IMPROVED TOOLS FOR EXAMINING LOW COPY NUMBER (LCN) DNA OBTAINED FROM CHALLENGED OR DEGRADED SAMPLES

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Abstract

There are limits to forensic DNA analysis. One important parameter is the amount of template DNA used in the polymerase chain reaction (PCR). When the amount of DNA is below a certain quantity, the results obtained from current forensic DNA typing methodology generally are not reproducible because low copy number (LCN) typing is not sufficiently robust. In order to improve LCN typing, several approaches were undertaken which include: 1) improvements to the robustness of the amplification through the use of PCR enhancers; 2) increasing DNA recovery using pressure cycling technology (PCT), improved silica columns, or synchronous coefficient of drag alteration technology (SCODA); and 3) more efficiently reducing inhibition. The data illustrate that each of these approaches can contribute to improving the efficacy of analysis either by increasing yield of sample, more effectively purifying a sample, or by increasing amplification efficiency (e.g., decreased stutter). The impact is that some samples that traditionally yield too little DNA for typing may become suitable for routine analysis or a more effective methodology may be developed that will enable analysis of samples that typically have not been typeable. Moreover, more challenged samples may be analyzed by combinations of better purification columns, PCT, SCODA, and PCR enhancement.

Introduction

Low copy number (LCN) typing is defined as the analysis of any DNA sample that yields exaggerated stochastic effects and, for STR typing, typically that is less than 200 pg of template DNA. Although this is a simplification of the criteria of a much more complex process, the general concept of the minimum amount of DNA is a good, reasonable first approximation for defining a sample as a LCN sample. When increasing the sensitivity of detection to type LCN samples, stochastic effects during PCR are so exacerbated that, for STR analysis purposes, peak height imbalance, allele drop-out, and increased stutter occur. Because of these vagaries, LCN typing cannot be considered a robust methodology for identity testing. However, typing of human remains (and other samples) requires the use of LCN methodologies because samples often contain low quantities of degraded DNA. Current LCN technical methodologies are not well-developed for its application and the statistical weight associated with a DNA profile is not well-defined. Thus, LCN typing needs to be better developed so genetic data from primarily missing persons evidence can be exploited to its full potential and those individuals making identifications will be able to use the genetic information effectively. One approach is to improve the technology for DNA typing so that LCN analysis can become more robust

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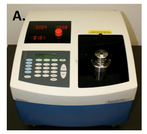
Pressure Cycling Technology (PCT)

Increasing DNA Recovery:

In an effort to increase DNA recovery from bones, hair, or from devices used for collecting crime scene biological evidence, such as cotton swabs, samples were processed with Pressure Cycling Technology (PCT). PCT (Pressure BioSciences, South Easton, MA) uses cycles of alternating high hydrostatic and ambient pressures to extract DNA from a variety of sample types, including but not limited to swabs, hairs, soft and hard tissues, and liquid samples. The severe changes in pressure allow for molecular interactions to be controlled and because of baroporation, DNA is released into solution while generally maintaining the sample's morphological integrity.



Barocycler NEP3229

B.

Specially designed multi-functional tube

Figure 1. A) Barocycler® NEP3229. The Barocycler® NEP3229 (Pressure BioSciences, South Easton, MA) is a commercially available bench top instrument
B) PULSE Tubes. Specially designed single use tubes (**P**ressure **U**sed to **L**yse **S**amples for Extraction) are available with and without lysis discs for sample shredding.

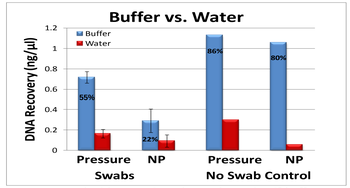


Figure 2. Buffer was compared with water in order to determine if buffer use during pressure cycling results in loss of DNA. For swabs and the no swab controls, 50µl (1.2 ng/µl) of cultured epithelial cells (200 cells/µl) were used (swabs were dried overnight prior to analysis). Swabs were barocycled (30 Cycles [20s at 35k psi and 10s at ambient psi]). Pressured and non-pressured swabs were extracted using the Maxwell 16® (Trace sample on swab protocol) and QIAamp® DNA Mini Kitand quantified using Quantifiler® Human DNA Quantification Kit (reduced volume protocol) on the ABI 7500 Real Time PCR System. Pressure samples were compared with non-pressured samples and swabs were compared with the no swab controls. Samples were performed in triplicate.

Inhibition Study:

DNA extracted from forensic samples can be degraded and also contain co-extracted contaminants that inhibit PCR. The Quantifiler™ Human DNA Quantification Kit internal PCR Control (IPC) contained within each reaction acts as an indicator of the possible presence of PCR inhibitors. In an effort to determine if pressure cycling technology will denature inhibitor compounds, thus rendering them incapable of inhibiting downstream DNA analyses, several concentrations of hematin and humic acid were tested. In the presence of hematin and humic acid, the IPC increases with concentration of inhibitor. However, following pressure cycling, IPC values are significantly lower for 5uM/ul Hematin and 2.5ng/ul HA. At concentrations greater than 7uM for Hematin and 5ng of Humic Acid, the IPC values were not determined for either PCT or non-pressure treated samples.

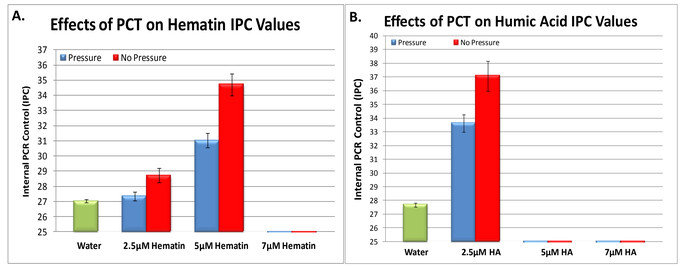


Figure 3. 1mL of Hematin (84.5µM/µl stock) and Humic Acid (500ng/µl stock) were barocycled (30 Cycles [20s at 35k psi and 10s at ambient psi]). Various concentrations of hematin (0, 2.5, 5, and 7 µM/µl) and humic acid (0, 2.5, 5, and 7 ng/µl), **Figures A and B** respectively, were then added to the Quantifiler master mix (reduced volume protocol) and quantified using ABI 7500 Real Time PCR System. The internal PCR control (IPC) values for each concentration are shown. Pressure samples were compared with non-pressured controls.

Improving Amplification with PCR Enhancers

One way samples containing less than 200 pg of DNA are amplified is by increasing PCR cycle number to increase the sensitivity of detection. When increasing the sensitivity of detection to type LCN samples, stochastic effects during PCR are so exacerbated that, for STR analysis purposes, peak height imbalance, allele drop-out, and increased stutter occur. Many of these artifacts may result from strand slippage or when the polymerase pauses during extension. Additives which alleviate the paused extension of primer, stabilize the enzyme, or reduce instability of the template strand may improve PCR amplification. For this study, we investigated two known PCR enhancers, betaine and dimethyl sulfoxide (DMSO). Both betaine and DMSO facilitate strand separation. Betaine acts as an isostabilizing agent, equalizing the contribution of GC- and AT-base pairing to the stability of the DNA duplex; while DMSO acts by disrupting base pairing. This study aims to determine if betaine and DMSO enhance the PCR such that stochastic effects are decreased (i.e., stutter) and the overall PCR efficiency is increased. Amplification reaction mixes were prepared using various concentrations of betaine and DMSO. Primers for D18S51 and D21S11, two loci known to present higher levels of stutter, were tested. Buccal swabs of ten individuals were extracted using the AutoMate *Express™* Forensic DNA Extraction System (Applied Biosystems, Foster City, CA) and PCR was performed at both 28 and 34 cycles. Samples were then analyzed on the ABI Prism® 3130xl Genetic Analyzer (Applied Biosystems) and data were analyzed with GeneScan® Analysis software (Applied Biosystems). Samples were performed in triplicate. Stutter percentages at both loci were then evaluated.

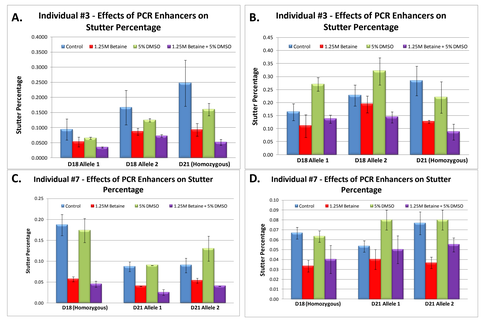


Figure 4. Buccal samples from two individuals were extracted using AutoMate *Express™*. DNA extracts were added to custom amplification reaction mixes containing, Control – no PCR enhancer, 1.25M Betaine, 5% DMSO, or a mixture of 1.25M Betaine and 5% DMSO, and amplified using TaqGold polymerase. Samples were then run on the 3130xl and analyzed with GeneScan©. Stutter percentages were then calculated.
A) 1ng/µl of DNA at 28 cycles. **B)** 1ng/µl of DNA at 34 cycles. **C)** 0.5ng/µl of DNA at 28 cycles. **D)** 0.5ng/µl of DNA at 34 cycles.

Synchronous Coefficient of Drag Alteration (SCODA)

Forensic analyses often deal with samples in which there are low amounts of nucleic acids, on substrates that often lead to inhibition of subsequent enzymatic reactions such as PCR amplification. These substrates include indigo dye (blue jean denim), hematin (blood), and humic acids (soil). These inhibitors can co-extract with nucleic acids in standard or bead based columns, leading to frequent failure of STR profiling.

Synchronous Coefficient of Drag Alteration (SCODA) (Boreal Genomics, Vancouver, Canada) is a novel instrument for DNA purification of forensic samples that is capable of highly effective concentration of nucleic acids from soil particulates, fabric, and other complex samples including solid components. The SCODA process is inherently selective for long-charged polymers such as DNA, and therefore is able to effectively remove known contaminants.

In order to determine if SCODA is capable of efficiently purifying and concentrating nucleic acids, various concentrations of purified DNA (0ng/µl, 0.62ng/µl, 0.21ng/µl, 0.68ng/µl, and 0.023ng/µl [50 µl total volume in TE^{-4} buffer]) were processed using SCODA. Post-SCODA DNA yields were then compared to original starting template quantities using the Quantifiler™ Human DNA Quantification Kit (Applied Biosystems, CA).

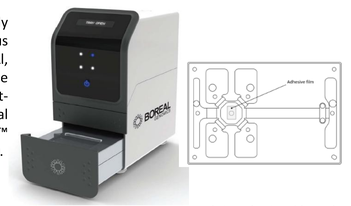


Figure 5. SCODA Instrument and Sample Cartridge. The custom cartridges consist of a 5 mL injection chamber (60 mm long, 7 mm deep and 12 mm wide), a concentration gel casting region, and a 15 mL electrophoretic buffer reservoir for each of the four electrodes that generate the rotating fields. Figure used with permission from Boreal Genomics.

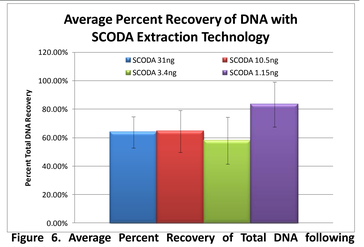


Figure 6. Average Percent Recovery of Total DNA following SCODA. 50µl of various concentrations of purified DNA (0.62ng/µl, 0.21ng/µl, 0.68ng/µl, and 0.023ng/µl) were placed into the SCODA sample cartridge. Experiments were performed in triplicate. Post SCODA DNA yields were compared to Pre-SCODA starting template quantities and results were given as a percentage of the starting quantity.

DNA from Skeletal Remains

Testing was carried out on two new prototype devices designed to enhance recovery of DNA from challenging samples. The first device is a 20 mL capacity tube that can be capped and sealed at both ends to facilitate agitation of large or bulky solid substrates in large volumes of extraction buffer. This will be particularly useful for larger items that likely only contain a few cells, such as fabric cuttings that have dilute stains or that may have been contacted by an individual's skin (gripped, rubbed, worn, etc.). These devices are also particularly promising for DNA extraction from swabs, as the entire swab head can be used for elution, rather than having to take a cutting from the swab and potentially leaving evidence behind. DNA can be extracted from the large volume of eluate obtained from the spin basket device using the Hi-Flow column.

The other prototype device is a small-scale silica column that can bind DNA from as much as 250 µl of crude extract and efficiently elute the DNA in as little as 1 µl. This device would allow for concentration of low-copy samples as well as low-signal cycle sequencing and STR amplification reactions that otherwise would have signal too low to be analyzed by current methodologies. The best performance of the column was achieved with 3 washes of 5 mL each, however, we found that the repetition of washes was time-consuming and unwieldy, so the wash protocol was tested with a single 15 mL wash.

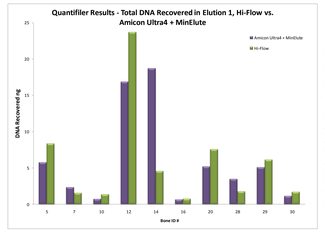


Figure 7. Total DNA recovery from elution one of the Amicon Ultra4 + MinElute method (purple) and the Hi-Flow method (green). Extractions were performed on bone powder from a single grind cycle from bones 5, 7, 10, 12, 14, 16, 20, 28, 29, and 30.

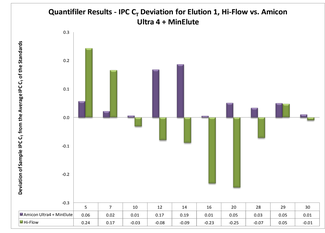


Figure 8. The deviation of the sample IPC C_T value from the average IPC C_T value of the standards is shown with the Amicon Ultra4 + MinElute method in purple and the Hi-Flow method in green. A positive shift in IPC C_T value indicates that the IPC reaction was inhibited.

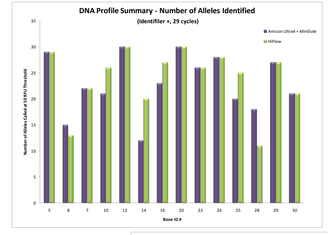


Figure 9. Average peak heights for every peak called in each profile. This is to allow for a relative comparison of signal strength. The Amicon Ultra4 + MinElute samples are represented in purple and the HI-Flow samples are in green.

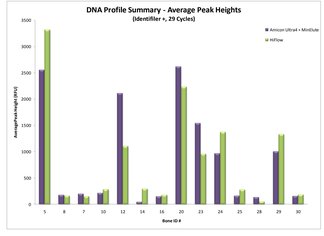


Figure 10. Total number of alleles identified using Identifiler Plus from DNA extracts generated with the Amicon Ultra4 + MinElute method (purple) or the Hi-Flow method (green).