Document Title:	**Use of Pressure Cycling Technology to Enhance DNA Yield and Profile Success in Touch Samples**
Author(s):	**Rebecca Schultheiss Mikulasovich M.S., Michael Donley, M.S., Mark Powell, M.S., Roger Kahn, Ph.D.**
Document Number:	**251813**
Date Received:	**July 2018**
Award Number:	**2011-DN-BX-K554**

Use of Pressure Cycling Technology to Enhance DNA Yield and Profile Success in Touch Samples

2011-DN-BX-K554

Rebecca Schultheiss Mikulasovich MS, Michael Donley MS, Mark Powell MS and Roger Kahn PhD

Abstract

Touch DNA samples can provide useful evidence in criminal cases and the potential for touch DNA samples to aid investigations cannot be overstated. Not surprisingly, submissions of touch DNA samples to forensic laboratories continue to increase across the United States. Unfortunately, in many instances touch DNA samples do not yield sufficient DNA to produce interpretable results. Many strategies have been examined to help solve this problem. To date, profile improvement approaches such as longer injections and extra-cycle amplifications have been most utilized leading to concerns related to stochastic effects. An appealing way to improve DNA profiles from touch DNA samples would be to increase the recovery efficiency from DNA extraction. Pressure cycling technology (PCT) has been suggested as a way to do that. This study was undertaken to assess whether PCT could improve DNA recovery from low yield samples. PCT (Pressure BioSciences, South Easton, MA) is done in a Barocycler instrument that uses alternating periods of high hydrostatic and ambient pressure during DNA extraction. PCT is known to alter conformations and interactions of biomolecules and destabilize their secondary structures. Preliminary studies by other groups suggested that DNA yield increases of 20% to 100% could be observed when PCT was used during DNA extraction.

This project evaluated the integration of a PCT step into an existing DNA extraction process in two ways, first by attempting to determine the optimal time and temperature of pressure cycling and second by performing an internal validation with tests of sensitivity, reproducibility, mock casework samples and effects of inhibitors. In all studies the PCT treated samples were compared to equivalent untreated samples. Throughout the study, low amounts of DNA were used to mimic touch DNA samples.

The optimization study evaluated the variables of time, pressure and temperature. Parameters selected for validation closely matched the results of those of the untreated control samples. No significant differences were noted between pressure treated samples and untreated samples when comparing DNA profile success. There were limited indications that PCT improved results from mock touch casework samples but we were unable to demonstrate significant yield improvements.

Table of Contents

Executive Summary

Forensic DNA samples can yield human DNA ranging from many nanograms down to amounts not detectable by available methods. It is of interest to optimize the recovery of DNA from every sample. Efforts to improve the recovery of DNA from forensic samples have targeted every step of the DNA analysis process. In this project, we focused on improving the efficiency of recovery of DNA from touch DNA samples prior to extraction by using Pressure Cycling Technology, or PCT.

PCT uses ultra-high hydrostatic, hyperbaric pressure to reversibly alter the partitioning of nucleic acids between adsorbed and solvated phases relative to partitioning at ambient pressure. The goal of this project was to explore the operating parameters of PCT and to apply optimized PCT conditions to touch DNA samples in an effort increase DNA yields in comparison with standard methods with no additional pressure.

The PCT instrument, known as a Barocycler, operates by applying 35,000psi of pressure to samples for a designated time, forcing samples through a membrane while cycling between ambient and high pressure. A comparison of identical sample sets, one subjected to PCT and another subjected only to the current casework extraction protocol, was made by comparing IPC values, total RFU (relative fluorescent units), and total alleles obtained from each.

The study goals included:

1. Determining optimal Barocycler parameters to process low level samples.
2. Determining optimal incubation temperature on the Barocycler.
3. Performing a formal validation of the instrument to include:
    A. Sensitivity study
    B. Reproducibility study
    C. Mock casework sample study
    D. Inhibition study

The current HCIFS extraction standard is the QIAsymphony pretreatment protocol which consists of incubation at 56°C on a Thermomixer R with the addition of the QIAgen ATL buffer, QIAgen Proteinase K, and DTT. Samples are purified on the QIAsymphony SP robot and quantified using Quantifiler Duo® on the ABI 7500. Amplification is with ABI Identifiler Plus® and samples are injected on an ABI 3130*xl* Genetic Analyzer. Data is analyzed using GeneMapper ID v3.2.1.

A Pressure Biosciences, Inc. Barocycler NEP3229 was used for application of pressure cycling prior to pretreatment. The Barocycler replaced the Thermomixer R for sample incubation of the PCT treated sample sets and incubation at 56°C was completed simultaneously with the performance of pressure cycling.

Determination of the optimal parameters compared time at pressure versus number of cycles (Table 1). A series of DNA amounts, ranging from 1,000pg to 100pg, was prepared and subjected to variations in the number of cycles and time at maximum pressure and compared to an identical untreated DNA series.

Table 1: Pressure Cycling Run Parameters on the Barocycler NEP3229

Pressure	35k psi
Time at Pressure (T1)	20, 40, 60, 80 sec
Time at Ambient Pressure (T2)	10 sec
Cycle Number	20, 40, 60, 80
Temperature	Water bath (59°C); barocycler (62°C); chamber (56°C)

Varying the amount of time at pressure and number of pressure cycles did not lead to any significant differences when compared to results obtained from untreated control samples. The initial comparison, although not significant, indicated an improvement over the control samples; however, closer inspection of the control indicated possible inhibition. Another control was incorporated into the comparison and improvement was not indicated. The 20-20 parameters were selected for validation as the IPC results closely matched those of the untreated control samples (p value of 0.9). These parameters were used previously in studies by Budowle at al.[xx], as well.

The effect of changing the sample chamber temperature in the Barocycler was tested to determine if increasing the incubation temperature would increase DNA yield. A circulating water bath was used to test the incubation temperatures of 56°C, 75°C and 95°C. DNA amounts of 500pg and 100pg were prepared from donor saliva. Comparison of the amplification success of the three temperatures to that of the corresponding untreated control samples indicated 56°C was significantly better than 75°C or 95°C.

Barocycler parameters of 20-20 and 56°C were used for the validation studies. Of great interest was the sensitivity study of DNA recovered from touch samples; these samples tend to have very little DNA. Target amounts of DNA from saliva were prepared at 3,000pg, 1,000pg, 300pg, 100pg, 30pg, 10pg, 3pg, and 1pg in triplicate. Comparison of treated and untreated samples measured as profile success was mixed. Treated samples appeared to yield higher total RFUs but presented fewer alleles on average. None

of the results were significant, primarily due to imprecision in the quantitation results. Interestingly, the pressure treated samples did lead to a significant improvement in IPC when compared to the untreated samples, suggesting an improvement in Quantifiler Duo amplification efficiency even though yield improvements were not significant.

Reproducibility studies using target amounts of 250pg of saliva DNA from five different donors, did not indicate a significant difference in total RFUs, total alleles or sample IPC between treated and untreated samples.

To examine the potential benefit of PCT in mock casework touch samples, swabs were collected from several typical objects encountered in casework such as cups, phones and keyboards. Although none of the results from the comparisons were significant, some samples appeared to indicate some improvement over the corresponding untreated samples. More mock casework samples would have to be tested to further test the significance of the results.

Objects and swabs collected at a crime scene may contain inhibitors that may appear to reduce the amount of DNA recovered from touch DNA samples. PCT was evaluated to assess its effects on an inhibitor. Humic acid was pressure treated, serial diluted and added to previously extracted samples. The same process was performed using untreated humic acid to serve as a control.

Results indicated the untreated humic acid samples fared somewhat better when measured as total RFUs and total number of alleles detected when compared to the pressure treated humic acid samples. However, the differences were not statistically significant.

A comparison of IPC results indicated the untreated samples performed significantly better, i.e., the IPC was lower, for all inhibitor inputs except for samples at 0.125ug. All had p values <0.05 indicating a significant difference from the treated samples. The 0.125ug had a p value of 0.99 indicating a strong similarity to the treated samples.

There was a consistent lack of significance for most the differences from most comparisons. One reason for this is the imprecision of the DNA quantitation system, especially for low level samples[xiii]. This might be overcome by running many more samples. Within the framework of this study, significant benefits were not observed.

Introduction

A. Statement of the Problem

Touch DNA evidence is commonplace in all types of criminal investigations, especially in high volume crimes such as property crimes. Many of these samples fail to yield interpretable DNA results above stochastic thresholds. One approach has been to increase the cycle number in the polymerase chain reaction (PCR) or to increase the DNA signal through increased injection voltage or injection time. These methods do not overcome stochastic limitations, however. This project assessed the application of pressure treatment as potential way to improve extraction efficiency. The possibility of improving yields through improvements to recovery efficiency is of interest.

Current methods are more than adequate at extracting large amounts of DNA from typical biological materials. DNA can be lost in the extraction techniques currently in use, but for high level samples this is not an issue. For low template samples, such touched items, this is an issue. Any loss of DNA at recovery or extraction can reduce the chances of obtaining usable DNA results.

Pressure Cycling Technology or Treatment (PCT) was evaluated as a means to improve the yield of DNA. Preliminary work by others using Pressure Cycling Technology applied to touch DNA samples showed some promising results.

B. Literature Citations and Review

1. Efforts to Improve Touch DNA Success Rates

Because of the low success rate and the potential to solve numerous crimes from touch samples, many efforts have been made to increase the success rate of touch DNA. Research has focused on all aspects of DNA testing work flow.

A variety of techniques for improving the efficacy of collection of samples at the crime scene have been evaluated for ways to increase the amount of DNA yield. Methods tested included swabbing once with a moistened swab followed by a second swabbing with a dry swab, or using a swab moistened with 0.01 % sodium dodecyl sulphate [i] [ii] [iii] [iv] [v]. Multiple swab types are also in use, ranging from cotton to foam, and even tape to collect potential touch material from surfaces. [vi] [vii]. Improvements have been observed when comparing a single moistened swab to the double swab technique and the method is now in common use. The other methods, while demonstrating improvements for varying evidence types and surfaces, have not shown improvements across-the-board. These results indicate that improvements in collection alone may not significantly improve success rates for touch DNA.

Another approach to improving the success of touch DNA analysis is to increase the number of cycles in amplification. Initially used by the Forensic Science Service in the UK, it is known as low copy number (LCN) or low template DNA analysis. The Forensic Science Service increased the cycle number to 34 from their standard 28 cycle amplification. [viii] While they were able to obtain DNA profiles from samples with too little DNA for success at 28 cycles, the method is not without controversy. Several issues come with the increased cycle number including allelic drop out, where one or both alleles at a heterozygous locus fail to amplify due to stochastic effects [ix] and allelic drop-in, which is a result of increased stutter or contamination from the crime scene or the laboratory.[x] [xi]

LCN was first called into question in testimony in 2007 when a United Kingdom court was reviewing a case from 1998. In that year, the city of Omagh in Northern Ireland experienced a terrorist bombing in a busy market area which killed 29 people and injured 200 others. Sean Hoey, a 38 year old electrician, was arrested and placed on trial for the murders based, in part, on DNA evidence. Justice Weir, the judge in the case, was critical of the handling of the DNA evidence. In particular, Justice Weir remarked that the crime scene investigators, police, and forensic laboratory did not take appropriate protective precautions for LCN typing[xii] [xiii]. The criticism illustrates the great care and expertise required of law enforcement and forensic science personnel when the power of LCN is brought to bear on criminal cases. Although LCN is in use in several crime laboratories throughout the world, its use in the United States is very limited. It has been described by some members of the field as less robust than conventional DNA typing and not reproducible, suggesting its use will remain limited. [xiv]

Along with increasing the cycle number to improve the DNA results, methods have also been developed to boost the signal detected from low yield samples. Improving signal is achieved by removing ions, unused dNTPs and primers prior to CE or concentrating PCR product prior to CE [xv]. Although signal is improved by these techniques, stochastic effects remain as these are introduced during PCR and cannot be overcome by increasing the amount of PCR product that enters the CE.

2. Improving the Efficiency of DNA Extraction

Another opportunity to improve DNA results from low yield samples is prior to DNA extraction. We proposed to investigate whether improved DNA yields would result by integrating Pressure Cycling Technology (PCT) as an additional step prior to extraction. PCT work by others had shown some potential for improving the recovery of DNA from evidence samples with low amounts of DNA, although only a limited number of samples were tested. [xx]

PCT is based on the observation that ultra-high hydrostatic, hyperbaric pressure reversibly alters the partitioning of nucleic acids between adsorbed and solvated phases relative to partitioning at ambient pressure. The first step of PCT involves placing the sample (swab, tissue, bone, etc.) into a PULSE tube (Figure 1). Specimens are placed in the chamber of the PULSE tubes, assembled with a ram responsible for modulating pressure in the tube and then placed in the body of the tube along with a selected buffer or master mix. The PULSE tube is closed with a screw cap and placed into the pressure chamber of the Barocycler® (Figure 2).

Figure 1: Pulse tubes (FT-500ND)

Figure 2: Barocycler® NEP 3229

Once the tubes are inside the Barocycler, the pressure chamber is filled with distilled water and pressurization with compressed air begins. As the pressure increases, the movement of the ram within the body of the tube increases the pressure inside the tube, breaking up the cellular structures and releasing nucleic acids, small molecules, and proteins. The temperature, pressure and number of cycles can all be varied. The eluted nucleic acid molecules are then purified with traditional methods. At the Harris County Institute of Forensic Science (HCIFS) Forensic Genetics laboratory, samples are extracted using a QIASymphony SP with the QIASymphony Investigator Kit.

Initial research was performed on this technology appeared promising; a 100% increase in DNA yield via PCT from extracted bones was reported. Hair shafts also displayed similar improvements when extraction of mtDNA. Work on a fixed number of cells was reported to improve yield from 21% to 40%. [xvi]

PCT has reportedly been success in improving recovery of proteins. One study reported that PCT was more effective at lysing cells and improving protein recovery when compared sonication or the use of organic chemicals. [xvii]

Recently, Marshall et al. examined the effect of pressure treatment on on DNA testing inhibition and concluded that PCT offered a reduction in the effects of two PCR inhibitors, Hematin and Humic acid.[xviii]

Methods

A. DNA analysis of Samples

1. DNA Extraction and Purification

In accordance with standard HCIFS protocol, all consumables, including microcentrifuge tubes, PCR plates and the PULSE FT-500ND tubes, were autoclaved for two hours to reduce DNA contamination. DNA was isolated using the QIASymphony DNA Investigator Kit protocol (QIAgen). Five hundred microliters of a digestion buffer comprised of 400µL ATL buffer, 50µL QIAgen Proteinase K and 50µL of 1M DTT were added to a microcentrifuge tube containing the sample to be extracted and incubated for 15 minutes at 56°C on a Thermomixer R (Eppendorf) at 900rpm. A reagent blank consisting of digestion buffer was tested with each sample set with which it was pretreated. After incubation, samples were briefly centrifuged in a Centrifuge 5424 (Eppendorf) at maximum speed, 21,000 x g. If removal of a substrate was necessary, it was transferred to a spin basket using an autoclaved wooden stick and again centrifuged at maximum speed for 5 minutes. The spin basket was then removed and the samples subsequently purified on the QIASymphony SP (QIAgen) using the previously validated robot protocol for evidence samples.

2. DNA Quantification by Real-Time PCR

The amount of DNA in each sample was quantified using the Quantifiler® Duo DNA Quantification Kit (Applied BioSystems). Eight standards (50.0 ng/µL, 16.7ng/µL, 5.56ng/µL, 1.85ng/µL, 0.62ng/µL, 0.21ng/µL, 0.068 ng/µL, and 0.023ng/µL) were created via serial dilution of the Quantifiler® Human DNA Standard in the Quantifiler® Duo Dilution Buffer according to the Quantifiler® Kit User Manual and run in duplicate simultaneously with samples in a 96-well plate. Each reaction consisted of 6.25µL of the Quantifiler® PCR Reaction Mix, 5.25µL of the Quantifiler® DUO Primer Mix and 2uL of DNA extract, standard, or dilution buffer, for a total reaction volume of 13.5µL. Quantification plates were prepared using the Freedom EVO® 150 liquid handling system (Tecan). Real-time PCR was conducted on an ABI Prism® 7500 Sequence Detection System (Applied BioSystems). Cycling conditions for this assay were 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for one minute. Data were analyzed using the associated SDS Software (Applied BioSystems).

3. Autosomal STR Analysis

Samples were amplified using an AmpFℓSTR Identifiler® Plus PCR Amplification Kit. Identifiler® Plus reactions included 10µL of AmpFℓSTR PCR Reaction Mix and 5µL of AmpFℓSTR Identifiler® Plus Primer Set plus appropriate volumes of DNA sample and TE buffer for a total reaction volume of 25µL.

The volume of DNA added to each reaction was based on Quantifiler® DUO DNA quantification values, with a target amount of 0.75ng of DNA. In some instances, the DNA volume was maximized for those samples with low or undetectable DNA quantities. Amplification plates were prepared using the Freedom EVO® 150 liquid handling system (Tecan). PCR was conducted on a 9700 Thermal Cycler (Applied BioSystems). PCR cycling conditions were 95°C for 11 minutes, 28 cycles of 94°C for 20 sec followed by 59°C for 3 minutes, and 60°C for 10 minutes. Cell line 9974A DNA was used for a positive control while TE buffer served as the negative control.

All PCR products were electrophoresed on a 3130xl Genetic Analyzer (Applied BioSystems) with a 36cm capillary array. One microliter of PCR product was added to 0.3uL of GeneScan™ –500 LIZ® Size Standard (Applied BioSystems) and 9uL of Hi-Di™ Formamide (Applied BioSystems) on a 96-well plate. One microliter aliquots of AmpFℓSTR Identifiler® Allelic Ladder were included as appropriate for each injection. POP-4™ Performance Optimized Polymer (Applied BioSystems) was used for electrophoresis, as well as a 1X preparation of 10X Genetic Analyzer Buffer with EDTA (Applied BioSystems). Foundation Data Collection Software v.3.0 was used to collect data from injections and sample analysis was performed using GeneMapper ID v.3.2. The instrument protocols for Identifiler® Plus injection are oven temperature 60°C, run times of 10, 6, or 7 seconds, run voltage of 15kV. Peaks above 30 relative fluorescence units (RFUs) were called for profile identification.

B. Sample Preparation

1. Preparation of Human Saliva Samples

One microliter of neat human saliva was pipetted directly into 10 microcentrifuge tubes, extracted, purified, and eluted according to HCIFS protocol as described previously. The samples were quantified in triplicate with the Quantifiler® DUO kit on the ABI Prism® 7500 Sequence Detection System and results were averaged. These averaged results were used to calculate a working concentration of saliva from each of the donors which was then used to target the specific DNA amounts used for each study. The working concentration was used to prepare samples for the remainder of the experiments.

2. Preparation of Experimental Samples with and without Substrate

For samples applied to a swab, the designated concentration of saliva was added directly to the substrate and allowed to dry overnight. One-half of the cotton swab was cut and added to the appropriate tube, either a 2.0mL microcentrifuge tube (Eppendorf) or a FT500-ND PULSE tube (Pressure BioSciences). For samples not applied to the substrate, the buffer solution was prepared and added to each tube and the designated amount of saliva was added directly to the buffer solution.

C. Development of Pretreatment Method Incorporating a Pressure Cycling Component on the Barocycler NEP3229

1. Optimization of Run Parameters

Seventeen sets of saliva samples were prepared on swabs. Each set comprised a range of target DNA amounts (1,000pg, 500pg, 250pg, and 100pg) prepared in triplicate. Samples were pretreated with or without pressure using the current QIAgen protocol modified to incorporate pressure cycling. A transfer step was added to the pressure treated samples to move the samples from the treatment tubes into 2mL Eppendorf microcentrifuge tubes for processing on the QIASymphony SP; sample within the PULSE tubes were accessed by unscrewing the tube three turns, keeping the seal of the O-ring against the interior of the tube intact. Tubes were then centrifuged for one minute at 2000rpm. The screw caps were then removed and the supernatant transferred to a corresponding 2mL microcentrifuge tube. A spin basket was inserted into the 2mL tube and an autoclaved wooden stick was used to transfer the remaining substrate into the basket, taking care not touch the interior threads of the tube to avoid loss of DNA sample during transfer. The 2ml tubes were then centrifuged for 5 minutes at 20,000 x g. An identical set of samples was prepared but not treated with pressure. Results of these 204 samples were compared. Pressure cycling parameters tested are as follows:

Table 1. Run Parameters Tested for Pretreatment of DNA Samples on the Barocycler NEP3229

Pressure	35k psi
Time at Pressure (T1)	20, 40, 60, 80 sec
Time at Ambient Pressure (T2)	10 sec
Cycle Number	20, 40, 60, 80
Temperature	Water bath (59°C); Barocycler (62°C); chamber (56°C)

Results were evaluated by IPC and amplification success (total RFUs across the profile and allele counts) to identify the optimal pretreatment conditions.

2. Evaluation of Increased Incubation Temperatures

Six sets of saliva samples applied to swabs with target DNA amounts of 500pg and 100pg, prepared in triplicate, were pretreated with and without PCT at 56°C (the current HCIFS protocol), 75°C, or 95°C, for a total of 36 samples. The Barocycler parameters applied for validation are as shown in Table 2.

Table 2. Pressure Cycling Run Parameters Used for Pretreatment of DNA Samples on the Barocycler NEP3229

Pressure	35k psi
Time at Pressure (T1)	20 sec
Time at Ambient Pressure (T2)	10 sec
Cycle Number	20
Temperature	56°C

Asdf[aopsidfsSomSomen Results were evaluated in terms of amplification success (total RFUs and allele counts) for identification of the optimal incubation temperature to use for pretreatment of DNA samples incorporating a pressure cycling treatment component.

D. Internal Validation of a Pretreatment Method Incorporating Pressure Cycling Component Using the Barocycler NEP3229

1. Sensitivity-Efficiency

Two sets of saliva samples applied to swabs were prepared in triplicate with eight amounts of DNA (3000pg, 1000pg, 300pg, 100pg, 30pg, 10pg, 3p, and 1pg). One set was subjected to pretreatment without PCT while the other set incorporated PCT with the parameters shown in Table 2.

Results were evaluated in terms of IPC and amplification success (total RFU and allele counts). These samples also served to further increase the data set for comparison of pretreatment methods with and without a pressure treatment component.

2. Reproducibility

Six identically prepared sets of samples were created for a total of 60 samples. Each set included 250pg human saliva samples from each of five saliva donors. Three sets were pretreated without PCT while the other three sets were pretreated with PCT under the parameters in Table 2.

This test was performed twice, once using saliva samples deposited on a uniform swab cutting (1/2 of a cotton swab) and again using a set of saliva samples without a swab substrate. The non-swab samples were prepared by adding the appropriate saliva sample directly to the extraction buffer prior to incubation and/or pressure cycling.

The data were evaluated in terms of IPC and amplification success (total RFUs and allele counts) and served to further increase the data set for comparison of pretreatment methods with and without a pressure treatment component.

3. Mock Casework Samples

Twenty-one mock casework samples were prepared, including duplicate preparations of blood samples and samples from buccal swabs and swabs collected from a variety of touched objects, including cup handles, keyboards, and phones . The paired sample sets were evaluated for a total of 42 samples tested. One set was extracted using the QIAgen protocol and one was pretreated using the modified version of the protocol incorporating a pressure cycling treatment using the parameters listed in Table 2.

Results were compared in terms of IPC and amplification success (total RFUs and allele counts) and further increased the data set for comparison of pretreatment methods with and without pressure treatment.

4. Effects of Pressure Cycling Treatment on a Common Inhibitor

Sixteen non-swab samples with 1000pg or 500pg DNA were prepared in triplicate for a total of 48 samples. All samples were pretreated and purified using the current HCIFS method. In order to test the effect of the inhibitor subsequent to purification, two sets of dilutions were prepared from a stock solution of 1 mg/ml humic acid. Humic acid was added at 1µg, 0.33µg, 0.2µg, or 0.125µg. One of the two sets of humic acid-containing samples was subjected to the pressure cycling protocol in Table 2, the other was not. One microliter of the pressure treated or non-pressure treated inhibitor was added at each dilution to each purified sample immediately after treatment.

Results were evaluated and compared in terms of IPC, total DNA recovered and amplification success (total RFUs and allele counts).

Results

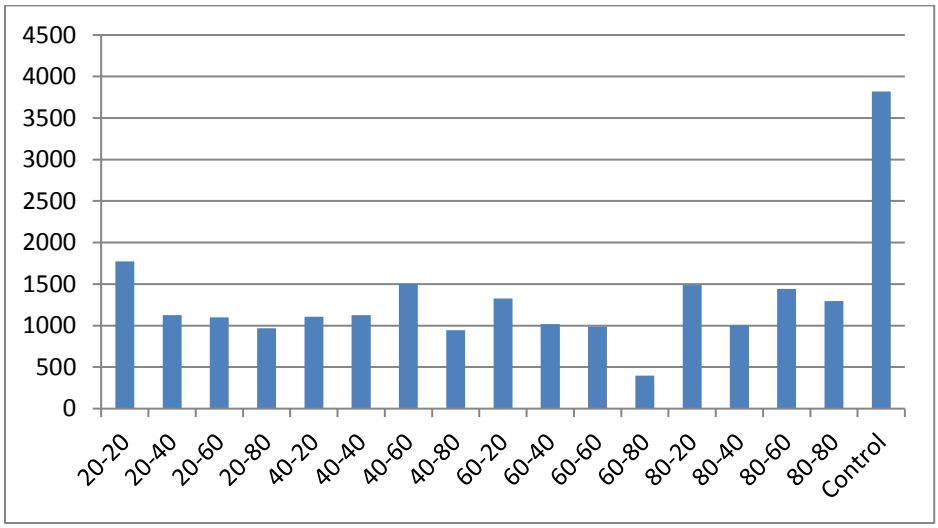
A. Development of Pretreatment Method Incorporating a Pressure Cycling Component on the Barocycler NEP3229

Triplicate samples at 1000 pg, 500 pg, 250 pg and 100 pg were run at varying pressure cycles. These sample results were pooled across all inputs and compared to the equivalent non-pressure treated sample results. The initial comparison of the pressure treated samples indicated an improvement in yield when compared to control; however, it was not statistically significant due to imprecision in both pressure treated and non-pressure treated samples. Four pressure cycling conditions, 20-20 (i.e., 20 seconds at

pressure and 20 pressure cycles), 40-20, 40-60 and 80-60 appeared to offer an improvement over non-pressure treated controls when measured by total RFUs and alleles recovered, with p values of approximately 0.3. When comparing IPCs, all samples at all pressure parameters were significantly better than the control sample. Taken together, these results suggested either an improvement linked to pressure treatment or a problem with the control sample. An additional control sample was prepared and tested. This time the results were reversed for every condition - the treated samples showed a decrease in yield when compared to the untreated control samples (Figure 3). Some of the decreases in total RFU values obtained were statistically significant and those that were not went no lower than a p value of 0.3. More total alleles were recovered from untreated samples than from treated; however, no results were statistically significant.

When comparing IPC values, the results were reversed as well. Nearly all of the treated samples had IPC values that were significantly higher than those of the control (Table 3). The 20-20 condition, however, was not significantly different from the control value, with a p value of 0.9. This parameter set was selected for further work.

Figure 3: Comparison of total RFU for 500 pg samples



IPC comparison	*20-20 Treatment*	*Untreated Controls*
Mean	30.01	30.09
Variance	0.01	0.01
Observations	12	9
Pooled Variance	0.01	
Hypothesized Mean Difference	0	
df	19	

t Stat	0.14	
P(T<=t) one-tail	0.44	
t Critical one-tail	1.73	
P(T<=t) two-tail	0.89	
t Critical two-tail	2.09	

Table 3: Comparison of IPCs from 20-20 treatment to untreated controls

Triplicate samples at estimated amounts of 500pg and 100pg were pressure treated under 20-20 conditions and 56°C, 75°C, and 95°C (the upper limit of the circulating water bath) temperatures applied. These sample results were pooled across all inputs and compared to non-pressure treated sample results run at 56°C. Comparison of both 75°C and 95°C results to that of the non-pressure treated samples showed a statistically significant decrease in total RFU values. Comparison of 56°C to non-pressure treated samples, while lower, was not statistically significant with a p value of 0.8. Incubation at 56°C was the chosen for validation purposes.

B. Internal Validation of Modified Pretreatment Method Incorporating Selected Pressure Cycling Run Parameters

1. Sensitivity

Triplicate samples at estimated amounts of 3000pg, 1000pg, 300pg, 100pg, 30pg, 10pg, 3pg and 1pg were pressure treated and total RFU and alleles detected results pooled and compared to the corresponding non-pressure treated sample results. No statistically significant difference (p value of 0.6 for RFU and 0.8 for alleles) was observed between the pressure and non-treated samples. Total RFU was slightly higher for pressure treated samples while the total number of alleles detected was higher for non-treated samples. Comparison of the sample IPC revealed a significantly higher IPC for the untreated samples compared to the pressure treated samples.

2. Reproducibility

Triplicate samples at estimated amounts of 250pg of saliva from five donors were added both directly to master mix microcentrifuge tubes and also to swabs. One set of these samples was pressure treated and the other left untreated. Total RFUs and alleles detected were compared to the equivalent non-pressure treated sample results. No statistically significant difference was observed between the pressure-treated and non-treated samples for any of the five sample donors, with swabs or without. In an attempt to gain significance, the results of the all the pressure treated samples were pooled and compared to the pooled non-treated samples. Again, no significant difference was observed between the treated and non-treated samples although for total RFU the p value was 0.2 which is closer to indicating a significant difference in favor of the non-treated samples. Comparison of the sample IPC revealed a slightly lower average IPC for non-treated samples, but not to a significant degree.

3. Mock Casework Samples

Mock casework samples collected from common touch DNA type items were prepared and run with and without pressure treatment. Items were swabbed and half of the swab was subjected to pressure treatment while the other half was left untreated. None of the comparisons displayed any statistically significant differences. The pressure treated keyboard samples did indicate an improvement over untreated keyboard samples in both total RFU and alleles detected (Tables 5 and 6); however, due to the variance inherent to the quantification system, the p value only approached 0.2. IPCs were slightly lower for untreated samples, but with a p value of only 0.5 and therefore not significant (Table 4).

Table 4: Comparison of IPCs from pressure to untreated

Keyboard	*Pressure IPC*	*Qiagen IPC*
Mean	29.95	29.92
Variance	0.0	0.0
Observations	3	3
Pooled Variance	0.00	
Hypothesized Mean Difference	0	
df	4	
t Stat	0.71	
P(T<=t) two-tail	0.52	
t Critical two-tail	2.78	

Table 5: Comparison of RFUs from pressure to untreated

Keyboard only	*Pressure RFU*	*Qiagen RFU*
Mean	12976.33	4225.33

This resource was prepared by the author(s) using Federal funds provided by the U.S. Department of Justice. Opinions or points of view expressed are those of the author(s) and do not necessarily reflect the official position or policies of the U.S. Department of Justice.

Variance	97794690.33	13776546.33
Observations	3	3
Hypothesized Mean Difference	0	
df	3	
t Stat	1.43	
P(T<=t) two-tail	0.25	
t Critical two-tail	3.18	

Table 6: Comparison of alleles from pressure to untreated

Keyboard only	*Pressure Allele*	*Qiagen Allele*
Mean	64.67	30.67
Variance	1084.33	36.33
Observations	3	3
Hypothesized Mean Difference	0	
df	2	
t Stat	1.76	
P(T<=t) two-tail	0.22	
t Critical two-tail	4.30	

Pressure treated swabbings of the phone handles again indicated an improvement over untreated phone handle samples in both total RFU and alleles detected (Tables 8 and 9); however, due to the variance inherent to the samples, the p value only approached 0.4. IPCs (Table 7) were slightly lower for the treated samples but, with a p value of only 0.5, the difference was not significant.

Table 7: Comparison of IPCs from pressure to untreated

Phone	*Pressure IPC*	*Qiagen IPC*
Mean	29.83	29.77
Variance	0.00	0.03
Observations	4	4
Pooled Variance	0.01	
Hypothesized Mean Difference	0	
df	6	
t Stat	0.75	
P(T<=t) two-tail	0.48	
t Critical two-tail	2.45	

Table 8: Comparison of RFUs from pressure to untreated

Phone	*Pressure RFU*	*Qiagen RFU*
Mean	5137	1045.67
Variance	44145727	378809.33
Observations	3	3
Hypothesized Mean Difference	0	
df	2	
t Stat	1.06	
P(T<=t) two-tail	0.40	
t Critical two-tail	4.30	

Table 9: Comparison of alleles from pressure to untreated

Phone	*Pressure Allele*	*Qiagen Allele*
Mean	36.33	16.67
Variance	1010.33	76.33
Observations	3	3
Hypothesized Mean Difference	0	
df	2	
t Stat	1.03	
P(T<=t) two-tail	0.41	
t Critical two-tail	4.30	

Pressure treated swabbings of cup bodies indicated the non-treated samples fared better in both total RFU and alleles detected, although, statistically, the p value of 0.8 points to a strong similarity between treated and untreated. IPC values were lower for untreated samples compared to treated samples with a level approaching significance, 0.08.

4. Effect of Pressure Cycling Treatment on a Common PCR Inhibitor

In general, when the results of all the samples were pooled and compared in an attempt to better evaluate significance, those that contained untreated inhibitor fared better in terms of alleles detected and total RFU value (Tables 10 and 11). Statistically the difference in favor of untreated inhibitor was not significant.

Table10: Comparison of alleles from pressure to untreated untreated

Inhibition	*Allele no treatment*	*Allele treatment*
Mean	26.33	25.46
Variance	6.32	8.78
Observations	24	24
Hypothesized Mean Difference	0	
df	45	
t Stat	1.10	
P(T<=t) one-tail	0.14	
t Critical one-tail	1.68	
P(T<=t) two-tail	0.28	
t Critical two-tail	2.01	

Table 11: Comparison of RFUs from pressure to

Inhibition	*RFU no treatment*	*RFU Treatment*
Mean	4113.63	3565.08
Variance	4146521.46	3318773.99
Observations	24	24
Hypothesized Mean Difference	0	
df	45	
t Stat	0.98	
P(T<=t) one-tail	0.17	
t Critical one-tail	1.68	
P(T<=t) two-tail	0.33	
t Critical two-tail	2.01	

Comparison of IPC results revealed the un-treated samples performed significantly better, meaning the IPC was lower, for all inhibitor inputs except for 0.125ug. All had p values <0.05 indicating a significant difference from the treated samples. The 0.125ug had a p value of 0.99 indicating a strong similarity to the treated samples.

Discussion

A. Development of Pretreatment Method Incorporating Pressure Cycling Component Using Barocycler NEP3229

The initial goal of the project was to identity cycling and temperature parameters optimal for processing touch type DNA samples. Due to the imprecision of quantitation at low levels of DNA (< 25 pg/ul) the goal of finding significantly beneficial PCT conditions was not met. An initial study comparing parameters indicated an improvement over non-treated control samples, however, the improvement was not significant. Further analysis of IPC results also suggested a problem with the control. Additional comparisons, made with another control sample, did not confirm initial results. Some results suggested

PCT interfered with DNA recovery, others suggested is improved it. Comparison of the IPC indicated a similar trend. The so-called 20-20 treatment was similar statistically to the untreated control samples and, for that reason, it was used throughout the remainder of the study.

In addition, three different incubation temperatures, 56°C, 75°C and 95°C, were compared under 20-20 parameters. Results indicated that 56°C was the optimum incubation temperature. Both 75°C and 95°C were significantly worse with respect to total RFU and alleles when compared to the equivalent untreated control samples. At 56°C, there was no showed no significant difference when compared to the equivalent untreated control samples.

B. Internal Validation of Modified Pretreatment Method Incorporating Selected Pressure Cycling Run Parameters

Sensitivity tests did not reveal significant differences between the pressure treated samples and the untreated control samples for total RFU values and alleles detected. The IPCs were significantly lower for the pressure treated samples when compared to the untreated samples, indicating a more efficient Quantifiler Duo amplification for the pressure treated samples. This efficiency did not translate to the Identifiler Plus amplification to a significant degree, however. Under the 20-20 conditions at 56°C, no significant improvement was indicated when samples were pressure treated. A promising trend was observed in total RFU for the pressure treated samples; however, many additional samples would be required to confirm whether this trend was significant.

The reproducibility study indicated much of the same. There was no significant difference noted between the treated and untreated control samples with respect to total RFU, alleles or IPC. A significant difference was almost reached (p value 0.2) when comparing all the pressure treated samples to all the non-pressure treated samples with respect to total RFU. This difference indicated greater total RFU for untreated samples. However, no conclusions can be reached regarding any differences between pressure treated and untreated samples. There are indications that no differences may be present between the two methods but, again, many more samples would be required to account for the wide variation seen in low level samples.

Perhaps the most promise for pressure treated samples was indicated in the mock casework samples. When swabbings were taken of keyboards and phones, the pressure treated samples indicated an improvement of non-treated samples in total RFU and alleles, although at a level that was not significant. When comparing IPCs, all of the treated samples were significantly lower than the equivalent untreated samples, indicating pressure treatment is effective at improving the efficiency of the Quantifiler Duo

reaction. More sample testing would be required to assess the significance of the trend, but indications are clear that for mock casework touch samples, the pressure treatment shows. These indications are different than observations in the other comparisons and could be a result of the sample types, as all the other comparisons were performed with saliva samples. If additional testing is performed it would be best to use touch type samples where the effect was observed.

The inhibition comparisons also lacked significance. Comparison of total RFU and alleles detected showed a slight advantage for untreated samples but the difference was not significant. Comparison of IPC values indicated the untreated samples are significantly better. The root cause of the result is unclear. It could be due to the timing of the humic acid addition to samples or pressure treatment could actually render humic acid a stronger inhibitor of PCR. A true test of inhibition was not possible as the QIAsymphony SP was previously demonstrated to remove the inhibitory effects of humic acid, so spiking extracted samples was the best available option but a wholly satisfying approach. Further testing, such as the effect of pressure on the inhibitor and DNA or adding aliquots of pressure-treated inhibitor directly to the Quantifiler Duo master mix in the absence of DNA, is necessary to study these results and make additional conclusions.

B. Implications of further research

Of all of the comparisons performed, the effect of pressure treatment on mock casework touch samples showed the most promise. While not significant, several of the sample types that were pressure treated outperformed the equivalent untreated samples. Many more samples are needed to assess significance. That is beyond the scope of this work.

References

[i] Sweet D, Lorente M, Lorente JA, Valenzuela A, Villaneuva E: **An improved method to recover saliva from human skin: the double swab technique.** *J Forensic Sci* 1997, **42:**320-322.

[ii] Pang BCM, Cheung BKK: **Double swab technique for collecting touched evidence.** *Legal Med* 2007, **9:**181-184.

[iii] Prinz M, Schiffner L, Sebestyen JA, Bajda E, Tamariz J, Shaler RC, Baum H, Caragine T: **Maximization of STR DNA typing success for touched objects.** *Int Congress Ser* 2006, **1288:**651-653.

[iv] Wickenheiser RA: **Trace DNA: a review, discussion of theory, and application of the transfer of trace quantities of DNA through skin contact.** *J Forensic Sci* 2002, **47:**442-450.

[v] Collopy C: **Mini-Popule developed to maximize DNA recovery for robotic forensic analysis.** [http:/ / www.forensicmag.com/ article/ mini-popule-developed-maximize-dna- recovery-robotic-forensic-analysis] *webcite* *Forensic Magazine* 2008.

[vi] Hansson O, Finnebraaten M, Knutsen Heitmann I, Ramse M, Bouzga M: **Trace DNA collection - performance of minitape and three different swabs.** *Forensic Sci Int Genet Suppl Ser* 2009, **2:**189-190.

[vii] Wickenheiser RA: **Trace DNA: a review, discussion of theory, and application of the transfer of trace quantities of DNA through skin contact.** *J Forensic Sci* 2002, **47:**442-450.

[viii] Gill P, Whitaker J, Flaxman C, Brown N, Buckleton J: **An investigation of the rigor of interpretation rules for STRs derived from less than 100 pg of DNA.** *Forensic Sci Int* 2000, **112:**17-40.

[ix] Ibid.

[x] Ibid.

[xi] Whitaker JP, Cotton EA, Gill P: **A comparison of the characteristics of profiles produced with the AmpFlSTR SGM Plus multiplex system for both standard and low copy number (LCN) STR DNA analysis.** *Forensic Sci Int* 2001, **123:**215-223.

[xii] The Queen v Sean Hoey. Neutral Citation Number [2007] NICC 49.
Available from: http://business.timesonline.co.uk/tol/business/law/article3083217.ece

[xiii] Budowle B, Eisenberg AJ, van Daal A: **Validity of low copy number typing and applications to forensic science.** *Croat Med J* 2009, **50:**207-217.

[xiv] Budowle B, Eisenberg AJ, van Daal A: **Validity of low copy number typing and applications to forensic science.** *Croat Med J* 2009, **50:**207-217.

[xv] Roeder AD, Elsmore P, McDonald A: **Maximising DNA profiling success form sub-optimal quantities of DNA: A staged approach.** *Forensic Sci Int Genet* 2009, **3:**128-137.

[xvi] **PRESSURE CYCLING TECHNOLOGY (PCT) AUGMENTS SENSITIVIY OF DETECTION AND ROBUSTNESS IN FORENSIC DNA ANALYSIS**
Bruce Budowle, PhD Executive Director of the Institute of Investigative Genetics, Professor in Department of Forensics and Investigative Genetics, University of North Texas Health Science Center, Fort Worth, Texas, 22nd Annual International Symposium on Human Identification, October 11 – 14, 2010, San Antonio, Texas.

[xxi] http://www.pressurebiosciences.com/pdf-new-2010/publications/USHUPO2010_Poster_IvanovAR_HSPH.pdf accessed March 30, 2011.

[xviii]Marshall P, King J, Lawrence N, Lazerev A, Gross V, Budolwe B: **Pressure cycling technology (PCT) reduces effects of inhibitors of the PCR.** Int J Legal Med 2013, 127:321-333.

Dissemination of Research Findings

1. Evaluation of Pressure Cycling Treatment on Barocycler NEP3229 for Extraction of Low-Template Forensic DNA samples. International Symposium of Human Identification, Nashville, TN. (2012)

2. Evaluation of Pressure Cycling Treatment on Barocycler NEP3229 for Extraction of Forensic DNA Samples, Washington, D.C. (2013)