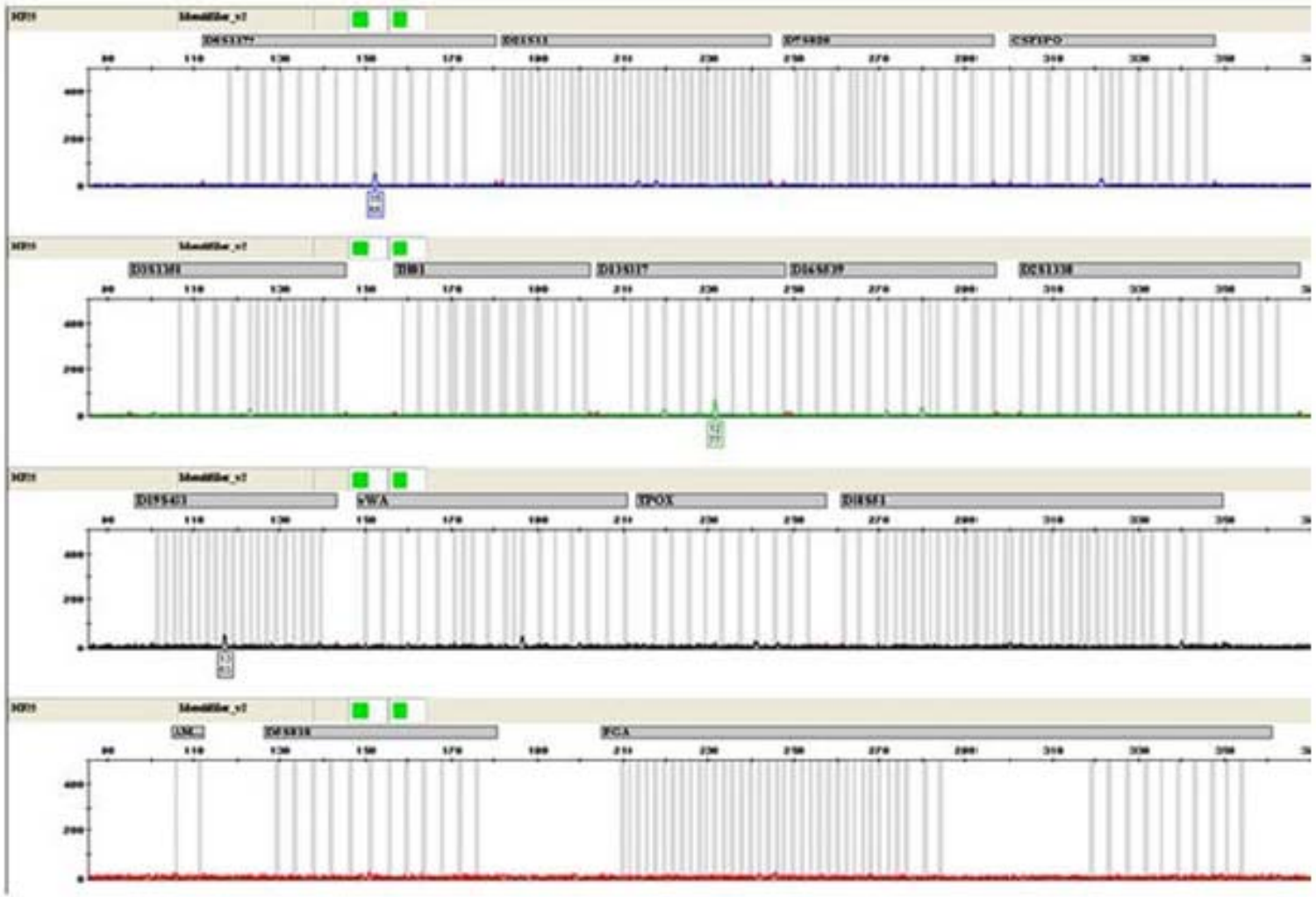
Effect of PCT on Human Bone Processing

Non Pressure Treated Sample Containing 0.2g Bone Powder



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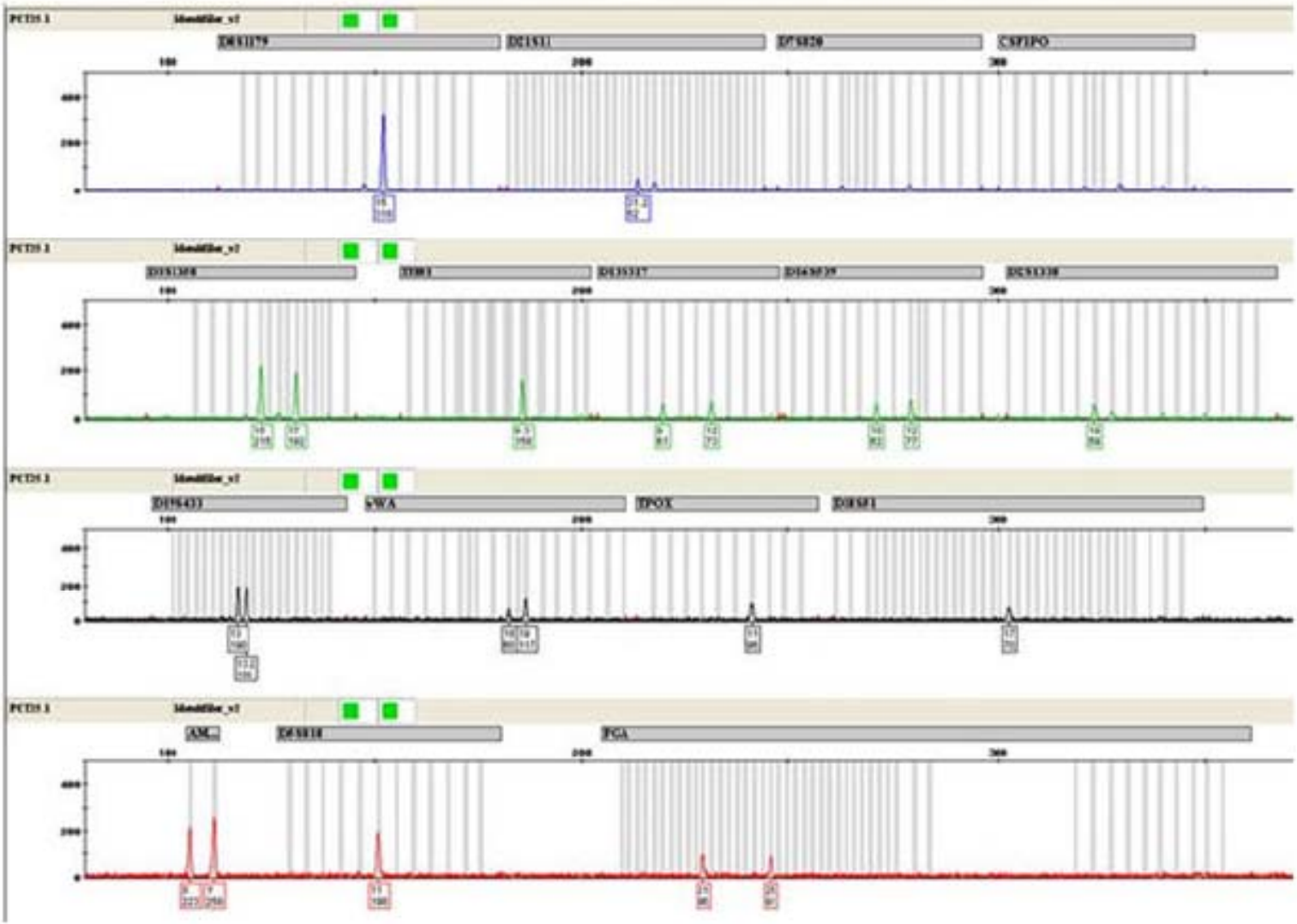
Effect of PCT on Human Bone Processing

Pressure Treated Sample Containing 0.2g Bone Powder



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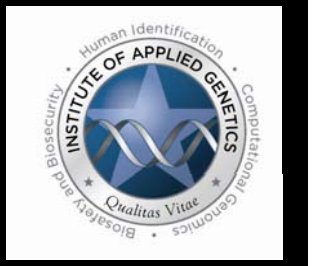
Discussion

- The results support that pressure cycling technology (PCT) reduces inhibitory effects and thus enhances yield of amplified products of both hematin and humic acid inhibited samples.
- Based on the results obtained in this study, this method can improve the recovery of alleles from challenged or inhibited DNA samples.

Conclusions



- Results demonstrate enhanced PCR efficiency for inhibited samples following PCT compared with those samples not exposed to pressure technology.
- These results indicate as a proof of concept that PCT is a viable method to overcome the inhibitory effects of hematin and HA on the PCR.
- This research study demonstrates the capabilities and potential of PCT applications for forensic DNA analysis of biological evidentiary samples.

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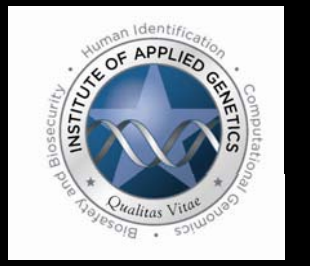
NIJ Acknowledgments



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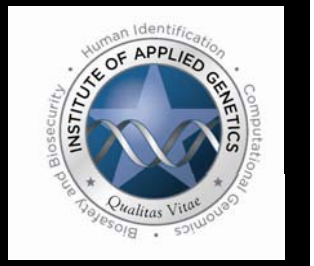

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- Alexander Lazarev
- Vera Gross

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