Marshall, Pamela L. Improved Tools for the Robust Analysis of Low Copy Number and Challenged DNA Samples. Doctor of Philosophy (Biomedical Sciences), May, 2014, 226 pp., 14 tables, 31 illustrations, 203 titles.

Low copy number (LCN) typing is a general technique used for analyzing low quantity DNA samples. Short tandem repeat (STR) testing on aged and extremely limited samples, such as "touch DNA" samples has increased over the past decade. These samples with low quantities of template DNA are typically subjected to exaggerated stochastic effects during the polymerase chain reaction (PCR), and these effects impact the reproducibility and reliability of DNA typing results. Current LCN methods are not analytically robust, and the confidence associated with a DNA profile and sample attribution is not well-defined. My research project was to develop and improve the analytical typing processes, creating a more robust system of LCN typing that is less refractory to stochastic effects, and given the more robust system, provide guidance on the statistical issues needed to assess the significance of a LCN typing result.

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.

KEYWORDS: Low Copy Number · DNA extraction · DNA inhibition · Stochastic Effects · Diomics X-Swab™ · Hi-Flow® silica column · Pressure Cycling Technology · SCODA · PCR additives · DNA transfer · DNA interpretation

IMPROVED TOOLS FOR THE ROBUST ANALYSIS OF LOW COPY NUMBER AND CHALLENGED DNA SAMPLES

DISSERTATION

Presented to the Graduate Council of the
Graduate School of Biomedical Sciences
University of North Texas
Health Science Center at Fort Worth
In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

Pamela L. Marshall, M.S.
Fort Worth, Texas
May, 2014

ACKNOWLEDGMENTS

"If I have seen further, it is by standing on the shoulders of giants." ~ Sir Isaac Newton

So many people have been a part of my journey at The University of North Texas Health Science Center. First, I would like to express my deepest gratitude to my mentor, major professor, role model, and friend, Dr. Bruce Budowle, for his unwavering faith in my abilities, his consummate guidance, and support throughout this process. I thank you, Bruce, for being an example of a humble, dedicated, and ethical forensic scientist. To my entire committee, Dr. Art Eisenberg, Dr. Ranajit Chakraborty, Dr. Angela van Daal, and Dr. Patricia Gwirtz, I thank you for your time, guidance and support with this project. I will forever consider myself blessed to have worked with my committee – thank you for your constructive criticisms that motivated me to become a better scientist. You are all "giants" in my eyes.

To my "Budowle Bunch" lab family, past and present, for always lending a helping hand, a supportive ear, or dropping everything to answer a question. To Angie Ambers, Sarah Schmedes, Monika Stoljarova, David Warshauer, and Jonathan King, you have helped me more than you know. To my GSBS family, Jan, Amanda, and Aleta, I love you all and thank you for your friendship.

To the wonderful companies and people I have worked with at Pressure Biosciences, Inc., Diomics Corporation, and Boreal Genomics, I thank you for the opportunity to perform cutting edge research with your products. To Nathan Lawrence, Matthew Potter, Leroy Chiao, Kirk Avery, John Steel, Franco Ferrari, Thomas Kindt, Joel Pel, and Jason Maydan, I thank you for your technical support and guidance. To Dixie Peters, Linda Larose, and the entire UNT Center for Human Identification group, I thank you for your assistance and technical guidance.

Last, but not least, to my family, I thank you – for always believing in me, for encouraging me to follow my dreams, and for showing me, from a very early age, that knowledge and education are power and will open more doors than even I could have realized. I also thank God, without whom none of this would have been possible.

This project was supported in part by award nos. 2009-DN-BX-K188 and 2012-IJ-CX-0016, awarded by the National Institute of Justice, Office of Justice Programs, U.S. Department of Justice. The opinions, findings, and conclusions or recommendations expressed in this publication are those of the authors and do not necessarily reflect those of the Department of Justice.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

LIST OF ILLUSTRATIONS (CONTINUED)

LIST OF ILLUSTRATIONS (CONTINUED)

INTRODUCTION

IMPROVED TOOLS FOR THE ROBUST ANALYSIS OF LOW COPY NUMBER AND CHALLENGED DNA SAMPLES

Low Template DNA Analysis: Since the introduction of the concept of 'low copy number' (LCN) DNA analysis (1), it has quickly become the focal point of forensic DNA applications and research. Other terms that exist for LCN DNA are low template DNA, touch DNA, and trace DNA. However, not all of these terms correctly describe or meet the criteria of LCN DNA samples (2). In fact, trying to define what exactly a LCN DNA sample represents is difficult and has become a point of contention among some scientists. LCN samples have been described by some as having less than 100 picograms (pg) of DNA (3, 4). 100 pg is the amount in approximately 16 diploid or 33 haploid cells.

Budowle et al. (2) have suggested that LCN DNA typing be re-defined as those samples which yield results "below the stochastic threshold for normal interpretation" and generally involves single-source samples containing less than 200 pg of template DNA for current STR typing systems. Although this is a simplification of the criteria of a more complex process, the general concept of a minimum amount of DNA is a good, reasonable first approximation for defining a LCN sample. For these samples to be typeable, the DNA must be amplified such that it can be analyzed for identification. Forensic PCR protocols typically specify 0.5 – 1.0 ng of DNA be used for optimal amplification, but samples with as little as 0.1 - 0.2 ng have yielded reliable results. However, LCN DNA samples fall below this DNA range requirement.

Most forensic approaches of analysis of LCN samples aim to increase sensitivity of detection, either through increased PCR cycle number or by post-PCR manipulation (e.g., increasing injection times) (2, 2, 4-9). The most common approach is to increase PCR cycle number from 28 cycles to 34 cycles (3, 9-14). One cautionary recommendation consistent in all of these studies is that concomitant with increased sensitivity is an increase in heterozygote imbalance, allele and/or locus drop out, increased stutter, and increased contamination. Because

of these unpredictable events, 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 they often contain low quantities of and/or degraded DNA.

Current LCN methods are not well-developed, and the confidence associated with a DNA profile and a sample is not well-defined. My research project was the development and improvement of the analytical typing processes, including DNA recovery, extraction efficiency, purification, and PCR enhancement, creating a more robust system of LCN typing that is less refractory to stochastic effects.

DNA Recovery: The first step in the analytical typing process of DNA is the collection of evidentiary samples. The device used to collect crime scene samples, either suspicious stains or areas suspected to contain touch DNA, is extremely important. Successful DNA analysis relies on the ability of a swab to absorb or adsorb materials from a stain or surface and then release the cells/DNA once extraction begins. There are a number of commercially available products for collection of evidence.

Prinz *et al*. (15) compared several collection devices in an attempt to improve cell/DNA removal from fingerprint evidence. Results indicated that a "specialized" swab yielded up to 2.5 times more alleles compared with cotton and Dacron swabs. Other studies have shown that a significant amount of the DNA collected is lost upon extraction. van Oorschot *et al*. (16) discussed how these losses may well be attributed to the processes of Chelex or organic extractions but also can be due to the collecting agent (swab, cloth, etc.) and the condition of the sample.

Hansson *et al*. (17) studied different swabs as well as minitape to determine the best collection device for LCN DNA recovery. The authors tested a cotton Dryswab™, 4N6 DNA Flocked Swab, a self-saturating swab, and the Scenesafe FAST™ minitape. While the results indicated no difference in DNA recovery among the swabs, the minitape showed higher DNA yields and gave full profiles for all experimental samples. Hansson *et al*. (17) suggested that investigators may want to consider the type of substrate when choosing a collection device and method. While their results demonstrated that minitape was better suited for textile sampling, there were no differences between swab collection devices on textiles. However, for trace DNA on plastic surfaces, the selection of swab devices may have an effect on DNA recovery. The authors suggested the use of a self-saturating swab (Puritan Medical Products) may be better suited for plastic surface recovery. They also recommended that further investigation into collection devices should be considered.

This project sought to define a more effective collection device. The novel collection device, the X-Swab™ (Diomics, La Jolla, California), was selected as this swab has high absorptive qualities and can be dissolved under DNA extraction conditions (one hour incubation at 56^{o}C). Thus, more DNA may be released from its matrix than other collection devices. Specifically, the potential of this device to improve the yield of DNA was studied. Preliminary data on known quantities of DNA demonstrated an average DNA recovery of 82% with the X-swabs compared with a liquid control. Improving the initial DNA yields from collection devices may result in some samples that traditionally yield too little DNA becoming suitable for routine analysis. Furthermore, this device may improve retrievable higher quality DNA as longer strands of DNA may remain trapped in the matrix of other collection devices. The recovery of higher quality DNA can impact positively the reliability of the LCN process.

***DNA Extraction*:** A second area of research effort was to increase the starting template molecule by improving the overall extraction of DNA from skeletal remains and other challenged samples. A large focus of my proposal was to improve the extraction methodology of LCN DNA samples. The concept is that the amount of DNA currently obtained from some LCN samples may be increased in quantity and/or quality so a more robust analysis (i.e., the standard or typical practices) could be carried out. Thus, improving sample extraction and being able to concentrate the sample can increase DNA yield.

To date, the three most established methods for DNA extraction, particularly from skeletal remains, are organic (i.e., phenol:chloroform), silica-based approaches, and ultrafiltration (18-25). The phenol:chloroform method is effective at removing proteins and lipids from DNA extracts, but tends to be ineffective for removal of hydrophilic compounds, a problem for skeletal remains in particular, as they have often been in prolonged contact with soil or water (and been exposed for example to the PCR inhibitor humic acid) (20, 21). Organic extraction solvents are a known health hazard. Therefore, much effort has been dedicated to development of non-organic extraction methods. The majority of these methods use the ability to reversibly bind DNA to silica via salt bridging and the use of ultrafiltration membranes to remove contaminants based on simple size exclusion with a concomitant reduction in extract volume.

In an effort to determine an effective extraction protocol for skeletal remains, a study was undertaken on a relatively new silica device, the Hi-Flow column (Generon Ltd., Maidenhead, UK). The Hi-Flow columns were constructed on the 20 mL capacity Proteus™ (AbD Serotec, Raleigh, NC) protein purification column platform (designed to be seated in a 50 mL conical tube during use), and contain a glass fiber filter. The chemistry for the Hi-Flow Protocol is

similar to the QIAquick® (Qiagen, Valencia, CA) silica gel columns (as modified from Yang *et al*. (23)). Preliminary results suggested that higher DNA yield and less inhibition can be obtained using the Hi-Flow method. Additionally, the Hi-Flow method resulted in more STR alleles being detected with generally higher relative fluorescent units (RFUs). The Hi-Flow method performed comparably with the Loreille, *et al*. (22) method, but required the use of only one device instead of two (resulting in reduced processing time). A goal of this study was to evaluate and validate the use of the Hi-Flow column as an effective extraction method.

A novel forensic tool, PCT (Pressure BioSciences Inc., South Easton, MA, USA), was investigated for its effectiveness in the DNA extraction process. PCT 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 (26-28). High pressure weakens hydrophobic interactions between aliphatic amino acid side chains, while electrostatic interactions are enhanced under pressure (29, 30). Moreover, primary pressure effects on biological macromolecules are attributed to pressure perturbation of the interactions of such molecules with the solvent, leading to reversible partial denaturation of proteins, weakening of lipid bilayers, and dissociation of multimeric protein complexes (31).

During exposure to multiple cycles of pressure, nucleic acids can, in theory, be expelled from cells and tissue, with a high degree of precision, reproducibility, convenience, speed, and safety. This novel approach has the potential to increase the recovery of the amount of template DNA entrapped in bone and other substrates, such as evidentiary collection swabs. An effort of this study was to improve DNA extraction from bone by applying PCT. However, swabs were evaluated first because they were easier to control and manipulate. The processes that may

improve yield from bone can be tested more readily with swabs. An ancillary benefit is swabs are often used as a collection device. Increasing the yield of DNA extracted from a swab may augment the amount of template DNA that can be placed in a PCR. Most extraction procedures from swabs are inefficient, such that portions of DNA are not removed from the swab. This residually retained DNA can be relatively quite substantial. Experiments were performed to determine if PCT can be an effective tool for increasing DNA yield.

Experiments were performed using a second novel extraction tool, synchronous coefficient of drag alteration (SCODA). While commonly used extraction methods, such as silica adsorption and phenol:chloroform extraction, have been successful in recovering LCN DNA and removing inhibitors, such methods at times fail to provide typing results with some challenged forensic samples. In addition, during sample manipulation with these methods there can be substantial loss of DNA and, other than the Hi-Flow columns described previously, only small volume samples are accommodated in the extraction process. A potential solution to this problem is SCODA (Boreal Genomics, Vancouver, BC), a technology that effectively removes inhibitors while simultaneously concentrating DNA. The SCODA process inherently selects for long, charged polymers, such as DNA, and by alternating electric fields drives DNA to the center of the opposing fields. Non-nucleic acid molecules are driven out of the focal field. DNA has been recovered from various environmental and bacterial samples (32-34) and contaminated forensic samples (33) using SCODA. Experiments were performed to determine if SCODA can be an effective tool for increasing DNA yield. However, one important test criterion of any extraction methodology is whether sufficient quantity, and quality, of DNA was recovered for successful profiling. The issue of DNA quality will be discussed in the next section.

Purification of DNA: Various methods have been used to attempt to overcome PCR inhibition, such as diluting the DNA sample, the addition of bovine serum albumin (BSA), and/or the addition of extra *Taq* polymerase to the PCR (35, 36). Dilution can reduce the concentration of the inhibitor in a PCR, but concomitantly decrease the amount of template DNA available for analysis. At times when attempts to remove or ameliorate the effects of inhibitors are not successful, the analysis either stops or additional DNA purification steps are sought.

Different methods of purification can be used to remove inhibitors from a sample and to further concentrate a DNA sample, such as silica-containing columns and phenol-chloroform extraction. The use of QIAquick® columns (Qiagen, Valencia, CA), which employs silica-membrane spin columns to bind and elute DNA, has proven to be successful for purifying DNA for the PCR. However, some inhibitors are not effectively removed from more challenging samples, and increased sample manipulation can lead to notable loss of nucleic acids.

During an investigation into the use of PCT to attempt to increase DNA yield from 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 inhibitory compounds which in turn may reduce the effects of the inhibitor, thus improving the yield of PCR products.

Pressure generally has no effects on covalent bonds. Natural compounds such as flavors, aromas, dyes, and pharmacologically active molecules typically are not altered by high-pressure treatment at ambient temperature (37, 38). 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 (39). This observation suggested

that reactivity of aromatic compounds, including porphyrins and polycyclic aromatics, such as tannins, humic acid, phenolic compounds, and terpenes, could be somewhat altered under pressure, especially at elevated temperatures. 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.

PCT and SCODA were evaluated for their ability to effectively remove contaminants that inhibit PCR and concomitantly concentrate the samples. These two functionalities can effectively improve the performance of the LCN analytical process to yield a more robust system that may be less refractory to stochastic effects.

DNA Amplification: A number of issues arise with the DNA template during the PCR. These issues become more problematic as the amount of template decreases. If the number of DNA template molecules introduced at the beginning of amplification is too few, it is possible that heterozygous alleles may amplify differentially. For example, primer binding may not occur equally for each allele at a locus during the first few cycles of the PCR, resulting in a notable imbalance between allelic products or, in extreme cases, the complete loss of one or both alleles (**Figure 1**, (2)). Simply, LCN DNA templates in the PCR will experience stochastic (random) amplification that may result in peak height imbalance, allelic drop out, and/or increased stutter (3, 6, 8, 9, 40, 41).

Differential amplification of one allele compared with another can occur during the PCR. In a DNA profile, this may present in the form of allelic imbalance of heterozygous peaks. For standard DNA profiling using 1 ng and 28 cycles of PCR, Whitaker *et al*. (40) estimated that

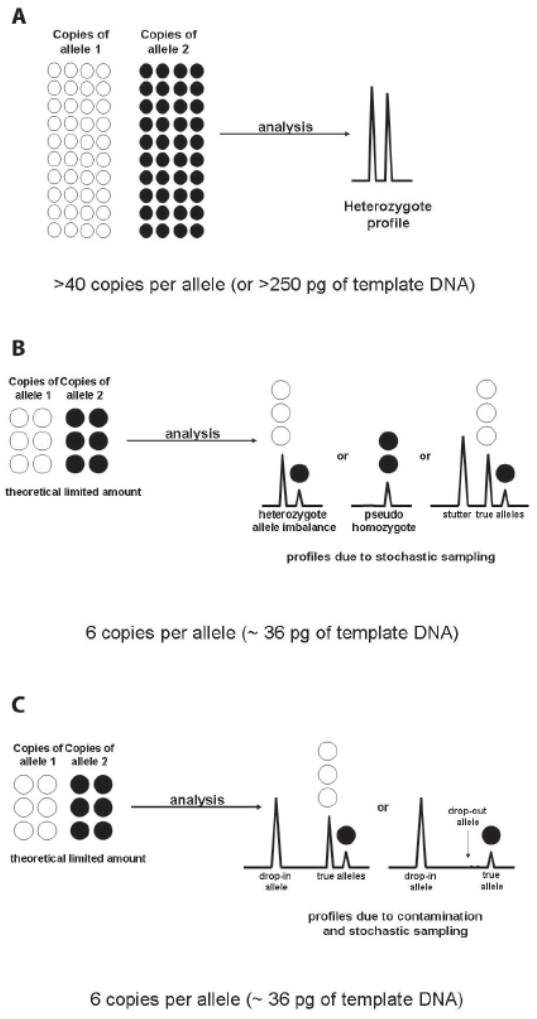


Figure 1. Stochastic Amplification Schematic. (A) With sufficient DNA, eg, 250 pg, faithful reproduction of the alleles can be generated. (B) Possible stochastic effects when a low copy number (LCN) sample containing approximately 36 pg of DNA is analyzed. The result can be heterozygote peak imbalance, allele dropout (or a pseudo-homozygote profile), increased stutter, or combinations thereof. (C) The possible stochastic effects when a LCN sample containing approximately 36 pg of DNA is analyzed. Due to increased sensitivity of detection the risk of allele dropin (and allele dropout) is exacerbated. Reprinted with permission (2).

heterozygous allele peaks are within 60% of each other. Under LCN DNA typing conditions (34 cycles; 25 pg), however, one of the two heterozygous alleles can measure just 20% or less of the height of its sister allele. Allele drop out, extreme allele imbalance, also may occur yielding only one allele at a heterozygote locus. The imbalance can be exacerbated by preferential amplification of shorter alleles over longer alleles. Thus, some alleles may not be replicated

sufficiently for detection. A locus may appear to be homozygous falsely due to the drop out of an allele. Allele drop out is related to sample quantity and quality. These features often are ill-defined in LCN samples and are sample-specific.

Stutter products are a common artifact in the PCR amplification of forensically-relevant STRs. The most plausible mechanism for generating stutter is strand slippage when the polymerase pauses during extension (42). Under increased sensitivity assays where there are few template molecules, a stutter event that occurs early in the PCR can be over-represented compared with the true allele. Stutter has been shown to increase in proportion to its parent allele under LCN typing conditions, causing increased complexity for interpretation even for single source samples. When a stutter peak's height is sufficiently high such that it could be considered a true allele, more uncertainty is created about the significance of a result.

It is possible that a peak due to stutter may be seen twice in replicate analyses and deemed a "true" allele. Reduction in stutter will facilitate genotyping of low level DNA samples. Additives which alleviate the paused extension of primer, stabilize the enzyme, or reduce instability of the template strand may reduce the stochastic effects observed with stutter and peak height imbalance generated during PCR amplification.

A potential approach to improve robustness of amplification of low template DNA is to modify the PCR by use of additives that effectively concentrate the target and enzyme. Robustness of amplification can be measured by reduced stutter values, better heterozygote balance, and increased DNA yield. PCR enhancers often are used to increase both yield and specificity or to overcome difficulties encountered in the PCR, such as spurious amplification products, uneven or no amplification of some target sequences, and complications in reproducing some results. Some additives may improve the balance and production of PCR products but may

not work effectively with a particular sequence or particular PCR conditions. There are several possible reasons for this shortcoming: GC content rich template, secondary structure formation of the template, or even the complexity of the components in the PCR (namely, K^+ and Na^+) (43-46).

Accordingly, a variety of PCR additives and enhancing agents have been the focus of efforts to improve amplification and stringency (47-61). The most successful of the additives tested have been dimethyl sulfoxide (DMSO), glycerol, polyethylene glycol (PEG), betaine and formamide (62). While these additives may have beneficial effects on some amplifications, it is impossible to predict which agents will be useful in a particular context and therefore, need to be thoroughly tested. Addition of PCR enhancing agents can increase yield of the desired PCR product or decrease of undesired products.

Weissersteiner *et al.* (62) first introduced the glycine betaine as a powerful PCR additive to counteract effects of NaCl and other high salt inhibition of *Taq* polymerase (63). Since then, betaine has been used as a PCR facilitator, not only as a single compound, but also in combination with other additives (43, 44, 46-55, 58, 59, 64-70). Most of these studies, however, failed to explain fully how betaine and the other additives work. Betaine is believed to facilitate PCR via strand separation, lowering melting temperature (Tm), and acting as an isostabilizing agent, equalizing the contribution of GC- and AT-base pairings to the stability of the DNA duplex (59, 67). Furthermore, certain DNA sequences can cause the DNA polymerase to pause, a phenomenon that can be counteracted by betaine. It has been suggested that betaine disrupts the contorted DNA helix without perturbing the polymerase-DNA interaction (68). In fact, betaine has been used to enhance formation of long PCR products, in diagnostic PCR, on GC rich template, and in low temperature PCRs. Another advantage of using betaine is that it acts as an

osmoprotectant, increasing the resistance of the polymerase to denaturation. Betaine also allows the PCR to overcome some low level of contaminants that can co-purify with DNA, allowing for PCR of low quality DNA samples.

Other potential PCR enhancers include PEG and DMSO. PEG is an additive which effectively concentrates the target and enzyme, acting as a volume excluder or molecular crowder (7, 71-74). DMSO is thought to assist in amplification by reducing secondary structure, facilitating strand separation by disrupting base pairing, which is particularly useful for GC rich templates (57, 64). However, in high amounts, DMSO can reduce *Taq* polymerase activity by up to 50% (67). Recently, PCRboost® (Biomatrica, San Diego, CA), a novel additive, became commercially available. Previous studies have shown that PCRboost® has the ability to enhance yield, specificity, and consistency of the PCR (75, 76). Amplifications from low quality and low quantity DNA samples containing inhibitors have shown increased allele detection.

However, even with the research studies on PCR additives, to my knowledge no one has explored the effects of these products on reducing stochastic effects during amplification of LCN samples employing increased PCR cycle number and STR loci. This project investigated the amplification enhancement of betaine, DMSO, PEG and PCRboost on low quantity and low quality DNA samples. Specifically, the potential of these additives on improving the sensitivity and robustness of the PCR of LCN samples based on reduction of stochastic effects, namely stutter and peak height imbalance, was studied.

DNA Transfer: Interpretation of LCN DNA profiles may be improved with a better understanding of DNA transfer. The examination of DNA transferred through contact has become a major subject of interest in the field of forensic genetics. It has direct bearing on the

interpretation related to the reconstruction of a case based on DNA profiles and the relevance of the information even for single source samples. Therefore, it is necessary to understand the potential impact of transfer DNA to a sample that may overwhelm the DNA on or in the sample or be interpreted as originating from that sample. Various studies have been performed to ascertain the characteristics of DNA transfer, secondary transfer, and persistence of DNA on evidentiary items (77-79). That research included the examination of an individual's shedder status, an individual's sex, and the effects of time, substrate, and environment on DNA recovery.

In an early study on DNA transfer, Wickenheiser (77) examined the generation of complete DNA profiles from skin cells while taking into consideration handling time, individual characteristics, and contact surface. The results demonstrated that DNA transfer occurred within ten seconds of contact. The results also showed that DNA transfer can be dependent on the individual donor and substrate.

Goray *et al*. (78) reported that various factors can affect DNA deposition from touched objects. These factors included substrates, types of contact (passive, pressured, and friction), type of biological substance deposited and the amount of moisture of the substance. van Oorschot *et al*. (79) provided several observations regarding DNA transfer. Their findings were that the type of substrate, the moisture level of the sample, and the type of contact between an individual and substrate have a significant role in the amount of DNA transfer. Results suggested that primary substrates which are porous and/or dry samples transfer less DNA compared with non-porous primary substrates and/or wet samples; secondary substrates which are porous yield increased DNA transfer; and friction contact increases DNA transfer (79). While this research has increased the general knowledge of DNA transfer, all of the factors discussed by the authors are uncontrollable in real world situations.

Most of the above mentioned studies to date have been somewhat limited in that they have focused on DNA deposited through epithelial cells sloughed off during contact with individuals' hands. Saliva is a bodily fluid commonly encountered and transferred between individuals (or objects) on a continuous basis. For instance, it is not uncommon for a person to hold a pen in his or her mouth for a small amount of time while studying or reading or a person to lick his or her thumb while turning pages of a book. The deposition of saliva-based DNA on the pen is a primary transfer event. If the pen is later handed to a second person, a secondary transfer event can occur when the first individual's DNA is passed from the pen to the second individual's hand. A goal of this study was to determine if, due to the inherent uncertainty of the manner in which DNA is deposited on a forensic sample, primary and secondary transfer involving saliva based DNA can be ruled out as a possible source of the recovered DNA of a LCN sample.

DNA Interpretation: Steele and Balding (80) stated "because there is no clear distinction between LTDNA and standard DNA profiling, any method of analysis for LTDNA profiles should return the same results as would a standard analysis when presented with profiles obtained using optimal DNA template." For LCN DNA analysis, however, the recovered DNA profiles often are difficult to interpret due to greater complexity and are especially problematic for mixed profiles (81-84). One of the most important tasks for a DNA analyst is the interpretation of DNA profile results. The main challenges are due to stochastic effects of the PCR:

1. Heterozygote imbalance – allele pairs are more prone to imbalance, and the variance in the distribution of the observed ratio of peak heights is increased, which means that standard interpretation guidelines (applicable for single source profiles generated from greater than 100 pg of template DNA) would no longer apply.
2. Allele dropout – Sampling and amplification stochastic effects with low level template can result in the failure of one or both alleles to amplify or produce a peak that does not exceed a defined threshold level.
3. Increased stutter peaks – stutter peaks are often relatively larger than those observed with higher template quantities.
4. Contamination (i.e., allele drop in) – peaks which cannot be identified as artifacts, but do not reside in the sample. Their origin can be postulated to derive from extraneous DNA present in the laboratory, in the sample matrix or introduced during collection and transfer of evidence.

If true DNA alleles can dropout and spurious alleles can drop in, one should ask how can such evidence ever be correctly interpreted? For some, the answer to this question is that probabilistic models can approximate the events in a particular case and such analyses may be informative. In some cases, computer software has been developed for in-house use or made available on the internet (85-90). These programs are based on two general approaches for the interpretation of LCN profiles: a biological model and a statistical model.

Statistical inferences are the weakest part of the LCN typing process, although strides have been made (91, 92). There are two methods for interpretation: a biological model and a statistical model. Both involve interpretation but only the statistical model provides more

comprehensive guidance on statistical weight to be applied to LCN typing results. Limitations of either model are the degree of confidence associated with a result and data to populate the variables. The latter often is supported predominately through modeling studies (90, 93, 94, 94-97). A number of statistical methods have become available for LCN DNA STR profile analysis. **Chapter 7 Table 1** summarizes the key features of available software programs, and the programs are briefly discussed.

Recently, the International Society for Forensic Genetics (ISFG) published recommendations based on discrete models for forensic analysis of single source DNA profiles (98). I support the recommendations from ISFG and make additional suggestions for a future model for single source sample interpretation that includes template sampling issues from a sample, stochastic effects during the PCR, and that the events may not be independent from locus-to-locus. Dimensions from the main statistical models proposed to date are incorporated and further dimensions to interpretation of LCN typing results have been added and are discussed.

REFERENCES

1. Findlay I, Taylor A, Quirke P, Frazier R, Urquhart A. DNA fingerprinting from single cells. Nature. 1997 10;389(6651):555-6.

2. Budowle B, Eisenberg AJ, van Daal A. Validity of low copy number typing and applications to forensic science. Croat Med J. 2009 6;50(3):207-17.

3. 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 7;112(1):17-40.

4. Gill P. Application of low copy number DNA profiling. Croat Med J. 2001 6;42(3):229-32.

5. Westen AA, Nagel JH, Benschop CC, Weiler NE, de Jong BJ, Sijen T. Higher capillary electrophoresis injection settings as an efficient approach to increase the sensitivity of STR typing. J Forensic Sci. 2009 5;54(3):591-8.

6. Forster L, Thomson J, Kutranov S. Direct comparison of post-28-cycle PCR purification and modified capillary electrophoresis methods with the 34-cycle "low copy number" (LCN) method for analysis of trace forensic DNA samples. Forensic Sci Int Genet. 2008 9;2(4):318-28.

7. Budowle B, van Daal A. Extracting evidence from forensic DNA analyses: Future molecular biology directions. BioTechniques. 2009 4;46(5):339,40, 342-50.

8. Smith PJ, Ballantyne J. Simplified low-copy-number DNA analysis by post-PCR purification. J Forensic Sci. 2007 7;52(4):820-9.

9. Kloosterman AD, Kersbergen P. Efficacy and limits of genotyping low copy number (LCN) DNA samples by multiplex PCR of STR loci. J Soc Biol. 2003;197(4):351-9.

10. Roeder AD, Elsmore P, Greenhalgh M, McDonald A. Maximizing DNA profiling success from sub-optimal quantities of DNA: A staged approach. Forensic Sci Int Genet. 2009 3;3(2):128-37.

11. Staiti N, Romano C, Trapani C, Ginestra E, Leo B, Schiavone S. Analysis of LCN DNA from synthetic ropes: A practical approach used in real homicide investigation. Forensic Science International: Genetics Supplement Series. 2008 8;1(1):446-7.

12. Staiti N, Giuffrè G, Di Martino D, Simone A, Sippelli G, Tuccari G, Saravo L. Molecular analysis of genomic low copy number DNA extracted from laser-microdissected cells. Int Congr Ser. 2006 4;1288:568-70.

13. Barbaro A, Cormaci P, Barbaro A. LCN DNA typing from touched objects. Int Congr Ser. 2006 4;1288:553-555.

14. Anjos MJ, Andrade L, Carvalho M, Lopes V, Serra A, Oliveira C, Batista L, Balsa F, Brito P, Corte-Real F, Vide MC. Low copy number: Interpretation of evidence results. Int Congr Ser. 2006 4;1288:616-8.

15. 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 Congr Ser. 2006 4;1288:651-3.

16. van Oorschot RA, Ballantyne KN, Mitchell RJ. Forensic trace DNA: A review. Investig Genet. 2010 12;1(1):14,2223-1-14.

17. Hansson O, Finnebraaten M, Heitmann IK, Ramse M, Bouzga M. Trace DNA collection—Performance of minitape and three different swabs. Forensic Science International: Genetics Supplement Series. 2009 12;2(1):189-90.

18. Amory S, Huel R, Bilić A, Loreille O, Parsons TJ. Automatable full demineralization DNA extraction procedure from degraded skeletal remains. Forensic Science International: Genetics. 2012 5;6(3):398-406.

19. Dukes MJ, Williams AL, Massey CM, Wojtkiewicz PW. Technical note: Bone DNA extraction and purification using silica-coated paramagnetic beads. Am J Phys Anthropol. 2012 7;148(3):473-82.

20. Jakubowska J, Maciejewska A, Pawlowski R. Comparison of three methods of DNA extraction from human bones with different degrees of degradation. Int J Legal Med. 2012 1;126(1):173-8.

21. Davoren J, Vanek D, Konjhodzic R, Crews J, Huffine E, Parsons TJ. Highly effective DNA extraction method for nuclear short tandem repeat testing of skeletal remains from mass graves. Croat Med J. 2007 8;48(4):478-85.

22. Loreille OM, Diegoli TM, Irwin JA, Coble MD, Parsons TJ. High efficiency DNA extraction from bone by total demineralization. Forensic Sci Int Genet. 2007 6;1(2):191-5.

23. Yang DY, Eng B, Waye JS, Dudar JC, Saunders SR. Technical note: Improved DNA extraction from ancient bones using silica-based spin columns. Am J Phys Anthropol. 1998 4;105(4):539-43.

24. Primorac D, Andelinovic S, Definis-Gojanovic M, Drmic I, Rezic B, Baden MM, Kennedy MA, Schanfield MS, Skakel SB, Lee HC. Identification of war victims from mass graves in croatia, bosnia, and herzegovina by use of standard forensic methods and DNA typing. J Forensic Sci. 1996 9;41(5):891-4.

25. Hochmeister MN, Budowle B, Borer UV, Eggmann U, Comey CT, Dirnhofer R. Typing of deoxyribonucleic acid (DNA) extracted from compact bone from human remains. J Forensic Sci. 1991 11;36(6):1649-61.

26. Smejkal GB, Witzmann FA, Ringham H, Small D, Chase SF, Behnke J, Ting E. Sample preparation for two-dimensional gel electrophoresis using pressure cycling technology. Anal Biochem. 2007 4;363(2):309-11.

27. Gross V, Carlson G, Kwan AT, Smejkal G, Freeman E, Ivanov AR, Lazarev A. Tissue fractionation by hydrostatic pressure cycling technology: The unified sample preparation technique for systems biology studies. J Biomol Tech. 2008 7;19(3):189-99.

28. Balny C. What lies in the future of high-pressure bioscience? Biochimica et Biophysica Acta (BBA) - Proteins & Proteomics. 2006 3;1764(3):632-9.

29. Delgado A, Kulisiewicz L, Rauh C, Benning R. Basic aspects of phase changes under high pressure. Ann N Y Acad Sci. 2010 2;1189:16-23.

30. Occhipinti E, Bec N, Gambirasio B, Baietta G, Martelli PL, Casadio R, Balny C, Lange R, Tortora P. Pressure and temperature as tools for investigating the role of individual non-covalent interactions in enzymatic reactions: Sulfolobus solfataricus carboxypeptidase as a model enzyme. Biochimica et Biophysica Acta (BBA) - Proteins & Proteomics. 2006 3;1764(3):563-72.

31. Winter R, Dzwolak W. Temperature-pressure configurational landscape of lipid bilayers and proteins. Cell Mol Biol (Noisy-le-grand). 2004 6;50(4):397-417.

32. So A, Pel J, Rajan S, Marziali A. Efficient genomic DNA extraction from low target concentration bacterial cultures using SCODA DNA extraction technology. Cold Spring Harb Protoc. 2010 10.

33. Broemeling DJ, Pel J, Gunn DC, Mai L, Thompson JD, Poon H, Marziali A. An instrument for automated purification of nucleic acids from contaminated forensic samples. JALA 2008 2;13:40-48.

34. Marziali A, Pel J, Bizzotto D, Whitehead LA. Novel electrophoresis mechanism based on synchronous alternating drag perturbation. Electrophoresis. 2005 1;26(1):82-90.

35. Alaeddini R. Forensic implications of PCR inhibition-a review. Forensic Sci Int-Gen 2012 5;6:297-305.

36. Kreader CA. Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. Appl Environ Microbiol. 1996 3;62(3):1102-6.

37. Lopes MLM, Valente Mesquita VL, Chiaradia ACN, Fernandes AAR, Fernandes PMB. High hydrostatic pressure processing of tropical fruits. Ann N Y Acad Sci. 2010;1189(1):6-15.

38. Considine KM, Kelly AL, Fitzgerald GF, Hill C, Sleator RD. High-pressure processing ? effects on microbial food safety and food quality. FEMS Microbiol Lett. 2008;281(1):1-9.

39. Van Eldik R, KlÃrner FG. High pressure chemistry: Synthetic, mechanistic, and supercritical applications. John Wiley & Sons; 2008.

40. Whitaker J, Tully G, Sullivan K. Response to low level DNA profiling. Forensic Sci Med Pathol. 2009;5(2):102,3; author reply 104-5.

41. Budowle B, Hobson DL, Smerick JB, Smith JAL. Low Copy Number - Consideration and Caution. Madison, WI: Promega Corporation; 2001. Report No.: 1-26.

42. Schlotterer C, Tautz D. Slippage synthesis of simple sequence DNA. Nucleic Acids Res. 1992 1;20(2):211-5.

43. Sahdev S, Saini S, Tiwari P, Saxena S, Singh Saini K. Amplification of GC-rich genes by following a combination strategy of primer design, enhancers and modified PCR cycle conditions. Mol Cell Probes. 2007 8;21(4):303-7.

44. Zhang Z, Yang X, Meng L, Liu F, Shen C, Yang W. Enhanced amplification of GC-rich DNA with two organic reagents. BioTechniques. 2009 9;47(3):775-9.

45. Zhang AH, Seo SB, Yi JA, Kim HY, Lee SD. Modification of a commercially available kit for the improvement of PCR efficiency. Human Biology. (3):343.

46. Chakrabarti R, Schutt CE. Novel sulfoxides facilitate GC-rich template amplification. BioTechniques. 2002 4;32(4):866, 868, 870,2, 874.

47. Chakrabarti R, Schutt CE. The enhancement of PCR amplification by low molecular weight amides. Nucleic Acids Res. 2001 6;29(11):2377-81.

48. Fazekas A, Steeves R, Newmaster S. Improving sequencing quality from PCR products containing long mononucleotide repeats. BioTechniques. 2010 4;48(4):277-85.

49. Haqqi T, Zhao X, Panciu A, Yadav SP. Sequencing in the presence of betaine: Improvement in sequencing of the localized repeat sequence regions. J Biomol Tech. 2002 12;13(4):265-71.

50. Henke W, Herdel K, Jung K, Schnorr D, Loening SA. Betaine improves the PCR amplification of GC-rich DNA sequences. Nucleic Acids Res. 1997 10;25(19):3957-8.

51. Horakova H, Polakovicova I, Shaik GM, Eitler J, Bugajev V, Draberova L, Draber P. 1,2-propanediol-trehalose mixture as a potent quantitative real-time PCR enhancer. BMC Biotechnol. 2011 4;11:41.

52. Hube F, Reverdiau P, Iochmann S, Gruel Y. Improved PCR method for amplification of GC-rich DNA sequences. Mol Biotechnol. 2005 9;31(1):81-4.

53. Jensen MA, Fukushima M, Davis RW. DMSO and betaine greatly improve amplification of GC-rich constructs in de novo synthesis. PLoS One. 2010 6;5(6):e11024.

54. Juen A, Traugott M. Amplification facilitators and multiplex PCR: Tools to overcome PCR-inhibition in DNA-gut-content analysis of soil-living invertebrates. Soil Biol Biochem. 2006 7;38(7):1872-9.

55. Mamedov TG, Pienaar E, Whitney SE, TerMaat JR, Carvill G, Goliath R, Subramanian A, Viljoen HJ. A fundamental study of the PCR amplification of GC-rich DNA templates. Computational Biology and Chemistry. 2008 12;32(6):452-7.

56. Nagai M, Yoshida A, Sato N. Additive effects of bovine serum albumin, dithiothreitol, and glycerol on PCR. Biochem Mol Biol Int. 1998 1;44(1):157-63.

57. Musso M, Bocciardi R, Parodi S, Ravazzolo R, Ceccherini I. Betaine, dimethyl sulfoxide, and 7-deaza-dGTP, a powerful mixture for amplification of GC-rich DNA sequences. J Mol Diagn. 2006 11;8(5):544-50.

58. Ralser M, Querfurth R, Warnatz H, Lehrach H, Yaspo M, Krobitsch S. An efficient and economic enhancer mix for PCR. Biochem Biophys Res Commun. 2006 9;347(3):747-51.

59. Rees WA, Yager TD, Korte J, von Hippel PH. Betaine can eliminate the base pair composition dependence of DNA melting. Biochemistry. 1993 1;32(1):137-44.

60. Sarkar G, Kapelner S, Sommer SS. Formamide can dramatically improve the specificity of PCR. Nucleic Acids Res. 1990 12;18(24):7465.

61. Spiess AN, Mueller N, Ivell R. Trehalose is a potent PCR enhancer: Lowering of DNA melting temperature and thermal stabilization of taq polymerase by the disaccharide trehalose. Clin Chem. 2004 7;50(7):1256-9.

62. Weissensteiner T, Lanchbury JS. Strategy for controlling preferential amplification and avoiding false negatives in PCR typing. BioTechniques. 1996 12;21(6):1102-8.

63. Favre N, Rudin W. Salt-dependent performance variation of DNA polymerases in co-amplification PCR. BioTechniques. 1996 7;21(1):28-30.

64. Kang J, Soog Lee M, Gorenstein DG. The enhancement of PCR amplification of a random sequence DNA library by DMSO and betaine: Application to in vitro combinatorial selection of aptamers. J Biochem Biophys Methods. 2005 8;64(2):147-51.

65. Koumoto K, Ochiai H, Sugimoto N. Structural effect of synthetic zwitterionic cosolutes on the stability of DNA duplexes. Tetrahedron. 2008 1;64(1):168-74.

66. Kovarova M, Draber P. New specificity and yield enhancer of polymerase chain reactions. Nucleic Acids Res. 2000 7;28(13):E70.

67. Kurz M. Compatible solute influence on nucleic acids: Many questions but few answers. Saline Systems. 2008 6;4:6.

68. Paul N. H. Optimizing multiplex and LA-PCR with betaine. Trends Biochem Sci. 1997 6;22(6):225-6.

69. Spiess A, Ivell R. A highly efficient method for long-chain cDNA synthesis using trehalose and betaine. Anal Biochem. 2002 2;301(2):168-74.

70. Vasudevamurthy MK, Lever M, George PM, Morison KR. Betaine structure and the presence of hydroxyl groups alters the effects on DNA melting temperatures. Biopolymers. 2009;91(1):85-94.

71. Ballantyne KN, van Oorschot RAH, John Mitchell R, Koukoulas I. Molecular crowding increases the amplification success of multiple displacement amplification and short tandem repeat genotyping. Anal Biochem. 2006 8;355(2):298-303.

72. Minton AP. The influence of macromolecular crowding and macromolecular confinement on biochemical reactions in physiological media. J Biol Chem. 2001 4;276(14):10577-80.

73. Zimmerman SB, Harrison B. Macromolecular crowding increases binding of DNA polymerase to DNA: An adaptive effect. Proc Natl Acad Sci U S A. 1987 4;84(7):1871-5.

74. Sasaki Y, Miyoshi D, Sugimoto N. Effect of molecular crowding on DNA polymerase activity. Biotechnology Journal. 2006;1(4):440-6.

75. Lee SB, Clabaugh KC, Silva B, Odigie KO, Coble MD, Loreille O, Scheible M, Fourney RM, Stevens J, Carmody GC, Parsons TJ, Pozder A, Eisenberg AJ, Budowle B, Ahmad T, Miller RW, Crouse CA. Assessing a novel room temperature DNA storage medium for forensic biological samples. Forensic Sci Int Genet. 2012 1;6(1):31-40.

76. Clement O, Whitney S, Muller-Cohn J, Muller R. Following nature's lead: Generating compounds for stabilizing biomolecules. Biopreservation and Biobanking. 2012;10(4):395-402.

77. 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 5;47(3):442-50.

78. Goray M, Eken E, Mitchell RJ, van Ooorschot RAH. Secondary DNA transfer of biological substances under varying test conditions. Forensic Sci Int Genet 2010 2;4(2):62-67.

79. van Oorschot RAH, Goray M, Eken E, Mitchell RJ. Impact of relevant variables on the transfer of biological substances. Forensic Science International: Genetics Supplement Series. 2009 12; 2(1):547–548.

80. Steele CD, Balding DJ. Statistical evaluation of forensic DNA profile evidence. Annu.Rev.Stat.Appl. 2014;1:20.1,20.24.

81. Cowell RG, Lauritzen SL, Mortera J. Probabilistic expert systems for handling artifacts in complex DNA mixtures. Forensic Science International: Genetics. 2011 6;5(3):202-9.

82. Weusten J, Herbergs J. A stochastic model of the processes in PCR based amplification of STR DNA in forensic applications. Forensic Science International: Genetics. 2012;6(1):17-25.

83. Michel S, De Bast A, Vandenbroere I, Froment O. Interpretation of low-copy-number DNA profile after post-PCR purification. Forensic Science International: Genetics Supplement Series. 2009;2(1):542-3.

84. Leclair B, Fregeau CJ, Bowen KL, Fourney RM. Systematic analysis of stutter percentages and allele peak height and peak area ratios at heterozygous STR loci for forensic casework and database samples. J Forensic Sci. 2004 9;49(5):968-80.

85. Gill P, Kirkham A, Curran J. LoComatioN: A software tool for the analysis of low copy number DNA profiles. Forensic Sci Int. 2007 3;166(2-3):128-38.

86. Mitchell AA, Tamariz J, O'Connell K, Ducasse N, Budimlija Z, Prinz M, Caragine T. Validation of a DNA mixture statistics tool incorporating allelic drop-out and drop-in. Forensic Science International: Genetics. 2012;6(6):749-61.

87. Perlin MW, Legler MM, Spencer CE, Smith JL, Allan WP, Belrose JL, Duceman BW. Validating TrueAllele® DNA mixture interpretation*,†. J Forensic Sci. 2011;56(6):1430-47.

88. Perlin MW, Szabady B. Linear mixture analysis: A mathematical approach to resolving mixed DNA samples. J Forensic Sci. 2001;46(6):1372-8.

89. Kelly H, Bright J, Buckleton JS, Curran JM. A comparison of statistical models for the analysis of complex forensic DNA profiles. Science & Justice. 2014 1;54(1):66-70.

90. Hinda H. Forensim: An open-source initiative for the evaluation of statistical methods in forensic genetics. Forensic Science International: Genetics. 2011 8;5(4):265-8.

91. Balding DJ, Buckleton J. Interpreting low template DNA profiles. Forensic Science International: Genetics. 2009 12;4(1):1–10.

92. Buckleton J. Validation issues around DNA typing of low level DNA. Forensic Sci Int Genet. 2009 9;3(4):255-60.

93. Hedman J, Nordgaard A, Rasmusson B, Ansell R, Radstrom P. Improved forensic DNA analysis through the use of alternative DNA polymerases and statistical modeling of DNA profiles. BioTechniques. 2009 11;47(5):951-8.

94. Gill P, Curran J, Neumann C, Kirkham A, Clayton T, Whitaker J, Lambert J. Interpretation of complex DNA profiles using empirical models and a method to measure their robustness. Forensic Sci Int Genet. 2008 3;2(2):91-103.

95. Tvedebrink T, Eriksen PS, Mogensen HS, Morling N. Statistical model for degraded DNA samples and adjusted probabilities for allelic drop-out. Forensic Science International: Genetics. 2012 1;6(1):97-101.

96. Gill P, Buckleton J. A universal strategy to interpret DNA profiles that does not require a definition of low-copy-number. Forensic Science International: Genetics. 2010 7;4(4):221-7.

97. Haned H, Egeland T, Pontier D, Pène L, Gill P. Estimating drop-out probabilities in forensic DNA samples: A simulation approach to evaluate different models. Forensic Science International: Genetics. 2011 11;5(5):525-31.

98. Gill P, Gusmao L, Haned H, Mayr W, Morling N, Parson W, Prieto L, Prinz M, Schneider H, Schneider PM, Weir BS. Recommendations on the evaluation of STR typing results that may include drop-out and/or drop-in using probabilistic methods. Forensic Sci.Int.Genet. 2012;5(6):679-88.

SECTION 1

Improving DNA Collection

Physical evidence cannot be wrong, it cannot perjure itself, it cannot be wholly absent. Only human failure to find it, study and understand it, can diminish its value." ~ Dr. Edmond Locard

CHAPTER 1

Evaluation of a Novel Material, Diomics X-Swab™, for Collection of DNA

Submitted to Forensic Science International: Genetics
February 14, 2014

Pamela L. Marshall
Monika Stoljarova
Bobby L. Larue
Jonathan L. King
Bruce Budowle

ABSTRACT

Success of DNA typing is related to the amount of target material recovered from an evidentiary item. Generally, the more DNA that is recovered, the better the chance is of obtaining a typing result that will be robust and reliable. One method of collecting stain materials is by swabbing. Recovery of DNA from a number of commercially-available swabs is not an efficient process. The X-Swab™ (Diomics Corporation, La Jolla, CA) is a unique bio-specimen collection material, which can be dissolved during certain extraction conditions. Therefore, more DNA may be collected from a substrate and be released from the swab matrix than other swabs. The ability to recover DNA from the X-Swab and success in STR typing were compared with the Copan 4N6FLOQSwab™ (Brescia, Italy), a device which utilizes a proprietary flocked-swab technology to maximize DNA collection and elution efficiency. Both types of swabs were impregnated with known amounts of DNA and body fluids and allowed to air dry. In addition, blood was placed onto glass slides, allowed to dry and collected using both types of swabs. DNA recovery was assessed by DNA quantification and by STR typing. Results demonstrated that the X-Swab material yielded greater DNA recovery, particularly of low quantity samples, compared with the 4N6FLOQSwab. Results also indicated that the X-Swab material itself enhances yield of PCR products.

KEYWORDS Diomics X-Swab™ · Copan 4N6FLOQSwabs™ · DNA Collection · DNA Recovery · DNA Quantification · STR typing

INTRODUCTION

The first step in the process of forensic DNA typing is the collection of evidentiary samples. Swabbing is one of several approaches for collecting biological evidence from stains. Successful DNA analysis relies on the ability of a swab to absorb and/or adsorb materials from a stain or surface and then release the cells/DNA by an extraction process. Swabs that are proficient at collecting materials often are less efficient at releasing DNA from the swab matrix, and vice versa. Indeed, it is well-known that recovery of DNA from a swab is inefficient (1-3). In fact, van Oorschot *et al.* (2) suggested that a significant proportion of DNA (20–76%) that is collected by a cotton cloth/swab is lost during the extraction phase which may be attributed to the collecting agent (swab, cloth, etc.) and the condition of the sample.

Prinz *et al*. (4) compared several collection devices in an attempt to improve cell/DNA removal from fingerprint evidence. A total of 109 touched objects, including 30 single fingerprint samples, were extracted and most of these samples provided less than 100 pg of DNA. However, the authors indicated that a "specialized" swab yielded up to 2.5 times more alleles compared with cotton and Dacron swabs. Hansson *et al*. (5) studied different swabs as well as minitape to determine the best collection device for low copy number (LCN) DNA recovery. The authors tested a cotton Dryswab™ (Medical Wire, Corsham, Wiltshire, England), Copan 4N6FLOQSwab™ (Brescia, Italy), a self-saturating swab (Puritan Medical Products Co., LLC, Guilford, ME), and the Scenesafe FAST™ minitape (Scenesafe LTD., Burnham on Crouch, Essex, England). While the results showed no difference in DNA recovery among the swabs, the minitape yielded more DNA and gave full profiles for all six experimental samples. The DNA recovered from flocked swabs produced only partial profiles, while the DNA from the self-saturating swab and the Dryswab produced three and four full profiles, respectively. Hansson *et*

al. (5) suggested that investigators may want to consider the type of substrate when choosing a collection device and also recommended further investigation into collection devices.

Brownlow *et al*. (6) studied DNA retrieval from a traditional cotton swab compared with that of the 4N6FLOQSwab using three different extraction platforms. The results suggested that while both swabs recovered greater than 50% of DNA, the extraction platform chosen had an impact on DNA recovery. Thus, the authors recommended careful consideration of an extraction method with the choice of swab. A more recent study carried out by Dadhania *et al*. (7) evaluated the 4N6FLOQSwab and cotton swab for DNA recovery using two different magnetic bead technologies (Prepfiler® (Life Technologies) and DNA IQ™ **(**Promega Corporation, Madison, WI)). It was determined that the 4N6FLOQSwab yielded higher recovery of DNA using Prepfiler compared with cotton swabs.

While the above studies suggested that the 4N6FLOQSwab yielded more DNA over the traditional cotton swab, recovery of DNA often was not greater than 50%. The novel collection material, The X-Swab™ (Diomics, La Jolla, California), was selected for potentially better DNA recovery as it has high absorptive qualities and can be dissolved under certain DNA extraction conditions (e.g., one hour incubation at 56^{o}C). Thus, more DNA may be released from its matrix than other collection devices. Improving DNA yield from collection devices may result in some samples that traditionally yield too little DNA becoming suitable for routine analysis. Furthermore, this device may improve retrieval of higher quality DNA as longer stranded molecules may be released from the matrix than with other collection devices.

MATERIALS AND METHODS

Samples:

DNA: Some experiments were performed using the Quantifiler® Human DNA Quantification Kit (Life Technologies) DNA Standard (Raji cell line; 200 ng/µL purified DNA). The standard was diluted in water to final concentrations of 0.1 ng/µL, 0.25 ng/ µL, 0.5 ng/µL, and 1 ng/µL. Negative controls were included with each experiment.

Whole Blood and Saliva: Whole blood and saliva were provided by UNTHSC faculty and staff. All samples were collected with informed consent and were anonymized to ensure the privacy of the contributing subjects in accordance with University of North Texas Health Science Center IRB. Dilutions of whole blood (1:10, 1:50, and 1:100) and saliva (1:10, 1:50, and 1:100) were prepared in 10 mM phosphate buffered saline (PBS). Negative controls were included with each experiment.

Swabs:

X-Swab: Samples of the X-Swab Diomat™ material (kindly provided by Diomics Corporation) were cut into squares (prototype format) weighing approximately 15 mg and measuring approximately 5 mm x 5 mm. Negative controls (no DNA on swab) were performed with every extraction.

4N6FLOQSwab: The 4N6FLOQSwab swab was purchased from Life Technologies. Negative controls (no DNA on swab) were performed with every extraction.

DNA Extraction:

Kits: DNA was extracted using either a modified QIAamp® DNA Micro (Qiagen, Valencia, CA) extraction protocol using MinElute® spin columns or PrepFiler Express™ DNA Extraction Kit (Life Technologies) according to manufacturer's instructions. The DNA extracts were stored at 4°C and −20°C for short- and long-term storage, respectively.

Phenol:Chloroform Organic Extraction: Extraction was performed using a standard protocol for phenol:chloroform organic extraction (8). The DNA extracts were stored at 4°C and −20°C for short- and long-term storage, respectively.

DNA Quantification: The quantity of extracted DNA was determined using a reduced volume (10 µL) protocol of the Applied Biosystems® Quantifiler™ Human DNA Quantification Kit (Life Technologies) on an Applied Biosystems® 7500 Real-Time PCR System (Life Technologies) with proper controls.

Amplification: For the AmpFℓSTR® Identifiler® Plus kit (Life Technologies), thermal cycling was performed on a GeneAmp® PCR System 9700 (Life Technologies) using 28 or 29 cycles (if quantity of DNA was below 0.5 ng) according to the manufacturer's recommendations. For the PowerPlex® ESI 17 Pro System (Promega Corporation, Madison, WI), thermal cycling was performed on a GeneAmp® PCR System 9700 according to manufacturer's recommendations. Positive (9947A), negative (no template DNA). Reagent blank controls were included on each assay plate.

Capillary Electrophoresis and Analysis: Capillary electrophoresis was performed on an Applied Biosystems® 3130xl Genetic Analyzer (Life Technologies) using POP-4™ polymer (Life Technologies). Data were analyzed using Applied Biosystems® GeneMapper® ID v3.2 software (Life Technologies).

RapidHIT™ Human DNA Identification System (IntegenX, Pleasanton, CA): 10 µL of whole blood from five individuals were applied to X-Swabs and allowed to dry overnight. The samples were run on the RapidHIT system according to the manufacturer's instructions. DNA results were analyzed with GeneMarker HID V2.4.0 (SoftGenetics; State College, PA, USA).

Massively Parallel Sequencing (MPS): A 1:10 dilution of whole blood was placed on an X-Swab, dried overnight, and extracted using the PrepFiler Express™ DNA Extraction Kit. The whole mitochondrial genomes from two replicates were sequenced using the MiSeq™ workflow (Illumina, San Diego, CA) as described by King *et al*. (9).

Data Analysis: The quantity of DNA recovered from the X-Swab was evaluated and compared with the 4N6FLOQSwab. PCR product yield (based on relative fluorescence units (RFUs)) and peak height ratio (PHR) for heterozygous loci were evaluated and compared with controls using a freely available in-house excel workbook, PHASTR (http://web.unthsc.edu/info/200210/molecular_and_medical_genetics/887/research_and_development_laboratory/3). Intra-locus PHRs were calculated for a given locus by dividing the peak height of an allele with a lower RFU value by the peak height of an allele with a higher RFU value, and then multiplying this value by 100 to express the PHR as a percentage. Unpaired 2-

tailed Student's T-tests were used to determine whether DNA yield was different between the swabs at a significance level of 0.05.

RESULTS AND DISCUSSION

Evaluation of Diomics X-Swab and Extraction Methods

Initial experiments examined the X-Swab Diomat material and the extraction method that would yield the greatest quantity of DNA. 5 µL of whole blood were placed onto X-Swab material and dried overnight. The samples, in replicates of ten, were then extracted using a modified MinElute extraction protocol, PrepFiler Express DNA Extraction Kit, and standard organic extraction. The quantity and quality of DNA recovered from the X-Swab material were evaluated and compared with no swab control (i.e., equivalent amount of liquid blood). The amount of DNA recovered for each extraction method is shown in **Table 1** and indicated that the PrepFiler Express yielded the highest recovery of DNA. Samples extracted using all three extraction methods produced the expected STR profiles using Identifiler Plus (28 cycles) with similar RFU values and peak height balance (data not shown). Due to these results, the PrepFiler Express was selected for the rest of the study.

Table 1. Extraction Efficiency of the X-Swab Impregnated with 5 µL Whole Blood (N=10) Using Three Different Extraction Methods

Extraction Method:	Average DNA Yield (ng/µL)	Standard Deviation	Elution Volume	Total DNA Yield (in ng)
MinElute	2.843	0.67	30	85.29
Prepfiler Express	2.676	0.63	50	133.8
Organic	0.7438	0.46	50	37.19

Comparison of X-Swab and the 4N6FLOQSwab

The effectiveness of DNA recovery using the X-Swab was compared with that of the 4N6FLOQSwab initially by placing known quantities of purified DNA (1 ng, 2 ng, and 5 ng) in a constant volume of 10 µL on both swabs and allowing them to dry overnight. The results suggested comparable yields for both swabs with 1 and 2 ng of input DNA (p value = 0.54 and p value = 0.78, respectively). However, with 5 ng of input DNA, the X-Swab material had an average DNA yield of (3 ng ± 1) total DNA, which was twice as much DNA as recovered with the 4N6FLOQSwab (1.6 ng ± 0.65). The difference in DNA yield was not significant but was approaching significance (p value = 0.053).

Known quantities of whole blood (1 µL and 5 µL) were placed onto X-Swab material and the 4N6FLOQSwab and allowed to dry overnight. While a comparable mean DNA yield was observed with 5 µL (115 ng ± 19 and 105 ng ± 51) for X-Swab and 4N6FLOQSwab respectively, the average DNA yield for 1 µL with the X-Swab was (57 ng ± 15) compared with (37 ng ± 8) DNA recovery from the 4N6FLOQSwab.

Dilutions of whole blood (1:5, 1:10, 1:50, and 1:100) were prepared in 10 mM PBS and10 µL placed onto the swabs and allowed to dry overnight. The 4N6FLOQSwab yielded slightly better DNA recovery at the 1:5 dilution (p value = 0.13) (**Figure 1a**). However, the X-Swab had a significantly higher DNA recovery at the 1:10 (p value = 0.008) and 1:50 dilutions (p value = 2.1×10^{-7}). The X-Swab had a higher DNA recovery at the 1:100 dilution but the difference in yield was not significant (p value = 0.55). One explanation for the comparable results at the greatest dilution of blood is that the amount of recoverable DNA is approaching the limit of detection for both samples. These overall results suggested that the X-Swab holds promise as an effective tool for low template DNA recovery.

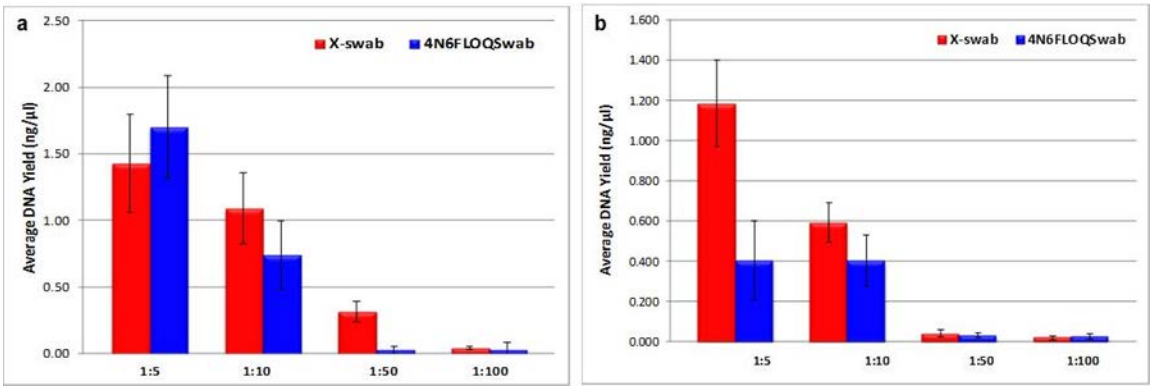


Figure 1. A dilution series of two different sample types, whole blood (1:5, 1:10, 1:50, and 1:100) and saliva (1:5, 1:10, 1:50, and 1:100) in 10 mM PBS was prepared. 10 µL of each sample were placed on both 4N6FLOQSwab and X-Swab (10 replicates). 1a shows average DNA yield for whole blood and 1b shows average DNA yield for saliva.

Based on the observations from the whole blood dilution analyses (i.e., a low quantity sample), a larger sample size (N=100) evaluation was carried out on the X-Swab material at whole blood 1:100 dilution. The average percent recovery was (80% ± 5) for the X-Swab material when DNA yield was compared with the no swab control. These data were consistent with the results previously obtained in the smaller-scale studies and indicated that more DNA may be recovered from low template DNA-type samples.

Next, a series of saliva dilutions (1:5, 1:10, 1:50, and 1:100) were placed onto the swabs and allowed to dry overnight. A significantly higher DNA yield was observed for the X-Swab for the 1:5 (p value = 1.1×10^{-7}) and 1:10 dilutions (p value = 0.002) compared with DNA recovery from the 4N6FLOQSwab (**Figure 1b**). DNA yields from the 1:50 (p value = 0.26) and 1:100 (p value = 0.23) saliva dilutions were comparable. The likely explanation of similar results at the greater dilutions of saliva is the amount of recoverable DNA is approaching or has reached a limit of detection.

Stain Study

DNA recovery from the X-Swab and the 4N6FLOQSwab were compared with laboratory-prepared stains. Using two dilutions of whole blood (1:10 and 1:100), 10 µL were placed on glass slides and allowed to dry (for three days). Because of the different size and surface area between the X-Swab and the 4N6FLOQSwab, different volumes of wetting agent were required. It was determined that 10 µL properly wetted the X-Swab while 30 µL were necessary for the 4N6FLOQSwab. For the 1:10 dilution stains, the X-Swab and the 4N6FLOQSwab had very similar average DNA yields (62 ng ± 17 and 73 ng ± 27, respectively), even though the X-Swab surface area and volume were considerably smaller. For the 1:100 dilution stains, the X-Swab yielded nearly twice as much DNA (6.6 ng ± 1.5) as the 4N6FLOQSwab (3.6 ng ± 1). Partial or full STR profiles were obtained for all samples for both swab types at both dilutions using the PowerPlex ESI 17 Pro System. For both the 1:10 and 1:100 dilutions, the X-Swab yielded higher RFU values at all loci (**Figures 2a** and **3a**, respectively). There were no observable differences in the PHR of the 1:10 or 1:100 dilutions comparisons (**Figures 2b** and **3b**, respectively).

An Examination of the X-Swab™ Polymer

STR typing results suggested that DNA extracted from the X-Swab tended to yield increased peak heights compared with DNA from the 4N6FLOQSwab. When DNA extracted from both swabs was normalized to 1ng and then typed for STRs with Identifiler Plus (28 cycles)**,** the X-Swab consistently yielded higher RFUs at all loci, some substantially higher, when compared with DNA from the 4N6FLOQSwab (**Figure 4a**). No differences in average

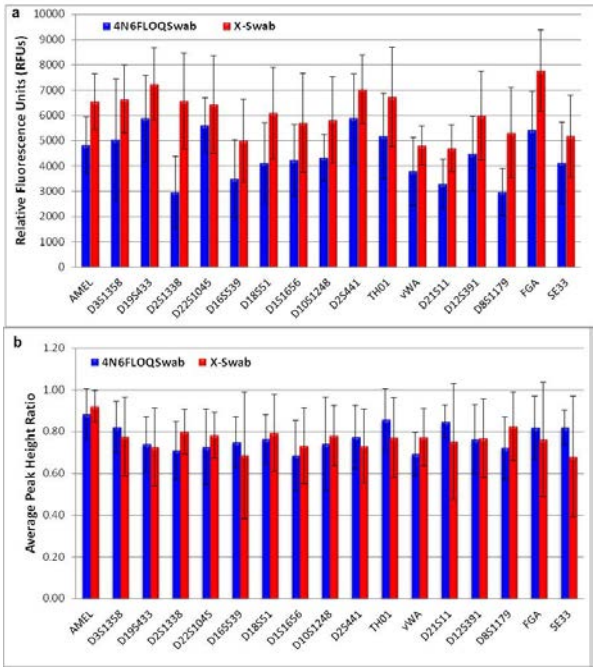


Figure 2. 10 µL of a 1:10 dilution of whole blood were placed on glass slides and allowed to dry. PBS was used to moisten X-Swab (10 µL) and 4N6FLOQSwab (30 µL) and the stains on slides were collected (10 replicate slides for each swab type). 1ng of DNA was amplified with PowerPlex ESI 17 Pro System. 2a shows average RFUs and 2b shows average PHRs.

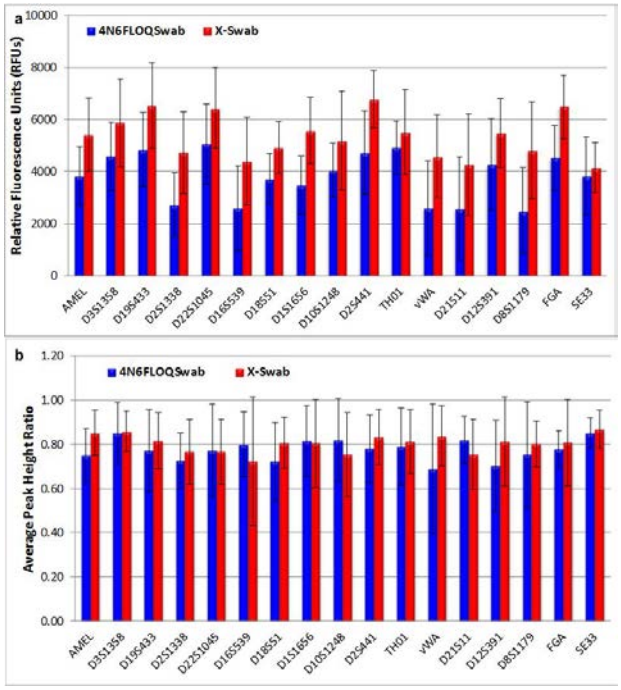


Figure 3. 10 µL of a 1:100 dilution of whole blood were placed on glass slides and allowed to dry. PBS was used to moisten X-Swab (10 µL) and 4N6FLOQSwab (30 µL) and stains on slides were collected (10 replicate slides for each swab type). 17.5 µL of the DNA extract were amplified with PowerPlex ESI 17 Pro System. 3a shows average RFUs and 3b shows average PHRs.

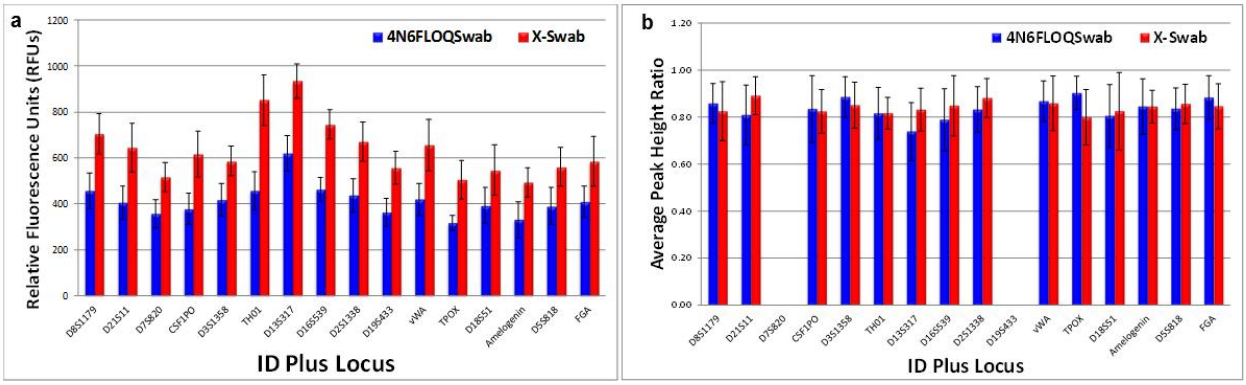


Figure 4. 10 µL of a 1:10 dilution of whole blood were placed on both the 4N6FLOQSwab and X-Swab (10 replicates). Swabs were extracted using PrepFiler Express. 1 ng of DNA was amplified with the AmpFℓSTR Identifiler Plus kit (28 cycles). 4a shows average RFUs and 4b shows average PHRs.

PHR were observed between the DNA from X-Swab material and the 4N6FLOQSwab (**Figure 4b**).

One possible explanation for the increase in peak heights may be that the Diomat polymer, which was co-purified with the DNA, could be associated with the signal increase. To test whether the solubilized polymer from the X-Swab material may be affecting PCR yield, clean X-Swab samples (i.e., no DNA) were subjected to the DNA extraction protocol. DNA (500 pg) from liquid whole blood and either 9 µL of sterile water or X-Swab polymer were placed in Identifiler Plus amplification reactions (29 cycles). The PCRs with X-Swab polymer yielded higher RFU values at all loci compared with those with water (**Figure 5a**) and no differences were observed in the average PHR (**Figure 5b**). The same experiment was carried out except the amount of template DNA was reduced to 100 pg. While the results were not as pronounced, samples with the X-Swab polymer additive yielded higher RFU values at 12 of the 16 loci compared with only water as an additive (**Figure 6a**) and no differences were observed in the average PHR (**Figure 6b**). These results supported the hypothesis that the presence of the polymer in the PCR increased PCR yield.

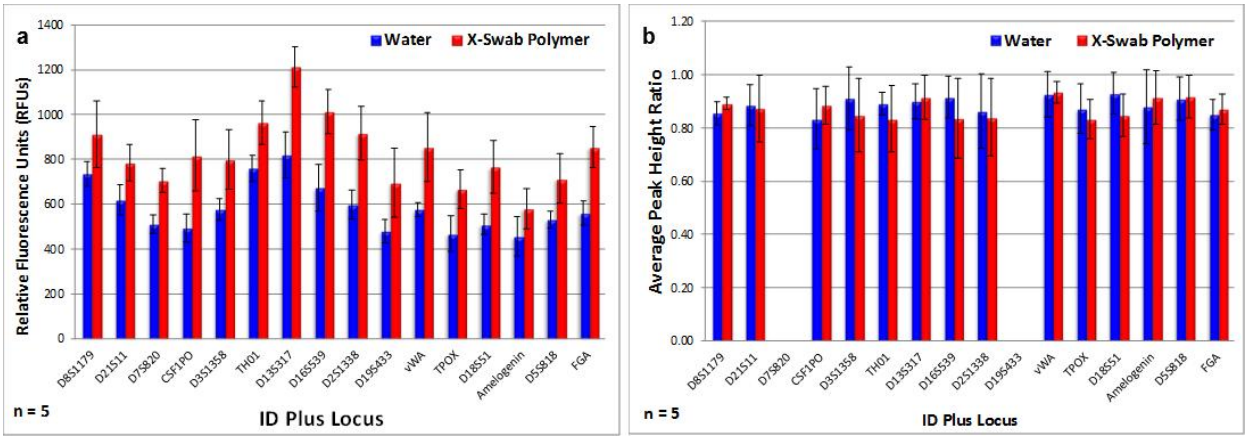


Figure 5. A whole blood sample was extracted using PrepFiler Express and normalized and 500 pg were placed in a PCR. Either 9 uL of water or X-Swab polymer were added. Amplification was performed using with the AmpFℓSTR Identifiler Plus kit (29 cycles). 5a shows average RFUs and 5b shows average PHRs.

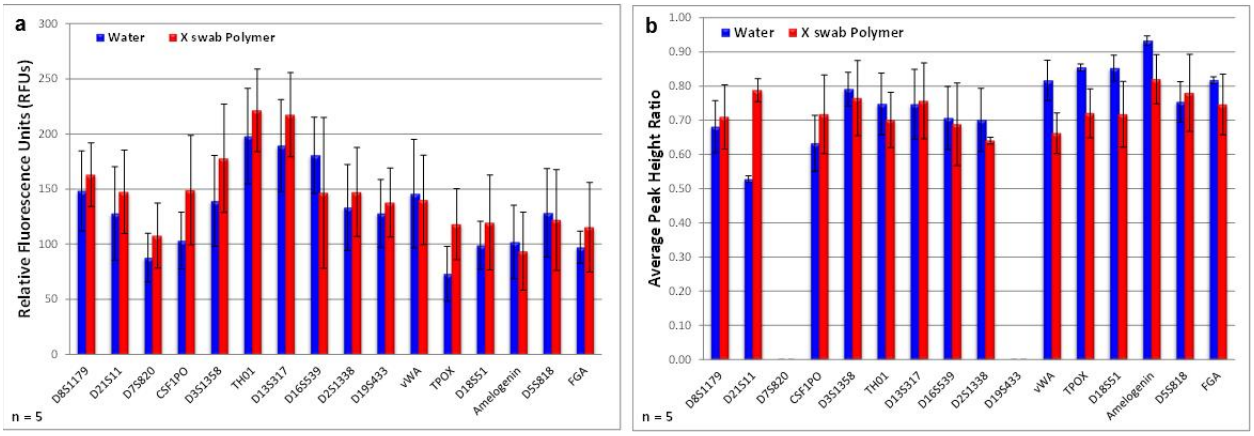


Figure 6. A whole blood sample was extracted using PrepFiler Express and 100 pg were placed in a PCR. Either 9 uL of water or X-Swab polymer were added. Amplification was performed with the AmpFℓSTR Identifiler Plus kit (29 cycles). 6a shows average RFUs and 6b shows average PHRs.

Compatibility of the X-Swab with New Technologies

The X-Swab material was compatible with alternate technologies, such as the RapidHIT™ Human DNA Identification System and Massively Parallel Sequencing (MPS). 10 µL of whole blood from five individuals were applied to X-Swab material and allowed to dry overnight. The samples were run on the RapidHIT system according to the manufacturer's instructions. **Figure**

7 shows a representative electropherogram. All five samples produced the expected DNA profiles, which demonstrated compatibility of a blood impregnated X-Swab with the RapidHIT system.

Because of the potential of the X-Swab polymer to co-purify with extracted DNA in the protocols described herein, the effects that polymer may have with MPS technology were tested. Haplotypes were generated for the two samples that had been sequenced previously (9). The range of coverage (753-17851X) and base calls were concordant. These results suggested that DNA extracted from the X-swab was compatible with MPS technology.

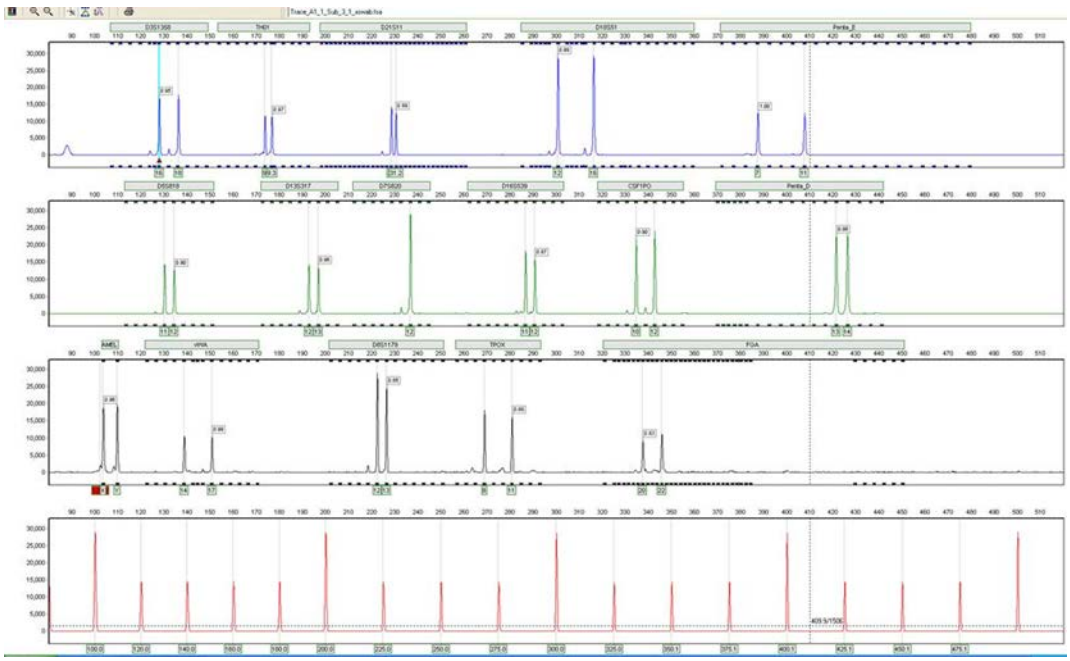


Figure 7. Representative electropherogram of a 10 µL whole blood sample applied to X-Swab and analyzed on RapidHIT Human DNA Identification System. DNA results were analyzed with GeneMarker HID V2.4.0.

CONCLUSIONS

Most collection swabs are inefficient in yielding DNA; that is, portions of DNA remain entrapped in the swab. This residually retained DNA can be relatively substantial. The results in this study demonstrated that the X-Swab yielded more DNA and higher average peak heights compared with DNA extracted from the 4N6FLOQSwab for both blood and saliva samples, particularly for low quantity samples. With a greater recovery of DNA, the X-Swab offers a potential solution in that there may be more "low level" samples that could yield sufficient DNA quantity for conventional STR typing protocols. The X-Swab was compatible with multiple extraction methodologies, although some performed better regarding yield than others. Lastly, the X-Swab and the DNA recovered were well-suited with the newer technologies of RapidHIT and MPS.

CONFLICT OF INTEREST

The authors PLM, MS, BLL, JLK, and BB declare that they have no conflict of interest.

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.

REFERENCES

1. Budowle B, van Daal A. Extracting evidence from forensic DNA analyses: Future molecular biology directions. BioTechniques. 2009 4;46(5):339,40, 342-50.

2. van Oorschot RA, Ballantyne KN, Mitchell RJ. Forensic trace DNA: A review. Investig Genet. 2010 12;1(1):14,2223-1-14.

3. Pang BCM, Cheung BKK. Double swab technique for collecting touched evidence. Leg Med 2007 7;9(4):181-4.

4. 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 Congr Ser. 2006 4;1288:651-3.

5. Hansson O, Finnebraaten M, Heitmann IK, Ramse M, Bouzga M. Trace DNA collection—Performance of minitape and three different swabs. Forensic Science International: Genetics Supplement Series. 2009 12;2(1):189-90.

6. Brownlow RJ, Dagnall KE, Ames CE. A comparison of DNA collection and retrieval from two swab types (cotton and nylon flocked swab) when processed using three QIAGEN extraction methods. J Forensic Sci. 2012 5;57(3):713-7.

7. Dadhania A, Nelson M, Caves G, Santiago R, Podini D. Evaluation of copan 4N6FLOQSwabs™ used for crime scene evidence collection. Forensic Science International: Genetics Supplement Series. 2013;4(1):e336-7.

8. Budowle B. DNA typing protocols: Molecular biology and forensic analysis. Natick, MA: Eaton Pub.; 2000.

9. King J, LaRue B, Novroski N, Parson W, Budowle, B. Application of Massively Parallel Sequencing for Whole Mitochondrial Genome Forensic DNA Analysis. Int J Legal Med, in submission.

SECTION 2

Improving DNA Efficiency and Quality

Science is a way of thinking much more than it is a body of knowledge.

~ Carl Sagan

CHAPTER 2

A High Volume Extraction and Purification Method for Recovering DNA from Human Bone

Submitted to Forensic Science International: Genetics
February 11, 2014

Pamela L. Marshall
Monika Stoljarova
Sarah E. Schmedes
Jonathan L. King
Bruce Budowle

ABSTRACT

DNA recovery, purity and overall extraction efficiency of a protocol employing a novel silica-based column, Hi-Flow® (Generon Ltd., Maidenhead, UK), were compared with that of a standard organic DNA extraction methodology. The quantities of DNA recovered by each method were compared by real-time PCR and quality of DNA by STR typing using the PowerPlex® ESI 17 Pro System (Promega Corporation, Madison, WI) on DNA from 10 human bone samples. Overall, the Hi-Flow method recovered comparable quantities of DNA ranging from 0.8 ng ± 1 to 900 ng ± 159 of DNA compared with the organic method ranging from 0.5 ng ± 0.9 to 855 ng ± 156 of DNA. Complete profiles (17/17 loci tested) were obtained for at least one of three replicates for 3/10 samples using the Hi-Flow method and from 2/10 samples with the organic method. All remaining bone samples yielded partial profiles for all replicates with both methods. Compared with a standard organic DNA isolation method, the results indicated that the Hi-Flow method provided equal or improved recovery and quality of DNA without the harmful effects of organic extraction. Moreover, larger extraction volumes (up to 20 mL) can be employed with the Hi-Flow method which enabled more bone sample to be extracted at one time.

KEYWORDS Hi-Flow® Silica Column · Bone · Organic Extraction · PCR Inhibition · DNA Quantity · STR Typing

INTRODUCTION

Bones are frequently encountered in the identification of individuals in mass disasters and missing person investigations. Bone is a challenging tissue for DNA extraction and purification due to potential environmental and microbial DNA damage, possible DNA degradation, the presence of co-extracting inhibitors, and low levels of DNA. Additionally, areas of extensive mineralization within the bone may present a physical barrier to extraction reagents, thus preventing release of DNA (1-3). An ideal DNA isolation protocol should provide a maximum yield of DNA free from inhibitory compounds that may affect downstream applications and does not employ harmful reagents. It also is important to minimize sample handling steps, as every manipulation provides an opportunity for contamination and increases the risk of loss of DNA through repeated extractions and transfers. The latter concern becomes even more critical in bones where there may be only low quantities of available DNA.

To date, the three most established methods for DNA extraction individually or in combination, particularly from skeletal remains, are organic (i.e., phenol:chloroform), silica-based approaches, and ultrafiltration (1, 4-10). The phenol:chloroform method is effective at removing proteins and lipids from DNA extracts. However the method tends to be ineffective for removal of hydrophilic compounds, which is a problem for skeletal remains in particular, as some samples may have been in prolonged contact with soil or water, and exposed, for example, to the PCR inhibitor humic acid (6, 7). Although recovery of DNA is effective with organic extraction, the solvents are known health hazards and require handling in a safety fume hood. Therefore, much effort has been dedicated to development of non-organic extraction methods. The majority of these methods use the ability to reversibly bind DNA to silica via salt bridging (4, 8, 11-14) and use ultrafiltration membranes to remove contaminants (1, 15-17).

Recent studies suggested that larger volumes of digestion buffer may be better at demineralization of pulverized bone samples (1, 11). In order to handle an increased volume of crude extract, one must employ ultrafiltration devices for, buffer exchange, removal of contaminants, and sample concentration. These devices are used in conjunction with another purification method, such as organic extraction or silica column purification, to sufficiently reduce co-purifying inhibitory compounds. Silica-based columns, slurries, and resins have long been available for DNA isolation, but the current methodology is geared toward extraction of DNA from small volumes. Small extraction volumes are a limitation of applying silica-based extraction methods to bone and dilute or diffuse samples.

A study was undertaken to develop a protocol that would combine large volume extraction with silica-based purification and ultrafiltration. The silica-based purification device, the Hi-Flow® column (Generon Ltd., Berkshire, UK), allows for extraction of samples in a volume up to 20 mL, so samples that normally require extraction in multiple tubes may be combined and extracted in a single tube, greatly reducing time and manipulation necessary to perform purification. The Hi-Flow protocol substantially reduces the number of handling steps and sample transfers compared with organic extraction (limiting risk of cross-contamination), as well as eliminating use of hazardous compounds such as phenol and chloroform.

MATERIALS & METHODS

Bone samples: 10 anonymized human bones were obtained from the University of North Texas Health Science Center (UNTHSC) Center for Human Identification.

Bone cleaning: The outer surfaces of the bone fragments were cleaned by immersing them in 50% commercial bleach (3% NaOCl) in a 50-mL conical tube for15 min. Next, the bones were washed briefly with nuclease-free water (4–5 washes). The bones then were immersed briefly in 95–100% ethanol and air dried overnight in a sterile hood. The bones were pulverized using a 6750 Freezer/Mill (SPEX SamplePrep L.L.C., Metuchen, NJ, USA), using a protocol of a 10-min rechill followed by 5 min of grind time at 15 impacts per second.

Hi-Flow® Silica-Column Extraction: The Hi-Flow columns (purchased from Generon Ltd.), were constructed on the 20 mL capacity Proteus™ (AbD Serotec, Raleigh, NC) protein purification column platform (designed to be seated in a 50 mL conical tube during use) and contain a glass fiber filter. The chemistry for the Hi-Flow protocol is similar to that with the QIAquick® (Qiagen, Valencia, CA) silica gel columns (as modified from Yang *et al*. (8)). Bone demineralization was carried out by mixing approximately 0.5 g bone powder with 3 mL digestion buffer (0.5 M EDTA pH 8.0; Invitrogen Corporation, Carlsbad, CA), 1% sodium N-lauroylsarcosinate (Sigma-Aldrich Corp., St. Louis, MO) and 200 µL of proteinase K (Roche Applied Science, Indianapolis, IN) (20 mg/mL), followed by incubation in a hybridization oven at 56°C under constant agitation overnight. After demineralization, the bone powder was pelleted via centrifugation at 2545 x g for 5 min. The supernatant was transferred to a sterile conical tube and mixed with five volumes of binding PB buffer (Qiagen). This mixture was vortexed thoroughly, transferred to a Hi-Flow DNA Purification Spin Column, and centrifuged at 2545 x g for 10 min. After discarding the eluate, the column was washed with 15 mL PE buffer (Qiagen), centrifuged at 2545 x g for 5 min and washing repeated for a total of three washes. The empty column was centrifuged at 2545 x g for 5 min to remove residual ethanol from the PE

buffer. The column was transferred to a sterile collection tube, and the DNA was eluted with 100 µL elution buffer (EB, Qiagen). Three or four elutions were performed for each sample for a total recovered volume of 300 µL or 400 µL for each bone. Each elution was transferred to a separate, sterile1.5mL microfuge tube. The DNA extracts were stored at 4°C and −20°C for short- and long-term storage, respectively.

Phenol:Chloroform Organic Extraction: Bone samples were extracted according to the method described by Ambers *et al*. (18), using approximately 0.5 g bone powder for each extraction. The final volume for each bone extract was 200 µL. The DNA extracts were stored at 4°C and −20°C for short- and long-term storage, respectively.

DNA Quantification: The quantity of extracted DNA was determined using a reduced volume (10 µL) protocol of the Applied Biosystems® Quantifiler™ Human DNA Quantification Kit (Life Technologies, Carlsbad, CA) on an Applied Biosystems® 7500 Real-Time PCR System (Life Technologies). The internal positive control (IPC) is incorporated into each reaction in the Quantifiler® Human DNA Quantification Kit (Life Technologies) as an indicator of amplification success and for the presence of inhibition (19). IPC results from each assay were monitored for the presence of PCR inhibition. Inhibition was indicated by comparing the cycle threshold (C_T) of the "sample" IPC to the C_T of a known uninhibited reaction, which was the DNA standard provided with the kit to create the standard curve for quantification (20). The average IPC cycle number of the standards was subtracted from the IPC cycle number of each sample to determine the IPC delta value. An IPC delta value of ≥1 cycle was considered indicative of inhibition (21).

Amplification: Bones 4 and 8 yielded sufficient DNA using the Hi-Flow and organic extraction methods to amplify 1 ng of DNA for comparison purposes. The remaining eight bones yielded less DNA, and thus, a standard typing protocol using increased cycle number was followed. The largest volume of extract possible (17.5 µL) was added to each PCR. For the PowerPlex® ESI 17 Pro System (Promega Corporation, Madison, WI), thermal cycling was performed on a GeneAmp® PCR System 9700 as follows: initial denaturation at 96°C for 2 minutes; 30 (three replicates for bones 4 and 8) or 36 cycles (all remaining bone samples and replicates) of 94°C for 30 seconds, 59°C for 2 minutes, and 72°C for 90 seconds; hold at 60°C for 45 minutes; and an indefinite hold at 4°C. Positive (9947A), negative (no template DNA), and reagent blank controls were included on each assay plate.

Capillary Electrophoresis and Analysis: Capillary electrophoresis was performed on an Applied Biosystems® 3130xl Genetic Analyzer (Life Technologies) using POP-4™ polymer (Life Technologies) according to manufacturer's recommendations, and data were analyzed using Applied Biosystems® GeneMapper® ID v3.2 software (Life Technologies).

STR Data Analysis: PCR product yield (based on relative fluorescence units (RFUs) and peak height ratio (PHR) for heterozygous loci were evaluated and compared with controls using a freely available in-house excel program, PHASTR (http://web.unthsc.edu/info/200210/molecular_and_medical_genetics/887/research_and_development_laboratory/3). Intra-locus PHRs were calculated for a given locus by dividing the peak height of an allele with a lower RFU value by the peak height of an allele with a higher RFU value, and then multiplying this value by 100 to express the PHR as a percentage.

RESULTS AND DISCUSSION

DNA recovery, purity and overall extraction efficiency of a protocol employing a novel silica-based column, Hi-Flow, were compared with that of a standard organic DNA extraction. Initial experiments focused on an evaluation of the Hi-Flow device and the elution volume and number of eluates collected regarding DNA yield. For elution buffer volume, a bone sample was extracted (3 replicates) with the Hi-Flow method using either four elutions, each with 100 µL of EB (Qiagen), or four elutions of varying volumes (140, 100, 100, and 60 µL) of EB. When 100 µL elution was used, the average first elution volume was 81 µL ± 7. Therefore, the first elution volume was increased to 140 µL so that final recovery would be approximately 100 µL. The results showed comparable quantities of DNA were recovered with the two elution volume strategies (4.7 ng ± 2.15 and 5.3 ng ± 1.8, respectively). Since it is simpler to perform extraction using the same volume for multiple elutions, four elutions of 100 µL were selected for the next phase of the study.

Four separate elutions were compared with three separate elutions on the same bone sample (3 replicates). When the DNA quantities of the four separate elutions were combined, the DNA yield was 7.5 ng ± 5, while DNA quantities of the three separate elutions combined yielded 8.2 ng ± 4. Due to the comparable DNA yield results, the use of three separate elutions of 100 µL was selected for the rest of the study to reduce sample handling and manipulation steps.

To evaluate the efficacy of the Hi-Flow protocol, DNA recovery was compared with that of organic extraction on ten human bone samples. The Hi-Flow method recovered comparable quantities of DNA ranging from 0.8 ng ± 1 to 900 ng ± 159 DNA compared with the organic method which ranged from 0.5 ng ± 0.9 to 855 ng ± 156 DNA (**Table 1**). Six of the ten samples yielded similar average DNA quantities with both bone extraction methods; bones 1 and 2

yielded higher amounts of DNA with the Hi-Flow method and bones 3 and 10 provided more DNA with the organic method. These results suggested that the two methods performed equivalently. The variation both within and between methods may be due to the fact that some bones (and their respective conditions) were more amenable to DNA extraction with one procedure or the other, or that the differences were merely stochastic.

The larger volume capacity of the Hi-Flow column allowed for more sample to be extracted and concomitantly manipulated with fewer steps. Four of the bones (5, 6, 7, and 9), which performed equally well between the extraction methods regarding DNA yield, were extracted with a modified Hi-Flow protocol that tripled the volume of EDTA buffer but maintained the amount of bone powder at a constant 0.5 g. Only one sample per test could be performed per bone because of limited material. DNA recovery results from the previous extraction were compared with the results from the modified Hi-Flow protocol. DNA yields increased for two of the bones (5 and 6, with 7 and 4.4 ng, respectively) while the other two bones (7 and 9) had comparable DNA yield with the standard Hi-Flow method, with 7.85 and 1.7 ng, respectively. Therefore, the modified Hi-Flow method also yielded more DNA for bones 5 and 6 compared with the organic extraction method.

TABLE 1. Amount of DNA Recovered from 10 Bone Samples (3 Replicates) with Two Extraction Methods.

Bone (in replicates of 3)	Hi-Flow Extraction			Organic Extraction		
	Total DNA Quantity (ng)	Average Total DNA Quantity (ng)	Standard Deviation	Total DNA Quantity (ng)	Average Total DNA Quantity (ng)	Standard Deviation
1	2.20	5.29	3.86	7.70	2.57	4.45
1	9.62			0.00		
1	4.05			0.00		
2	5.91	8.55	4.57	0.00	0.54	0.93
2	5.93			0.00		
2	13.83			1.62		
3	0.48	0.79	0.99	2.58	2.85	1.65
3	0.00			4.62		
3	1.90			1.36		
4	1083.40	900.28	158.59	856.00	854.67	156.00
4	809.53			1010.00		
4	807.91			698.00		
5	4.30	5.32	0.93	3.26	5.16	2.36
5	5.54			7.80		
5	6.12			4.42		
6	1.68	3.47	1.97	4.56	2.64	2.36
6	3.15			3.36		
6	5.57			0.00		
7	3.20	6.99	3.86	9.96	8.09	2.54
7	6.84			5.20		
7	10.92			9.12		
8	190.48	178.23	14.19	151.40	147.40	40.35
8	181.52			185.60		
8	162.69			105.20		
9	1.05	2.05	0.87	3.32	2.12	1.84
9	2.52			3.04		
9	2.58			0.00		
10	3.26	6.71	4.43	8.30	13.04	4.61
10	5.17			17.50		
10	11.71			13.32		

In addition, approximately 1 g (twice as much material) of bones 3 and 10 were subjected to the Hi-Flow protocol to determine if DNA yield could be increased. The test could only be performed once for each sample because of limited material. DNA recovery results from the previous extraction were compared with the results from the modified Hi-Flow protocol. DNA recovery was enhanced with bone 3 yielding 2 ng (highest yield of a replicate 1.9 ng, mean of 0.8 ng using initial Hi-Flow protocol) compared with the original DNA recovery using organic extraction (highest yield of a replicate 4.62 ng, mean of 2.85 ng). Bone 10, however, yielded over 15 ng of DNA with the modified Hi-Flow method (highest yield of a replicate 11.7 ng, mean of 6.7 ng using initial Hi-Flow protocol) compared with the original DNA recovery using organic extraction (highest yield of a replicate 17.5 ng, mean of 13.04 ng). Thus, bones 3 and 10, with the modified Hi-Flow method, had DNA recovery more comparable with that of the standard organic method, although the quantity of initial bone sample differed. The results supported that larger sample volumes and greater amount of bone could increase DNA recovery without additional steps or manipulations.

For determining whether to use DNA collected solely from the first eluate or if the three eluates were to be combined, the percent recovery of DNA in each of the three elutions was compared to total DNA recovered for a bone sample. On average, the first elution yielded approximately 60% ± 22 of the total DNA recovered compared with 24% ± 16 and 12% ± 14 in the second and third elutions, respectively. The maximum amount of DNA which could be amplified from the first elution then was compared with the amount of DNA which could be amplified from a pooling of the elution extracts. For example, for bone sample 1, the first elution for replicate 1 yielded 0.976 ng in a total elution volume of 80 µL or 12.2 pg/µL. For the PowerPlex ESI amplification, up to 17.5 µL of extract may be added, and the maximum amount

of DNA amplified would be 213.5 pg. If the elutions were pooled, the total DNA in the elutions was 2.196 ng in 267 µL or 8.2 pg/µL and the amount of DNA which could be amplified was 143.5 pg.

For all ten bone samples and their replicates, the amount of DNA which could be amplified was greater if taken from the first elution than from pooled elutions. The data suggested that pooling of elutions may not benefit downstream applications since the pooling would reduce the concentration of DNA in the extract. An additional concentration step would be necessary to overcome the reduction in DNA concentration, and that process would require determining the amount of DNA loss versus an increase in DNA concentration. Additional concentration of extract was not tested in this study but could be considered for future developments. Due to these results, the first elution for each bone sample was used for amplification and STR typing in the next phase of the study.

DNA recovery is only one component of an evaluation of protocol performance; another parameter is the ability to remove inhibitors that may impact downstream analyses. The IPC in the qPCR can indicate the presence of an inhibitor that is directly correlated with the degree of purity of an extracted sample. The IPC delta values for all DNAs extracted by Hi-Flow and organic methods were well below 1, which indicated little or no inhibition detected by the qPCR assay. In addition, the average IPC values for the first, second, and third elutions were comparable (26.5, 26.4, and 27.2, respectively), suggesting that there was no detectable difference in purity of the DNA extract from elution to elution. However, Amory *et al*. (4) observed that an inhibitor may be co-purified from some bone samples but not be indicated by a change in the IPC. Therefore, STR typing of normalized quantities of template DNA may be a better indicator of purity of extracted DNA.

TABLE 2. DNA Concentration (ng/µL), Amount of Total DNA Amplified, and the Number of Alleles Detected from DNA Extracted from 10 Human Bone Samples (3 Replicates) with Two Extraction Methods.

Bone (3 Replicates)	Hi-Flow Extraction					Organic Extraction				
	Concentration (ng/µL)	Total DNA Added to Amplification	# of Alleles Detected	Average # of Alleles Detected	Standard Deviation	Concentration (ng/µL)	Total DNA Added to Amplification	# of Alleles Detected	Average # of Alleles Detected	Standard Deviation
1A‡	0.0122	0.2135	28	27	1	0.0385	0.6738	23	11	10
1B‡	0.0875	1.5313	27			0.0200	0.3500	6		
1C‡	0.0283	0.4953	26			0.0090	0.1575	4		
2A‡	0.0647	1.1323	31	27	6	0.0000	0.0000	15	13	3
2B‡	0.0582	1.0185	31			0.0000	0.0000	14		
2C‡	0.1240	2.1700	20			0.0347	0.4000	9		
3A‡	0.0061	0.1071	3	3	3	0.0129	0.2258	4	3	1
3B‡	0.0000	0.0000	0			0.0231	0.4043	3		
3C‡	0.0203	0.3553	5			0.0068	0.1192	3		
4A†	7.8000	1.0000	29	29	0	4.2800	1.0000	29	29	0
4B†	5.2000	1.0000	29			5.0500	1.0000	29		
4C†	5.3700	1.0000	29			3.4900	1.0000	29		
5A‡	0.0320	0.5600	9	12	5	0.0163	0.2853	17	16	2
5B‡	0.0314	0.5495	9			0.0390	0.6825	14		
5C‡	0.0299	0.5233	17			0.0221	0.3868	16		
6A‡	0.0101	0.1768	7	6	6	0.0228	0.3990	11	9	4
6B‡	0.0276	0.4830	12			0.0168	0.2940	11		
6C‡	0.0241	0.4218	0			0.0000	0.0000	4		
7A‡	0.0485	0.8488	0	16	14	0.0498	0.8715	29	27	2
7B‡	0.0463	0.8103	24			0.0260	0.4550	28		
7C‡	0.0589	1.0308	23			0.0456	0.7980	25		
8A†	1.2700	1.0000	28	28	0	0.7570	1.0000	28	26	3
8B†	1.1600	1.0000	28			0.9280	1.0000	22		
8C†	1.0400	1.0000	28			0.5260	1.0000	28		
9A‡	0.0099	0.1740	1	3	4	0.0166	0.2905	0	1	1
9B‡	0.0311	0.5443	0			0.0152	0.2660	2		
9C‡	0.0239	0.4183	7			0.0000	0.0000	1		
10A‡	0.0210	0.3675	12	11	9	0.0415	0.7263	15	16	1
10B‡	0.0576	1.0080	1			0.0875	1.0000	17		
10C‡	0.0857	1.4998	19			0.0666	1.1655	15		

A notable difference in peak heights of STR alleles can indicate inhibition and thus the purity of DNA extract. **Table 2** shows the amount of DNA recovered in the organic extraction and the first elution of Hi-Flow extraction, the amount of DNA which was placed in the amplification (30 cycles for bones 4 and 8 and 36 cycles for all remaining bone samples), and the number of alleles detected. Two of the 10 bone samples yielded assumed complete profiles (17/17 loci tested) in at least one of the three replicates for both extraction methods. One bone sample yielded an assumed complete profile with Hi-Flow extraction but not with organic

extraction. Partial profiles were obtained for all other bone samples and replicates with DNA extracted with the Hi-Flow and organic extraction methods.

Only bone samples 4 and 8 had sufficient DNA to compare at optimum amounts. For bone 8, an assumed full profile was obtained for the three replicates extracted with the Hi-Flow method (28 alleles) and 2 of 3 replicates extracted with the organic method. A full profile was obtained for all three replicates of bone 4 for both extraction methods but the results indicated a slight difference in DNA purity between extraction methods (**Figure 1**). While approximately 1 ng of DNA from both extraction methods was amplified, average peak height RFUs for bone 4 DNA extracted using the Hi-Flow method were 746 RFU ± 94 compared with 645 ± 155 for the organic method. The results support the hypothesis that an inhibitor may be present in the organic extract which may have been removed with the Hi-Flow procedure (4). The second and third elutions were not typed (because of limited DNA); therefore there was no inference on purity for these portions of the extracts.

For the remaining bone samples and replicates, the full amount of DNA extract (17.5 µL) was placed into the amplification, the same protocol which is followed in routine casework, and the results were compared to evaluate performance in a practical application manner. Across the ten bone samples and their replicates, the Hi-Flow method resulted in more alleles called above the threshold of detection, 483, compared with 451 for the organic method. However, because different amounts of DNA were amplified, no inferences can be drawn regarding performance and average peak height RFUs detected. The different results could be due to a number of factors, including quantity of DNA placed in the PCR, the quality of DNA recovered, and/or difference in presence concentration of inhibitors.

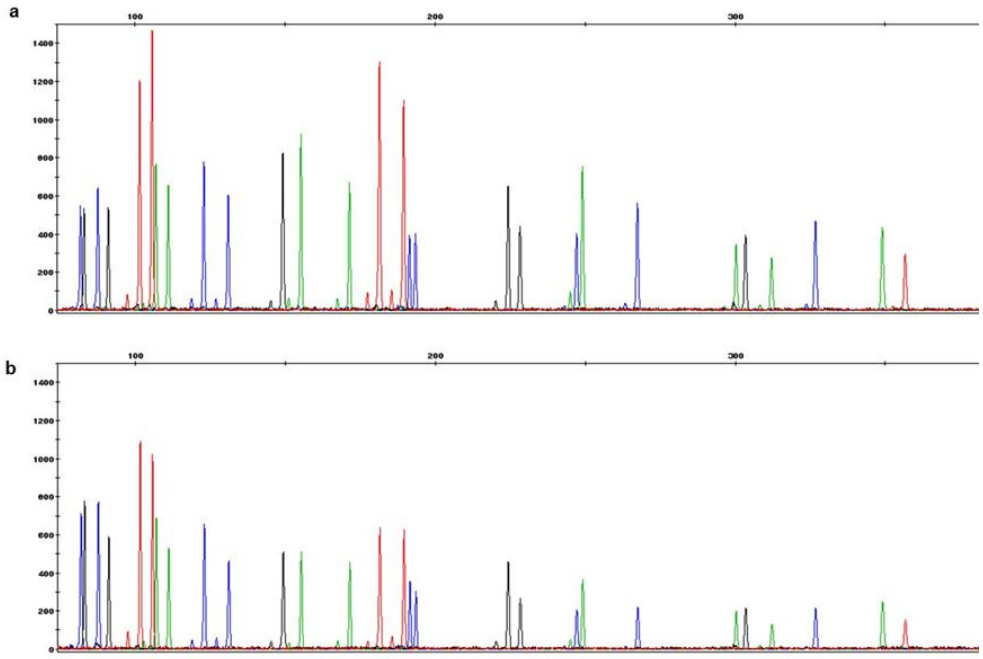


Figure 1. Electropherograms of 1 ng of DNA from bone 4 (amplified with 30 cycles). 1a shows STR profile from DNA extracted using Hi-Flow method and 1b shows STR profile from DNA extracted using organic method. DNA was amplified with the PowerPlex ESI 17 Pro System.

CONCLUSIONS

Overall, the general Hi-Flow method recovered comparable quantities of DNA compared with the organic method for the majority of the bones studied. In contrast with the organic method, however, the Hi-Flow protocol reduced the number of handling steps and sample transfers required (thereby limiting risk of cross-contamination). Furthermore, the Hi-Flow device enabled processing of samples in a larger volume (i.e., increased buffer and bone powder capacity), if needed, which can increase DNA yield over the general method; allowed extraction in a single tube; and reduced time and manipulation necessary to perform purification. Finally, the Hi-Flow method eliminated the use of hazardous compounds such as phenol and chloroform. The more flexible Hi-Flow method is an attractive alternative to the standard organic method for extracting DNA from bone.

ACKNOWLEDGMENTS

This project was supported in part by award nos. 2009-DN-BX-K188 and 2012-IJ-CX-0016, awarded by the National Institute of Justice, Office of Justice Programs, U.S. Department of Justice. The opinions, findings, and conclusions or recommendations expressed in this publication are those of the authors and do not necessarily reflect those of the Department of Justice.

The authors would like to thank Dixie Peters for providing technical support, and Linda LaRose and the UNTHSC Center for Human Identification for providing assistance during the course of this project.

CONFLICT OF INTEREST

The authors PLM, MS, SES, JLK, and BB declare that they have no conflict of interest.

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.

REFERENCES

1. Loreille OM, Diegoli TM, Irwin JA, Coble MD, Parsons TJ. High efficiency DNA extraction from bone by total demineralization. Forensic Sci Int Genet. 2007 6;1(2):191-5.

2. Hoff-Olsen P, Mevag B, Staalstrom E, Hovde B, Egeland T, Olaisen B. Extraction of DNA from decomposed human tissue. an evaluation of five extraction methods for short tandem repeat typing. Forensic Sci Int. 1999 11;105(3):171-83.

3. Pagan F, Lim C, Keglovic M, McNevin D. Comparison of DNA extraction methods for identification of human remains. Australian Journal of Forensic Sciences. 2012 06; 2014 1;44(2):117-27.

4. Amory S, Huel R, Bilić A, Loreille O, Parsons TJ. Automatable full demineralization DNA extraction procedure from degraded skeletal remains. Forensic Science International: Genetics. 2012 5;6(3):398-406.

5. Dukes MJ, Williams AL, Massey CM, Wojtkiewicz PW. Technical note: Bone DNA extraction and purification using silica-coated paramagnetic beads. Am J Phys Anthropol. 2012 7;148(3):473-82.

6. Jakubowska J, Maciejewska A, Pawlowski R. Comparison of three methods of DNA extraction from human bones with different degrees of degradation. Int J Legal Med. 2012 1;126(1):173-8.

7. Davoren J, Vanek D, Konjhodzic R, Crews J, Huffine E, Parsons TJ. Highly effective DNA extraction method for nuclear short tandem repeat testing of skeletal remains from mass graves. Croat Med J. 2007 8;48(4):478-85.

8. Yang DY, Eng B, Waye JS, Dudar JC, Saunders SR. Technical note: Improved DNA extraction from ancient bones using silica-based spin columns. Am J Phys Anthropol. 1998 4;105(4):539-43.

9. Primorac D, Andelinovic S, Definis-Gojanovic M, Drmic I, Rezic B, Baden MM, Kennedy MA, Schanfield MS, Skakel SB, Lee HC. Identification of war victims from mass graves in croatia, bosnia, and herzegovina by use of standard forensic methods and DNA typing. J Forensic Sci. 1996 9;41(5):891-4.

10. Hochmeister MN, Budowle B, Borer UV, Eggmann U, Comey CT, Dirnhofer R. Typing of deoxyribonucleic acid (DNA) extracted from compact bone from human remains. J Forensic Sci. 1991 11;36(6):1649-61.

11. Lee HY, Park MJ, Kim NY, Sim JE, Yang WI, Shin K. Simple and highly effective DNA extraction methods from old skeletal remains using silica columns. Forensic Science International: Genetics. 2010 10;4(5):275-80.

12. Ye J, Ji A, Parra EJ, Zheng X, Jiang C, Zhao X, Hu L, Tu Z. A simple and efficient method for extracting DNA from old and burned bone. J Forensic Sci. 2004 7;49(4):754-9.

13. Kemp BM, Monroe C, Smith DG. Repeat silica extraction: A simple technique for the removal of PCR inhibitors from DNA extracts. Journal of Archaeological Science. 2006 12;33(12):1680-9.

14. Rohland N, Siedel H, Hofreiter, M. A rapid column-based ancient DNA extraction method for increased sample throughput. Molecular Ecology Resources. 2010;10(4):677-83.

15. Kloosterman AD, Kersbergen P. Efficacy and limits of genotyping low copy number (LCN) DNA samples by multiplex PCR of STR loci. J Soc Biol. 2003;197(4):351-9.

16. Alonso A, Martin P. A real-time PCR protocol to determine the number of amelogenin (X-Y) gene copies from forensic DNA samples. Methods Mol Biol. 2005;297:31-44.

17. Deng YJ, Li YZ, Yu XG, Li L, Wu DY, Zhou J, Man TY, Yang G, Yan JW, Cai DQ, Wang J, Yang HM, Li SB, Yu J. Preliminary DNA identification for the tsunami victims in thailand. Genomics Proteomics Bioinformatics. 2005 8;3(3):143-57.

18. Ambers A, Gill-King H, Dirkmaat D, Benjamin R, King J, Budowle B. Autosomal and Y-STR analysis of degraded DNA from the 120-year-old skeletal remains of ezekiel harper. Forensic Science International: Genetics. 2014 3;9(0):33-41.

19. Green RL, Roinestad IC, Boland C, Hennessy, LK. Developmental validation of the Quantifiler™ real-time PCR kits for the quantification of human nuclear DNA samples. J Forensic Sci 2005 7;50(4):809-825.

20. King C, Debruyne R, Kuch M, Schwarz C, Poinar H. A quantitative approach to detect and overcome PCR inhibition in ancient DNA extracts. BioTechniques. 2009 11;47(5):941-9.

21. Davis CP, King JL, Budowle B, Eisenberg AJ, Turnbough MA. Extraction platform evaluations: a comparison of Automate Express™, EZ1® Advanced XL, and Maxwell® 16 Bench-top DNA extraction systems. Legal Medicine 2012 1;14:36-39.

CHAPTER 3

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

International Journal of Legal Medicine, March 2013, 127(2), 321-333

Pamela L. Marshall
Jonathan L. King
Nathan P. Lawrence
Alexander Lazarev
Vera S. Gross
Bruce Budowle

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 co-purify 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 (PCT) 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 qPCR and evaluating STR typing results. The results support that pressure cycling technology reduces inhibitory effects and thus, in effect enhances yield of 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

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 co-purify 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 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 is 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 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) uses hydrostatic pressure alternating between ambient and ultra-high 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 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 cyclo-addition reactions, involving conjugated double bonds and a substituted alkene, that 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* PULSE (Pressure Used to Lyse Samples for Extraction; Pressure BioSciences Inc., South Easton, MA) 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 five minutes with agitation. Tubes and caps then were washed three times with Nanopure water for five minutes with agitation. Tubes and caps were then washed in 70% ethanol solution for five minutes 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) DNA Standard (Raji cell line; 200 ng/µL purified DNA). The standard was diluted to a final concentration of 1ng/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) was dissolved in 0.1 N NaOH (Fisher Chemical, Fairlawn, NJ) 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) was dissolved in 0.1 N NaOH (Fisher Chemical, Fairlawn, NJ) to a final stock concentration of 100 mM. Three concentrations of hematin, 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) was dissolved using TE^{-4} buffer (10 mM Invitrogen UltraPure™ Tris-HCL, pH 8.0; Invitrogen Corporation, Carlsbad, CA, and 0.1 mM GIBCO UltraPure™ EDTA, pH 8.0; GIBCO Products, Grand Island, NY) 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 10mg/mL in molecular grade water. Humic acid was added to samples to final concentrations of 0, 0.01 mg, and 0.02 mg.

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 35kpsi for 20 seconds and ambient pressure for 10 seconds.

Non-pressure treated controls (NPC) controls 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 (10mM Tris-Cl pH 8.0, 0.1 mM Na_2EDTA) per manufacturer's instructions. 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. 0.8 μL sample volume were 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 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 minutes 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), filled with liquid nitrogen, using a protocol of a 10 minute re-chill followed by 5 minutes of grind time at 15 impacts per second. Approximately 0.2 g of bone powder was placed in a PULSE™ Tube. 1 mL of extraction buffer containing 0.5 M EDTA pH 8.0 (Invitrogen Corporation, Carlsbad, CA), 1% sodium lauroyl sarcosinate (sarkosyl, n-lauroyl sarcosine), and 100 μg/mL Proteinase K (Invitrogen Corporation, Carlsbad, CA), 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 two hours or overnight. Following incubation, samples were either subjected to PCT (30 Cycles; 20s at 35k psi and 10s at ambient psi) or no pressure. Each sample was centrifuged at 2545 x *g* for 5 minutes, and the supernatant transferred to a 50 mL conical tube. Five volumes of buffer PB (QIAGEN Inc., Valencia, CA) then were 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 2545 x *g* for 10 minutes. The flow through buffer was discarded and 5 mL of buffer PE (QIAGEN Inc., Valencia, CA) were added to the column. Each sample was then centrifuged at 2545 x *g* for 5 minutes and the flow through buffer discarded. This step was repeated two more times. The "empty" column for each sample was then centrifuged at 2545 x *g* for 5 minutes to remove residual alcohol from the column. Each column was then transferred to a new 50 mL conical tube and 100 μL of buffer EB (QIAGEN Inc., Valencia, CA) were placed directly on the column membrane. Each tube was incubated at room temperature for 5 minutes and then centrifuged at 2545 x *g* for 1 minute. 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

One µL 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 3130xl 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 1500 s run time. Data were collected using the AB 3130xl 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). 1 mL 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 µM and 5 µM hematin and 2.5 ng/µL humic acid, the C_T value of the IPC increased with concentration of inhibitor (**Figures 1a and 1b,** 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 ng/µL and 7 ng/µL of humic acid, the IPC was not amplified for either PCT or NPC samples.

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. 1 mL of 84.5 µM hematin or 1 ml of 500 ng/µL humic acid solutions were 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. 0.8 µL of 1 ng/µL DNA (Raji cell line) were 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.

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
2.5µM Hem - PCT	0.76	0.18	27.90	0.09
2.5µM Hem - NPC	0.56	0.03	27.63	0.26
5µM Hem - PCT	0.23	0.13	34.33	1.42
5µM Hem - NPC	0.14	0.03	Undetermined	0
7µM Hem - PCT	Undetermined	0	Undetermined	0
7µM Hem - NPC	Undetermined	0	Undetermined	0

Sample	Average Quant (ng/µl)	StDev Quant	Average IPC	StDev IPC
No HA - PCT	0.99	0.09	27.43	0.13
No HA - NPC	0.90	0.16	27.41	0.05
2.5ng HA - PCT	0.75	0.03	33.21	0.12
2.5ng HA - NPC	Undetermined	0	Undetermined	0
5ng HA - PCT	Undetermined	0	Undetermined	0
5ng HA - NPC	Undetermined	0	Undetermined	0

Abbreviations Used: Quant = Quantifiler Results; StDev = Standard Deviation; IPC = Internal PCR Control; Hem = Hematin; HA = Humic Acid; PCT = Pressure Cycling Technology; NPC = No Pressure Cycling

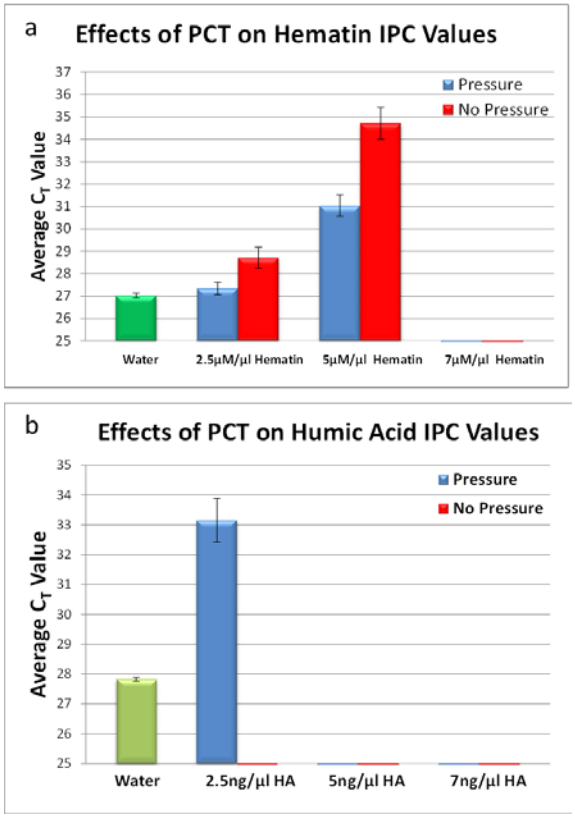


Figure 1. Effect of PCT on IPC Values of Inhibitors Hematin **(a)** Humic Acid (**b**)

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 ng/µL 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. 1 mL of 84.5 µM hematin or 1 mL of 500 ng/µL humic acid were 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 non-pressured samples, the negative effect of hematin was more pronounced in the non-pressured 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).

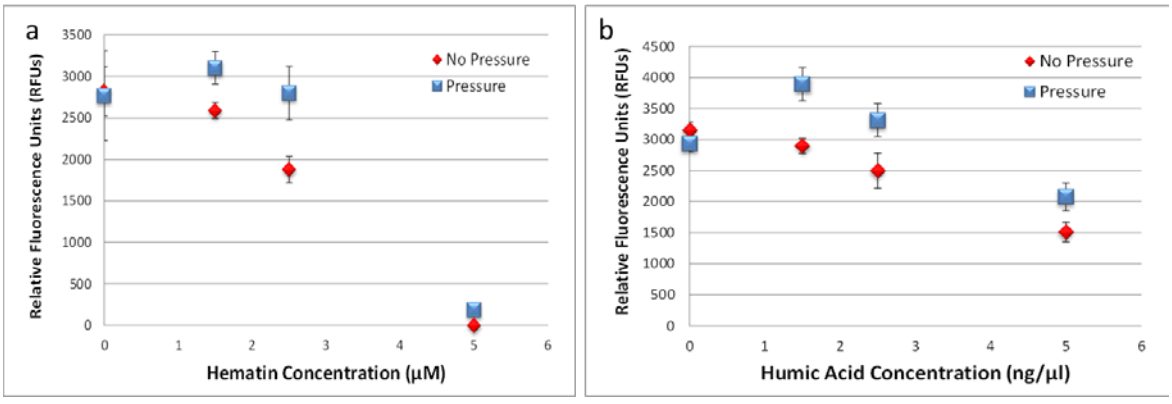


Figure 2. Effect of PCT on Average STR Peak Heights of Inhibited Samples. Hematin (**a**) and Humic Acid (**b**) Pressure-treated samples (squares) were compared with non-pressure treated controls (diamonds).

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), (**Figures 2a and 2b** 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 (Figure 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 **Figures 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 (Figure 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 (Figure 4). At a concentration of 2.5 ng/µL humic acid, dropout of larger loci in the NPC samples occurred. No 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) (**Figures 5a and 5b** 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 (Figure 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 compared with NPC samples.

Electropherograms are representative of STR typing results for no pressure treated and pressure-treated humic acid treated samples, shown in **Figures 6a and 6b** respectively. Significantly increased RFU values for pressure treated samples (Figure 6b) at concentrations of 0.01 mg/µL were observed compared with NPC samples (Figure 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).

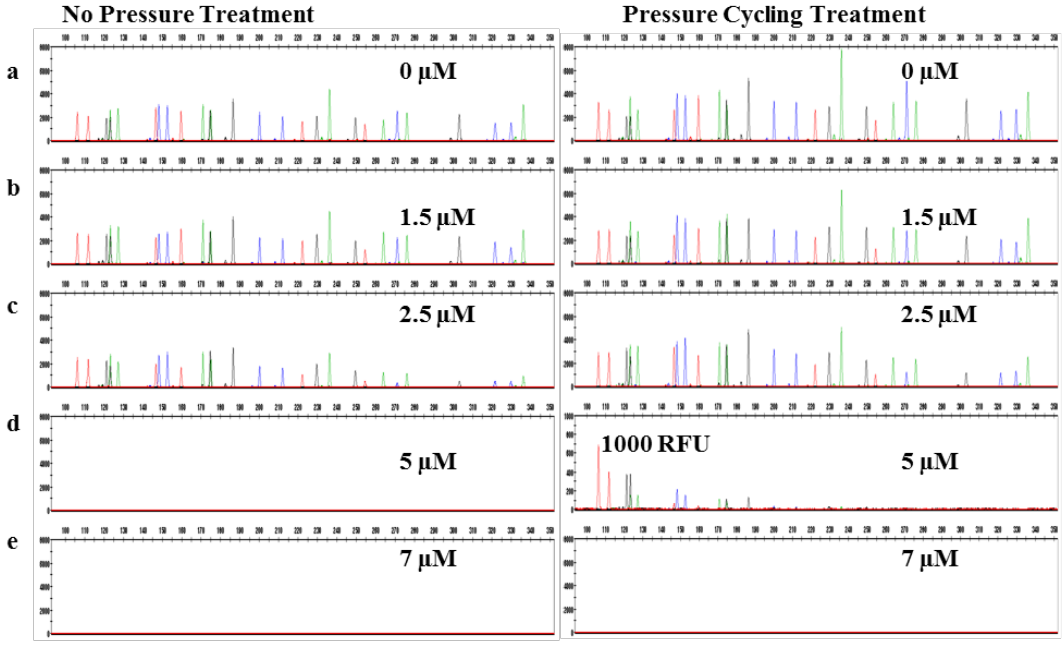


Figure 3. Effect of PCT on Hematin Inhibition**.** Non-pressure treated samples (left panel) and pressure treated samples (right panel). Concentration of hematin is: 0 µM (**a**) 1.5 µM (**b**) 2.5 µM (**c**) 5 µM (**d**) and 7 µM (**e**)

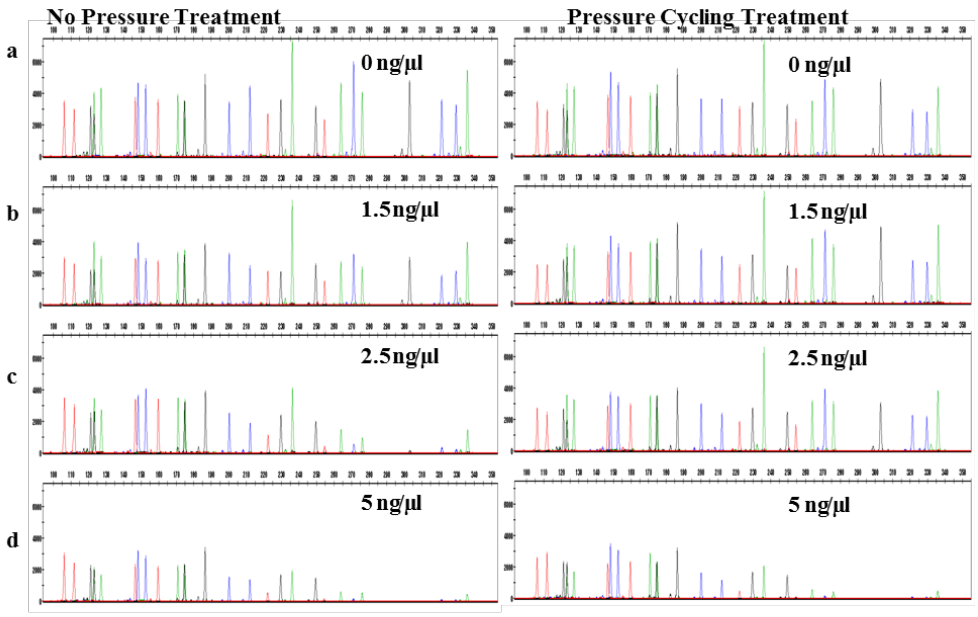


Figure 4. Effect of PCT on Humic Acid Inhibition**.** Non-pressure treated samples (left panel) and pressure treated samples (right panel). Concentration of humic acid is: 0 ng/µL (**a**) 1.5 ng/µL (**b**) 2.5 ng/µL (**c**) and 5 ng/µL(**d**)

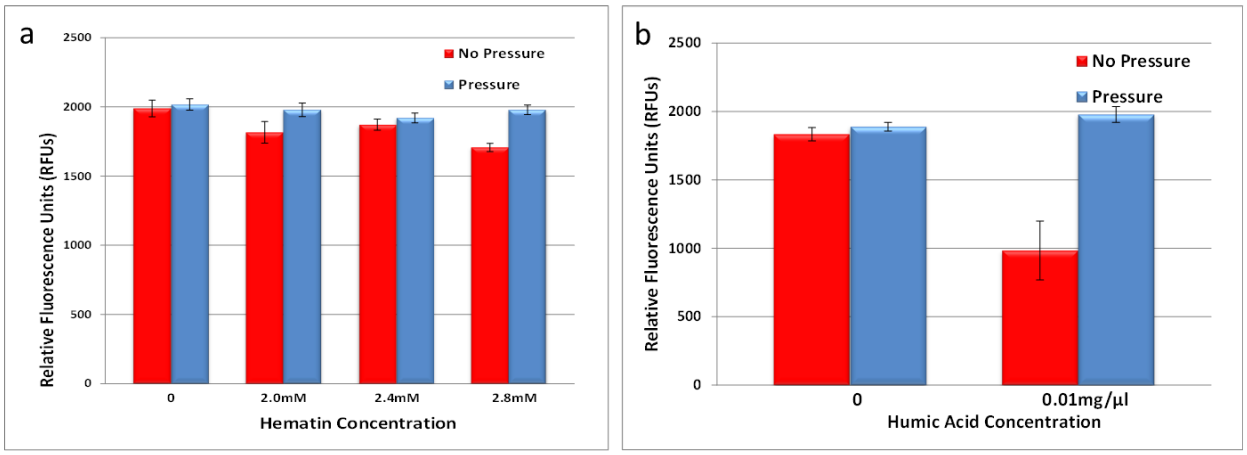


Figure 5. Effect of PCT on Average STR Peak Heights of Inhibited Samples. Hematin (**a**) and Humic Acid (**b**) Pressure-treated samples (blue) were compared with non-pressure treated controls (red).

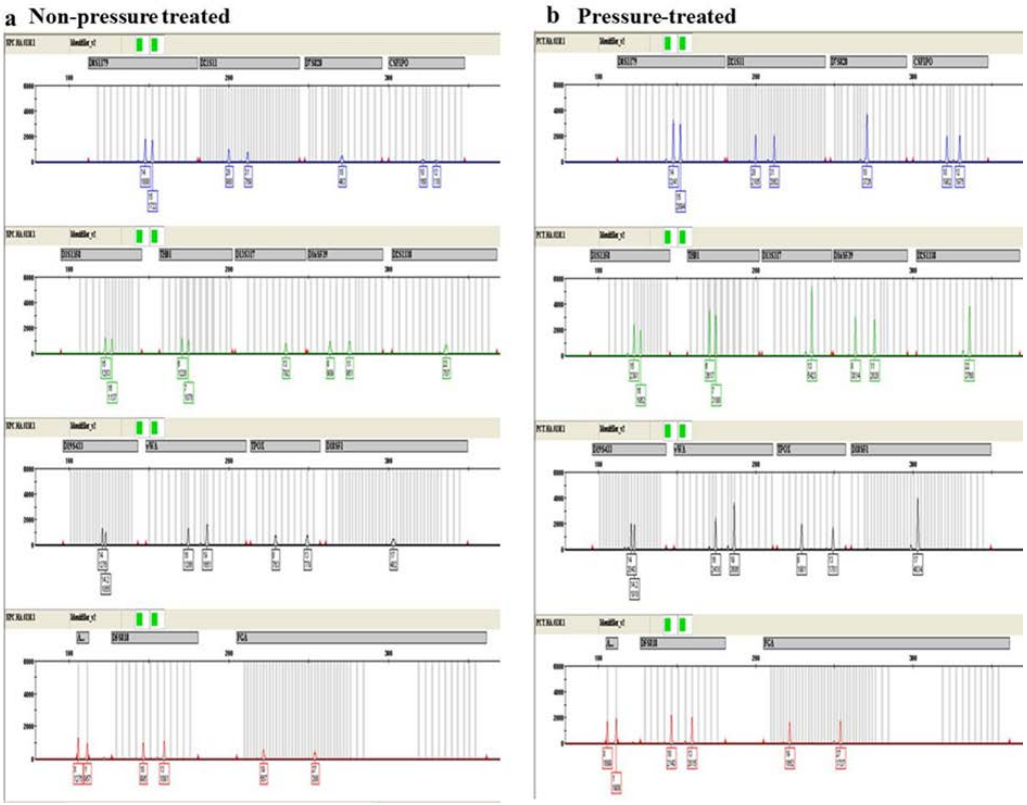


Figure 6. Effect of PCT on Humic Acid Inhibition**.** Non-pressure treated sample (**a**) and pressure-treated sample (**b**). Concentration of humic acid is 0.01 mg/µL.

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.

Elucidating a Possible Mechanism of Increased DNA Tolerance with Pressure Cycling

Considering possible Diels-Alder 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 of 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 (Figure 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 (Figure 8). These observations support the hypothesis, but more rigorous mechanistic studies are needed to elucidate the mechanism.

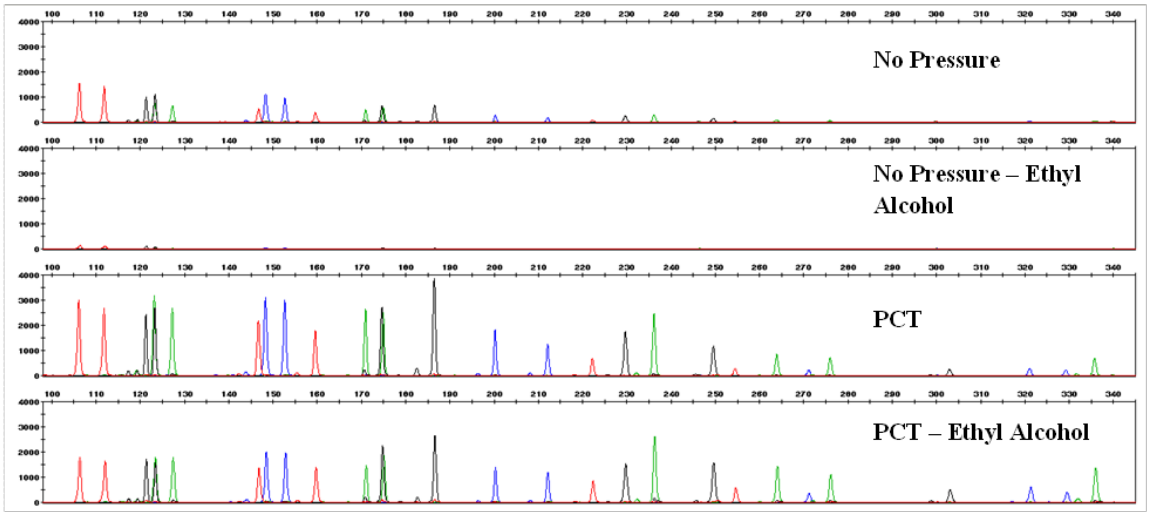


Figure 7. Effect of Ethyl Alcohol Addition on Hematin Inhibition. Non-pressure treated samples containing 1 ng/µL control DNA and 2.5 µM hematin, without (**a**) and with (**b**) addition of 10% Ethyl Alcohol. Pressure treated samples (PCT) containing 1 ng/µL DNA and 2.5 µM hematin, without (**c**) and with (**d**) addition of 10% Ethyl Alcohol

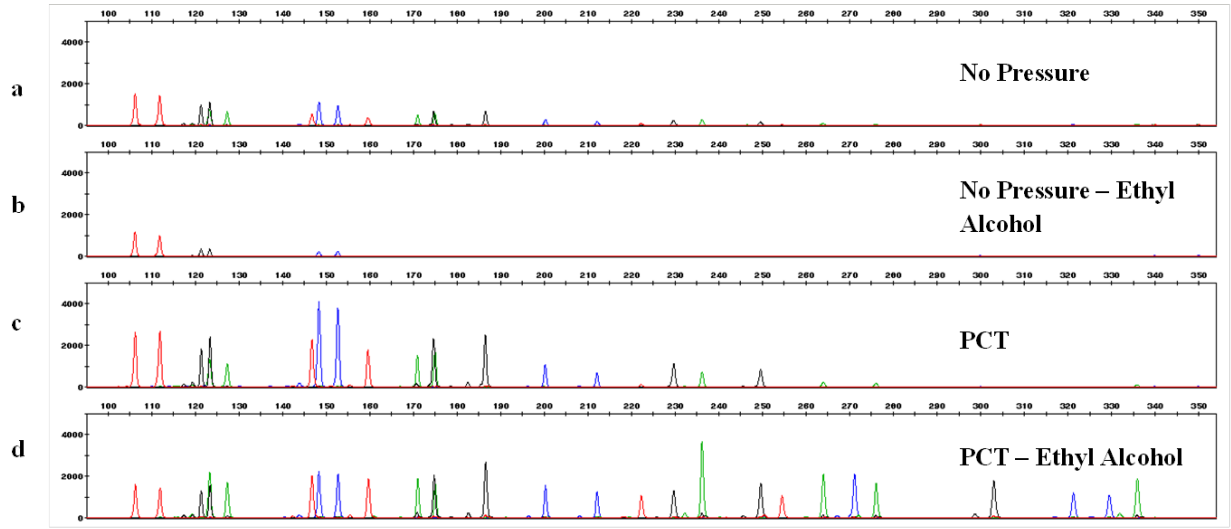


Figure 8. Effect of Ethyl Alcohol Addition on Humic Acid Inhibition. Non-pressure treated samples containing 1 ng/µL DNA and 2.5 ng/µL humic acid, without (**a**) and with (**b**) addition of 10% Ethyl Alcohol. Pressure treated samples containing 1 ng/µL DNA and 2.5 ng/µL humic acid exposed to PCT, without (**c**) and with (**d**) addition of 10% Ethyl Alcohol

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 with and without PCT and the results compared. Approximately 0.2 g of bone powder for each of the three bones were 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 two hours 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 for all three bones (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 less than 0.1 ng/µL, 10 µL were 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 two hour 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 hour 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 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 NPC samples, 0.2 g 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 less than 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 **Figure 9** for 0.5 g NPC (Figure 9a), 0.2 g NPC (Figure 9b), and 0.2 g PCT (Figure 9c). For bone 5, 0.2 g of bone powder

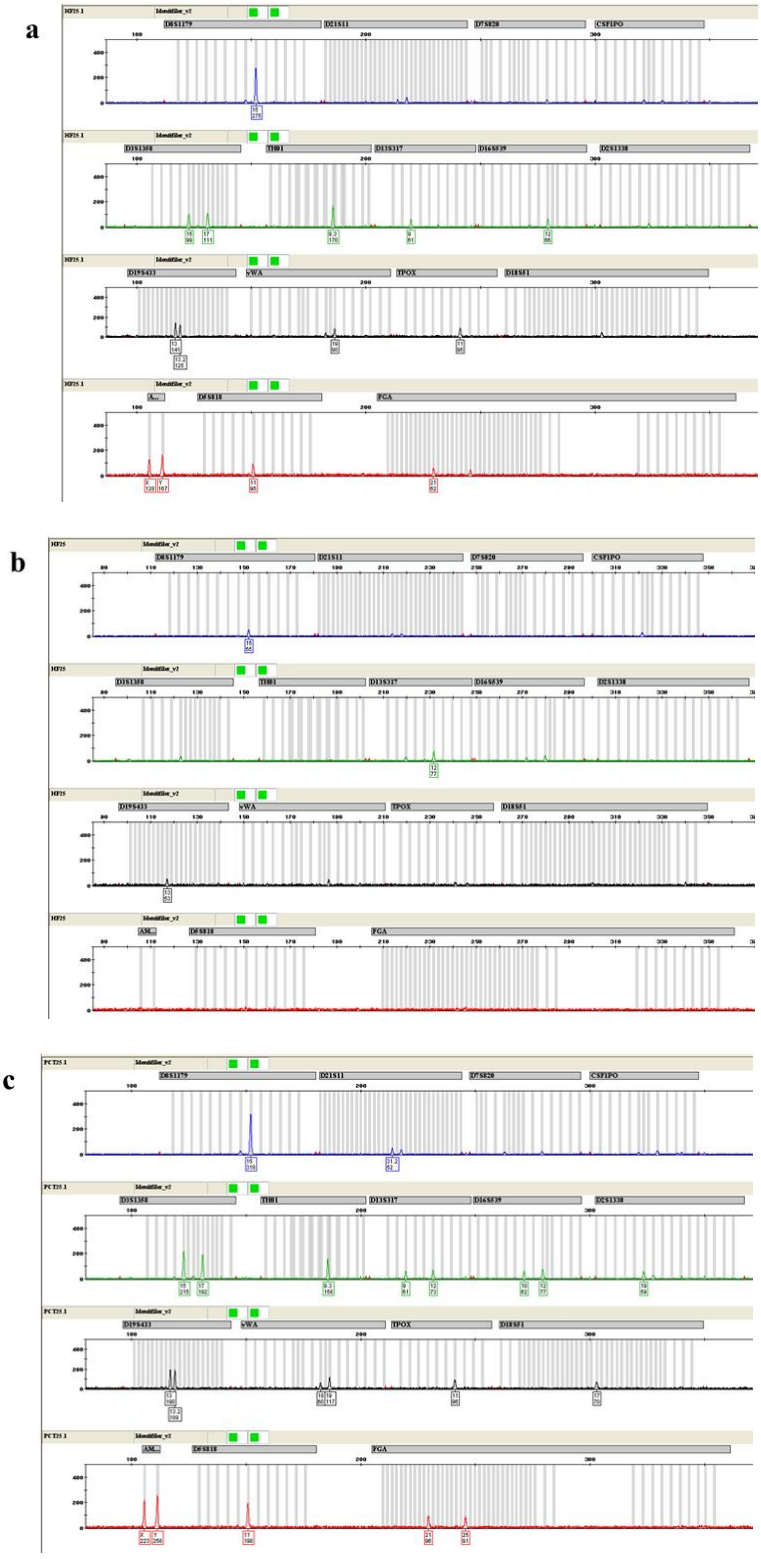


Figure 9. Effect of PCT on Human Bone Processing. Non-pressure treated samples containing approximately (**a**) 0.5 g bone powder and (**b**) 0.2 g bone powder. Pressure-treated sample containing approximately (**c**) 0.2 g bone powder

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).

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.

SPECIAL ACKNOWLEDGMENTS

This project was supported in part by Award No. 2009-DN-BX-K188, awarded by the National Institute of Justice, Office of Justice Programs, U.S. Department of Justice. The opinions, findings, and conclusions or recommendations expressed in this publication are those of the authors and do not necessarily reflect those of the Department of Justice.

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.

REFERENCES

1. Alaeddini R. Forensic implications of PCR inhibition-a review. Forensic Sci Int-Gen 2012 5;6:297-305.

2. Eilert KD, Foran DR. Polymerase resistance to polymerase chain reaction inhibitors in bone. J Forensic Sci. 2009;54(5):1001-7.

3. Ramakers C, Ruijter JM, Deprez RH, Moorman AF. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci Lett. 2003 3;339(1):62-6.

4. Funes-Huacca ME, Opel K, Thompson R, McCord BR. A comparison of the effects of PCR inhibition in quantitative PCR and forensic STR analysis. Electrophoresis. 2011;32(9):1084-9.

5. Kontanis EJ, Reed FA. Evaluation of real-time PCR amplification efficiencies to detect PCR inhibitors. J Forensic Sci. 2006;51(4):795-804.

6. Radstrom P, Lofstrom C, Lovenklev M, Knutsson R, Wolffs P. Strategies for overcoming PCR inhibition. CSH Protoc. 2008 3;2008:pdb.top20.

7. Hudlow WR, Chong MD, Swango KL, Timken MD, Buoncristiani MR. A quadruplex real-time qPCR assay for the simultaneous assessment of total human DNA, human male DNA, DNA degradation and the presence of PCR inhibitors in forensic samples: A diagnostic tool for STR typing. Forensic Science International: Genetics. 2008 3;2(2):108-25.

8. King C, Debruyne R, Kuch M, Schwarz C, Poinar H. A quantitative approach to detect and overcome PCR inhibition in ancient DNA extracts. BioTechniques. 2009 11;47(5):941-9.

9. Huggett JF, Novak T, Garson JA, Green C, Morris-Jones SD, Miller RF, Zumla, A. Differential susceptibility of PCR reactions to inhibitors: An important and unrecognised phenomenon. BMC Res Notes. 2008 8;1:70.

10. Graham EA. DNA reviews: Low level DNA profiling. Forensic Sci Med Pathol. 2008;4(2):129-31.

11. Budowle B, Eisenberg AJ, van Daal A. Validity of low copy number typing and applications to forensic science. Croat Med J. 2009 6;50(3):207-17.

12. Irwin JA, Leney MD, Loreille O, Barritt SM, Christensen AF, Holland TD, Smith BC, Parsons TJ. Application of low copy number STR typing to the identification of aged, degraded skeletal remains. J Forensic Sci. 2007 11;52(6):1322-7.

13. Smith PJ, Ballantyne J. Simplified low-copy-number DNA analysis by post-PCR purification. J Forensic Sci. 2007 7;52(4):820-9.

14. Kloosterman AD, Kersbergen P. Efficacy and limits of genotyping low copy number (LCN) DNA samples by multiplex PCR of STR loci. J Soc Biol. 2003;197(4):351-9.

15. Gross V, Carlson G, Kwan AT, Smejkal G, Freeman E, Ivanov AR, Lazarev A. Tissue fractionation by hydrostatic pressure cycling technology: The unified sample preparation technique for systems biology studies. J Biomol Tech. 2008 7;19(3):189-99.

16. Smejkal GB, Robinson MH, Lawrence NP, Tao F, Saravis CA, Schumacher RT. Increased protein yields from escherichia coli using pressure-cycling technology. J Biomol Tech. 2006 4;17(2):173-5.

17. Smejkal GB, Witzmann FA, Ringham H, Small D, Chase SF, Behnke J, Ting E. Sample preparation for two-dimensional gel electrophoresis using pressure cycling technology. Anal Biochem. 2007 4;363(2):309-11.

18. Occhipinti E, Bec N, Gambirasio B, Baietta G, Martelli PL, Casadio R, Balny C, Lange R, Tortora P. Pressure and temperature as tools for investigating the role of individual non-covalent interactions in enzymatic reactions: Sulfolobus solfataricus carboxypeptidase as a model enzyme. Biochimica et Biophysica Acta (BBA) - Proteins & Proteomics. 2006 3;1764(3):563-72.

19. Delgado A, Kulisiewicz L, Rauh C, Benning R. Basic aspects of phase changes under high pressure. Ann N Y Acad Sci. 2010 2;1189:16-23.

20. Winter R, Dzwolak W. Temperature-pressure configurational landscape of lipid bilayers and proteins. Cell Mol Biol (Noisy-le-grand). 2004 6;50(4):397-417.

21. McCoy J, Hubbell WL. High-pressure EPR reveals conformational equilibria and volumetric properties of spin-labeled proteins. Proc Natl Acad Sci U S A. 2011 1;108(4):1331-6.

22. Considine KM, Kelly AL, Fitzgerald GF, Hill C, Sleator RD. High-pressure processing ? effects on microbial food safety and food quality. FEMS Microbiol Lett. 2008;281(1):1-9.

23. Paschke A. Aspects of food processing and its effect on allergen structure. Molecular Nutrition & Food Research. 2009;53(8):959-62.

24. Lopes MLM, Valente Mesquita VL, Chiaradia ACN, Fernandes AAR, Fernandes PMB. High hydrostatic pressure processing of tropical fruits. Ann N Y Acad Sci. 2010;1189(1):6-15.

25. Van Eldik R, KlÃrner FG. High pressure chemistry: Synthetic, mechanistic, and supercritical applications. John Wiley & Sons; 2008.

26. Masson P, Tonello C, Balny C. High-pressure biotechnology in medicine and pharmaceutical science. J Biomed Biotechnol. 2001;1(2):85-8.

27. Balny C. What lies in the future of high-pressure bioscience? Biochimica et Biophysica Acta (BBA) - Proteins & Proteomics. 2006 3;1764(3):632-9.

28. Balny C, Masson P, Heremans K. High pressure effects on biological macromolecules: From structural changes to alteration of cellular processes. Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology. 2002 3;1595(1–2):3-10.

29. Opel KL, Chung D, McCord BR. A study of PCR inhibition mechanisms using real time PCR. J Forensic Sci. 2010 1;55(1):25-33.

30. Puntharod R, Webster GT, Asghari-Khiavi M, Bambery KR, Safinejad F, Rivadehi S, Langford SJ, Haller KJ, Wood BR. Supramolecular interactions playing an integral role in the near-infrared raman "excitonic" enhancement observed in beta-hematin (malaria pigment) and other related heme derivatives. J Phys Chem B. 2010 9;114(37):12104-15.

31. Zahra N, Hadi S, Smith JA, Iyengar A, Goodwin W. Development of internal amplification controls for DNA profiling with the AmpFlSTR((R)) SGM plus((R)) kit. Electrophoresis. 2011 6;32(11):1371-8.

32. Akane A, Matsubara K, Nakamura H, Takahashi S, Kimura K. Identification of the heme compound copurified with deoxyribonucleic acid (DNA) from bloodstains, a major inhibitor of polymerase chain reaction (PCR) amplification. J Forensic Sci. 1994 3;39(2):362-72.

33. Comey CT. DNA extraction strategies for amplified fragment length polymorphism analysis. J Forensic Sci. 1994;39:1254.

34. de Franchis R, Cross NC, Foulkes NS, Cox TM. A potent inhibitor of taq polymerase copurifies with human genomic DNA. Nucleic Acids Res. 1988 11;16(21):10355.

35. Kreader CA. Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. Appl Environ Microbiol. 1996 3;62(3):1102-6.

36. Watson RJ, Blackwell B. Purification and characterization of a common soil component which inhibits the polymerase chain reaction. Can J Microbiol. 2000 7;46(7):633-42.

37. Amory S, Huel R, Bilić A, Loreille O, Parsons TJ. Automatable full demineralization DNA extraction procedure from degraded skeletal remains. Forensic Science International: Genetics. 2012 5;6(3):398-406.

CHAPTER 4

Effective removal of co-purified inhibitors from extracted DNA samples using synchronous coefficient of drag alteration (SCODA) technology

International Journal of Legal Medicine, July 2013, 127(4), 749-755

Sarah E. Schmedes
Pamela L. Marshall
Jonathan L. King
Bruce Budowle

ABSTRACT

Various types of biological samples present challenges for extraction of DNA suitable for subsequent molecular analyses. Commonly used extraction methods, such as silica-membrane columns and phenol-chloroform, while highly successful may still fail to provide a sufficiently pure DNA extract with some samples. Synchronous coefficient of drag alteration (SCODA), implemented in Boreal Genomics' Aurora Nucleic Acid Extraction System (Boreal Genomics, Vancouver, BC), is a new technology that offers the potential to remove inhibitors effectively while simultaneously concentrating DNA. In this initial study, SCODA was tested for its ability to remove various concentrations of forensically and medically-relevant PCR inhibitors naturally found in tissue, hair, blood, and plant and soil samples. SCODA was used to purify and concentrate DNA from intentionally contaminated DNA samples containing known concentrations of hematin, humic acid, melanin, and tannic acid. The internal positive control (IPC) provided in the Quantifiler™ Human DNA Quantification Kit (Life Technologies, Foster City, CA) and short tandem repeat (STR) profiling (AmpFℓSTR® Identifiler® Plus PCR Amplification Kit; Life Technologies, Foster City, CA) were used to measure inhibition effects and hence purification. SCODA methodology yielded overall higher efficiency of purification of highly contaminated samples compared with the QIAquick® PCR Purification Kit (Qiagen, Valencia, CA). SCODA purified DNA yielded no cycle shift of the IPC for each sample and yielded greater allele percentage recovery and relative fluorescence unit (RFU) values compared with the QIAquick® purification method. The Aurora provided an automated, minimal-step approach to successfully remove inhibitors and concentrate DNA from challenged samples.

KEYWORDS SCODA · Nucleic acid purification · STR DNA typing · QIAquick · Inhibition

INTRODUCTION

Analysis of some forensic samples can be problematic due to the lack of quality and quantity of DNA. Biological forensic samples may contain inhibitors that co-purify with the DNA and can interfere with DNA amplification by the polymerase chain reaction (PCR). PCR inhibition can be caused by various substances such as heme (1-3), found in blood, and melanin (1, 4), found in tissue and hair. Collagen and calcium phosphate are known inhibitors found in bone (1, 5), which can play a role in failed nucleic acid amplification (6). In addition, buried bones and other biological evidence in contact with soil may contain the potent inhibitor humic acids (1, 6-8). Substances found in different types of clothing, such as tannins and indigo dyes, have been known to cause difficulties with forensic short tandem repeat (STR) typing, as well (1).

Various methods have been used to attempt to overcome PCR inhibition, such as diluting the DNA sample, the addition of BSA (bovine serum albumin), and/or the addition of extra Taq polymerase to the PCR (9-10). Dilution can reduce the concentration of the inhibitor in a PCR, but at that the same time decreases the amount of template DNA available for analysis, thus potentially increasing stochastic effects in the PCR and/or reducing the chance of obtaining successful typing results. At times when attempts to remove or ameliorate the effects of inhibitors are not successful the analysis either stops or additional DNA purification steps are sought. Different methods of purification can be used to remove inhibitors and further concentrate DNA from a sample, such as phenol-chloroform and silica-containing columns. The use of QIAquick® columns (Qiagen, Valencia, CA), which utilizes silica-membrane spin columns to bind and elute DNA, has proven to be successful for purifying DNA for the PCR from samples containing inhibitors (11-14). However, some inhibitors are not effectively removed from the more challenging samples using silica-based methods (14-15), and the increased sample manipulation can lead to notable loss of nucleic acids. Synchronous coefficient

of drag alteration (SCODA; Boreal Genomics, Vancouver, BC) is a novel technology used to extract DNA and remove inhibitors from different types of samples while concomitantly concentrating the DNA (16-17). SCODA employs the use of alternating electric fields that concentrates DNA into the center of the electrophoretic field while driving non-nucleic substances out of the field (16-17). DNA has been recovered from various environmental and bacterial samples (17-18), contaminated forensic samples (19), and samples for metagenomic studies (20) using SCODA.

The study herein was carried out to determine the efficacy of purifying DNA by removing inhibitors typically encountered in forensic and medical settings. The results indicated that SCODA can remove inhibitors at levels not possible by a commonly used silica-column based purification technique (i.e., QIAquick®). In addition, the concentrating process enables large volume samples (up to 5 mL) to be extracted that would facilitate recovering DNA from more challenging samples such as bone and diluted stains.

MATERIALS AND METHODS

Sample Preparation

Stock concentrations of inhibitors were prepared as follows: hematin (Sigma-Aldrich, St. Louis, MO), 100 mM in 0.1 M NaOH; humic acid (Sigma-Aldrich), saturated at 10 mg/mL in molecular grade water; melanin (Sigma-Aldrich), 10 mg/mL in 0.5 M NaOH; and tannic acid (Sigma-Aldrich), 10 mg/mL in molecular grade water. All dilutions of inhibitor stocks were made using molecular grade water. DNA used for each sample was the human DNA standard, from the Raji cell line, provided in the Quantifiler® Human DNA Quantification Kit (Life Technologies,

Carlsbad, CA). A total of 10 ng DNA was added to each sample. Spiked samples contained each of the following input amounts of inhibitor: melanin – 30 µg, 60 µg, 120 µg, 200 µg, 220 µg; tannic acid – 600 µg, 800 µg, 1000 µg; humic acid – 150 µg, 300 µg, 600 µg, 1000 µg; and hematin – 440 µg (0.7 µmol), 890 µg (1.4 µmol), 1010 µg (1.6 µmol). From the initial input amount, subsequent inhibitor amounts tested were doubled until either reaching maximum SCODA capability or until an excessive level of inhibitor was reached (excessive level defined as 1mg input). A minimum of three quantities were tested for each inhibitor. For melanin samples, only 220 µg were reached (maximum Aurora capability) due to conductivity issues starting at 240 µg melanin. Since the Aurora and QIAquick® each accommodate different input sample volumes, 5 mL for SCODA and up to 750 µL column capacity for QIAquick®, input quantities instead of concentrations of DNA and inhibitors were provided throughout for effective comparison; equal concentrations would be misleading as the total capacity of each approach would be different.

Initial inhibitor input amounts added to each purification method were determined based on the STR typing dropout threshold of each inhibitor with the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit (Life Technologies). The inhibitory effect on the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit (Life Technologies) of each inhibitor tested was determined by measuring the degree of allele dropout in each STR profile generated by amplifying 1 ng control DNA with varying amounts of each inhibitor. The concentrations for each inhibitor, in a 25 µL amplification reaction, were: melanin,0.04 - 0.28 µg/µL at 0.04 µg/µL increments; tannic acid, 0.4 - 2.8 µg/µL at 0.4 µg/µL increments; humic acid, 0.4 - 0.6 µg/µL at 0.04 µg/µL increments; and final concentrations of hematin were 0.4 mM - 2.8 mM at 0.4 mM increments. Initial inhibitor input amounts for each purification method were ten-fold higher than

the initial concentration of each inhibitor that produced partial (at least 25% of the alleles missing) or complete dropout.

Purification Methods-SCODA and QIAquick®

Spiked samples were purified with SCODA using the Aurora Nucleic Acid Extraction System (Boreal Genomics, Vancouver, BC), per manufacturer instructions. Each sample was loaded into the 5 mL sample chamber (sample volume was brought up to 5 mL with molecular grade water) of a disposable Aurora cartridge containing a 4.5 mm thick, 1% agarose gel in 0.25X TBE buffer. The Aurora DNA Clean-up Protocol was used which included an Injection, Wash, and Focus block. The total run time per sample was 4 hours. Purified SCODA samples were recovered in a volume of ~60 µL, and all samples were brought up to 100 µL with molecular grade water. A comparable sample set was purified using the QIAquick® PCR Purification Kit (Qiagen) according to the manufacturer instructions with the exception of performing two centrifugation steps (instead of one) for the application of sample and PB buffer (the binding buffer included in the kit) to the column due to the higher sample and PB volume of up to 1.02 mL, to accommodate the inhibitor input amounts being tested. Final elution volumes for each sample were 50 µL and all elutions were brought up to 100 µL with the EB buffer (10 mM Tris-Cl, pH 8.5) supplied in the kit. Purified DNA was stored at -20ºC until used. Ideally replicates of each spiked sample would have been run for both SCODA and QIAquick® methods; however, due to limited cartridge availability and the 4 hour run time of each SCODA run it was not feasible to perform replicates. Instead, a range of input inhibitor amounts were run for each method to provide data on multiple samples.

qPCR for Assessing Inhibition

Levels of possible inhibition were measured by amplifying each purified DNA sample using the Quantifiler® Human DNA Quantification Kit (Life Technologies) run on the 7500 Real-Time PCR System (Life Technologies), using a modified protocol with a reduced reaction volume. Each amplification reaction contained 4 µL primer mix, 5 µL reaction mix, and 1 µL template DNA. The performance of the internal positive control (IPC) incorporated into each amplification reaction was measured to determine the impact of inhibition in each sample. The average IPC C_T value of the standards was subtracted from the IPC C_T value of each sample to determine the IPC delay value (ΔC_T). An IPC ΔC_T of ≥1 cycle was considered indicative of inhibition. For samples in which the IPC amplification completely failed due to inhibition, the IPC delay value was calculated by subtracting the average IPC C_T for the DNA standards from 40 (the total number of cycles in each reaction). An IPC ΔC_T of >11 cycles was representative of complete IPC amplification failure, since the C_T threshold was not crossed.

STR Amplification and Profiling for Assessing Inhibition

STR amplification was performed on 10 µL of each 100 µL-purified product using the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit (Life Technologies), per manufacturer's instructions. STR amplicons were separated and detected on the 3500*xl* Genetic Analyzer (Life Technologies). Data were analyzed using GeneMapper® ID-X software (Life Technologies). Percentage allele recovery and average relative fluorescence unit (RFU) values were measured for each purified product.

Real-time analysis of collagen-contaminated DNA during SCODA

To evaluate the capability of SCODA purification of a DNA-bound inhibitor, the runs were visualized real-time to track the movement or lack of movement of DNA in the gel. Real-time analysis of collagen-contaminated DNA was visualized by staining collagen/DNA mixtures with 1X SYBR Gold (Life Technologies) and images were recorded using the Aurora software (Boreal Genomics) during the following intervals: Injection - at 10 second intervals; Wash - at 1 minute intervals; and Focus - at 1 minute intervals. The following samples were visualized to determine the effect collagen has on DNA migrating through the SCODA gel: (1) 400 ng DNA, no collagen (positive control); (2) 400 ng DNA, 15 µg collagen; and (3) 400 ng DNA, 150 µg collagen. Stock concentration of 10 mg/mL collagen was prepared by diluting 10 mg collagen (Sigma-Aldrich) in 1 mL 0.1N acetic acid. Purified DNA was collected from each run and 10 µL of each sample were amplified using the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit to generate STR profiles for each sample to determine the purity and amount of DNA that was able to migrate through the SCODA gel in the presence of collagen.

RESULTS AND DISCUSSION

SCODA and QIAquick® DNA purification

This study assessed the ability of the SCODA technology to purify DNA samples that contained inhibitors commonly seen in forensic and medical biological specimens. The inhibitory effect of melanin, tannic acid, hematin, and humic acid on the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit (Life Technologies) was measured by amplifying 1ng DNA with varying concentrations of each inhibitor (data not shown). The degree of allele dropout in each STR

profile provided a maximum input threshold for each inhibitor with the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit (Life Technologies), thus providing a baseline of inhibitor input for SCODA and QIAquick® purification (**ESM Table 1**). Initial inhibitor input added to DNA to be subjected to each purification method was ten-fold higher than the total amount of each inhibitor that produced partial or complete STR allele dropout with the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit (Life Technologies). A range of input amounts of melanin, tannic acid, humic acid, and hematin was added to 10 ng DNA, and each sample was then subjected to the SCODA and QIAquick® purification methods. Purification levels were measured for each sample by qPCR (i.e., monitoring performance of the IPC) and by STR typing performance (i.e, monitoring allele recovery and average RFU values). Because SCODA and QIAquick® each have different volume capacities, it is likely that these respective volumes will be exploited by an analyst to purify a sample of interest. Thus, regardless of the method, the same amount of materials (equal DNA and inhibitors) could be extracted but within different volumes. Therefore, due to the different volumes, equal concentrations for comparison was considered misleading as the total amounts of DNA and inhibitors would be substantially different. Hence total input amounts were used to provide an equal comparison (ESM Table 1).

Remaining levels of inhibition in each purified product were evaluated by amplifying post-SCODA and post-QIAquick® product using the Quantifiler® Human DNA Quantification Kit. Detection of inhibition was evaluated by measuring the IPC ΔC_T for each purified sample. The IPC is incorporated into each reaction in the Quantifiler® Human DNA Quantification Kit (Life Technologies) as an indicator of amplification success and for the presence of inhibition (21). Inhibition is detected by comparing the C_T of the "sample" IPC to the C_T of a known

ESM Table 1. Input quantities and concentrations for SCODA and QIAquick® purification.

	Identifiler Plus STR Profile Dropout		Purification Input		
Inhibitor	Total Quantity (µg)	Concentration (µg/µL)	Total Quantity (µg)	Concentration SCODA**	Concentration QIAquick***
Melanin	3	0.12	**30**	0.006 µg/µL	0.029 µg/µL
			60	0.012	0.067
			120	0.024	0.133
			200	0.040	0.196
			220	0.044	0.216
Tannic Acid	60	2.4	**600**	0.120	0.588
			800	0.160	0.889
			1000	0.200	1.111
Humic Acid	15*	0.6*	**150**	0.030	0.147
			300	0.060	0.333
			600	0.120	0.667
			1000	0.200	0.980
Hematin	44.3 (0.07µmol)	1.772 (2.8mM)	**440 (0.7µmol)**	0.088	0.431
			890 (1.4µmol)	0.178	0.988
			1010 (1.6µmol)	0.202	0.990

*Only 25% allele dropout was observed

**SCODA total volumes are 5 mL

***Total volumes differed by 0.9 mL or 1.02 mL to accommodate equal volumes for centrifugation balance and minimum volume needed to input quantity per batch of samples processed

uninhibited reaction, such as the DNA standard provided with the kit to create the standard curve for quantification (22). Positive deviations or shifts of IPC ΔC_T (>1 ΔC_T) are indicative of inhibition (23). DNA purified using SCODA did not display any indications of inhibition for each input amount tested. All IPC ΔC_T values were less than one cycle difference from the IPC ΔC_T values of the DNA standards. For DNA samples purified by the QIAquick® method there was complete IPC amplification failure for all concentrations of melanin and humic acid (**Figure 1**). Inhibitor presence after extraction was suspected in post-QIAquick® purified 1mg-humic acid and 220 µg-melanin input spiked samples because these samples still contained a dark color, whereas the same samples extracted using SCODA were clear. The QIAquick®-purified 1.10mg-hematin sample also contained a faint color as well. QIAquick® purification was able to remove all levels of inhibition for all input amounts of tannic acid and hematin. Since the Quantifiler® kit contains a different buffering system than that of the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit (Life Technologies) (24), performance may vary slightly between the two diagnostic systems. Regardless, the trends of impact of inhibitors were similar. These findings support that substantially challenged samples may be cleansed of inhibitors more thoroughly with SCODA than with the QIAquick® method.

The Quantifiler® Human DNA Quantification Kit was used as a tool to detect remaining inhibition presence, however, human-specific DNA quantification yields are reported here as additional information (**Table 1**). Exact DNA percentage quantification yields for each purification method could not be directly compared since the presence of inhibitor could misrepresent actual DNA recovery. However, inferences can be made by quantification values that were recorded for samples in which the IPC did not fail. SCODA purified DNA yields ranged from 44.45% for 890 µg-hematin to >100% yield for 8 out of the 15 total samples run.

Over 100% yield could be indicative of SCODA's ability to clean the DNA samples beyond the level of the standard DNA used for the assay, and also may reflect variation within the assay. The DNA yield for the remaining SCODA samples, that did not include >100% or the 44.45% outlier, ranged from 61.45% to 81.03% with an average of 73.28%. Over 100% yields were observed at least once for each inhibitor tested. QIAquick® purified DNA yields ranged from 57.68% to 78.16%, with an average of 67.39%, excluding a 26% outlier. SCODA purified DNA yields were higher than all QIAquick® purified DNA yields with the exception of a slightly lower yield with 440 µg and 890 µg hematin samples.

STR amplification and profiling using the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit also was used as a means to detect remaining inhibition post-purification. SCODA purified DNA yielded 100% allele recovery for all input amounts of melanin, tannic acid, humic acid, and hematin (**Table 1**). The QIAquick® method, however, was unable to recover STR profiles for the upper limit quantities tested for melanin and humic acid. When DNA was purified using the QIAquick® method, there was complete STR profile loss for 200 µg and 220 µg melanin input, complete STR profile loss for 1mg humic acid, and partial STR profile recovery for 600 µg humic acid samples. DNA from the QIAquick® method yielded 14.29% allele recovery for 600 µg humic acid (**Figure 2**). In addition to allele recovery, SCODA purified DNA yielded relatively balanced allele profiles with higher average RFU values than those using QIAquick®. SCODA had a range of 4183 to 6877 average profile RFU across all samples. For samples that displayed detectable alleles, QIAquick® purified DNA yielded RFU averages ranging from 873 to 4155. Although displaying lower average RFU values, QIAquick® tannic acid and hematin samples yielded 100% allele recovery for each inhibitor input amount.

Table 1 Total DNA yields, STR allele typing success and average RFU values of inhibited samples purified with SCODA and QIAquick®

Inhibitor		Total DNA Yield(%)		Allele Recovery (%)		Profile Average RFU (SD)			
	Input (µg)	SCODA	QIAquick	SCODA	QIAquick	SCODA		QIAquick	
Melanin	30	117.95	N/A	100	100	4183.21	(1430.09)	3800.29	(1459.43)
	60	78.46	N/A	100	100	4234	(1562.86)	2887.54	(1066.15)
	120	73.33	N/A	100	100	5249.57	(1914.74)	2503.75	(781.92)
	200	102.7	N/A	100	0	6848.64	(2445.57)	0	0
	220	140.54	N/A	100	0	5550.68	(1887.50)	0	0
Tannic Acid	600	122.05	57.68	100	100	4764	(1439.89)	1491.29	(647.40)
	800	76.41	59.83	100	100	4753.25	(1680.25)	1353.18	(517.09)
	1000	143.24	26	100	100	5476.5	(1952.39)	873.21	(354.87)
Humic Acid	150	81.03	N/A	100	100	4320.89	(1388.74)	2139.79	(767.57)
	300	69	N/A	100	100	4820.86	(1708.06)	1337.61	(458.55)
	600	116.26	N/A	100	14.29	5423.5	(1983.24)	534.75	(129.13)
	1000	105.69	N/A	100	0	6877.5	(2585.82)	0	0
Hematin	440 (0.7µmol)	61.45	78.16	100	100	4467.11	(1731.35)	2494.89	(893.36)
	890 (1.4µmol)	44.45	74.42	100	100	5851.82	(2142.47)	1749.71	(591.51)
	1010 (1.6µmol)	100.9	66.88	100	100	4883.79	(1694.39)	4155.39	(1430.09)
Positive Control (No inhibitor)		124.32	62.51	100	100	4751.61	(1904.91)	2768.95	(1094.14)

[1]Each sample loaded in SCODA and QIAquick had a total DNA input of 10ng

[2]N/A indicates non-amplifiable DNA due to inhibition

[3]The QIAquick positive control is an average of two positive controls

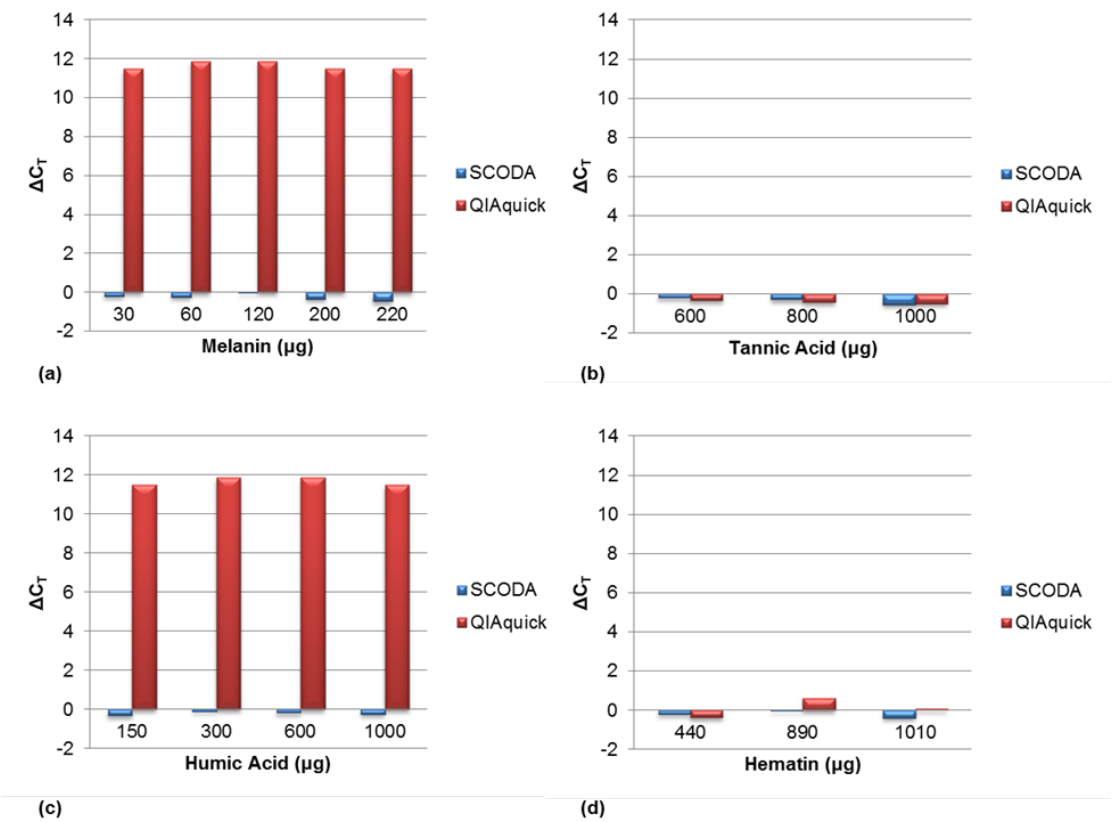
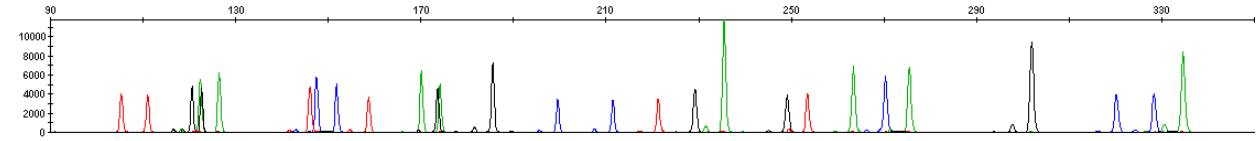
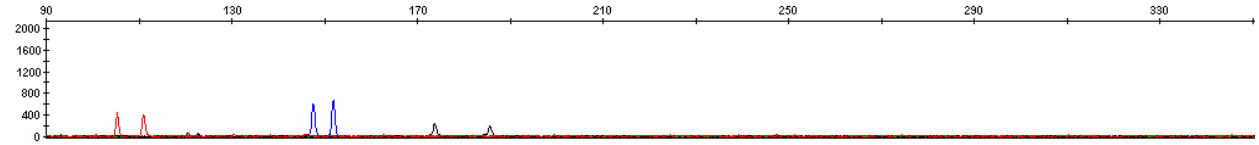


Figure 1. Real-time amplification using the Quantifiler® kit depicting IPC delay values for (a) melanin, (b) tannic acid, (c) humic acid, and (d) hematin-purified samples using SCODA (blue) and QIAquick® (red)

As inhibitor input increased, SCODA samples overall did not show any trend of decrease in RFU values with increasing tested amounts of inhibitors. In contrast, QIAquick® purified DNA displayed decreased RFU values as inhibitor input increased, with the exception of the hematin samples. Although we were not able to run replicates because of the run time and number of gels for testing and instead opted for testing across a range of values, the consistency of results across the various concentrations demonstrated that extreme levels of a number of inhibitors can be removed by SCODA.



(a) Post-SCODA



(b) Post-QIAquick®

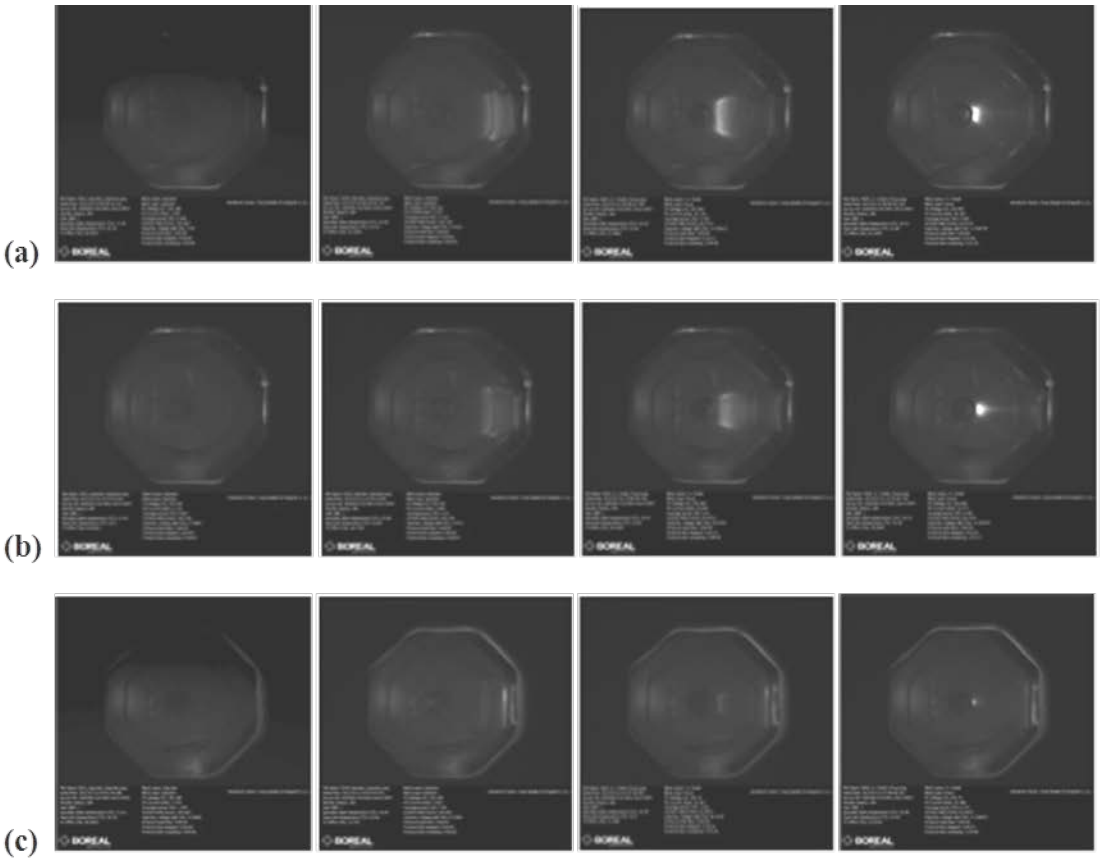
Figure 2. STR profile comparison of (a) post-SCODA and (b) post-QIAquick® DNA sample originally spiked with an input of 600µg humic acid. (Post-QIAquick® Y-axis scale was adjusted to better visualize allele peaks).

Evaluation of SCODA to address a known DNA-bound inhibitor

Inhibitory compounds such as melanin, tannic acid, humic acid, hematin, and collagen, likely have different mechanisms of inhibition (1, 4, 9, 25). Collagen has been shown to inhibit PCR by directly binding to the DNA template (1, 25-27). Purification of samples containing 150 µg collagen failed to yield a STR profile. One hypothesis is that the collagen in the sample has an inhibitory effect on the migration of the DNA in the SCODA gel. Therefore, real-time images were taken during SCODA purification runs to visualize the effects collagen could have on the migration of DNA. Two input amounts of collagen, 15 µg and 150 µg, were run with 400 ng DNA to visualize the general amount of DNA that was allowed to travel through the SCODA gel and eventually concentrate in the extraction well. A positive control containing 400 ng and no collagen was run for comparison. Each sample contained 1X SYBR Gold to visualize the DNA. Images were taken at 10 second intervals during the injection and 1 minute intervals in the wash and focus blocks throughout the course of the 4-hour SCODA run. As seen in **ESM Figure 1**,

noticeably less DNA was injected and migrated through the SCODA gel in which the sample contained 150 µg collagen as compared with only 15 µg collagen or no collagen. In addition, each sample was run without the addition of 1X SYBR Gold to amplify STRs to measure allele recovery and average RFU values. The sample containing 400 ng DNA and 15 µg collagen generated a full STR profile with an average RFU value of 2310 (±785). The sample containing 400 ng and 150 µg failed to generate an STR profile. Real-time images indicated that collagen-contaminated DNA appeared to inject and focus notably lower DNA quantities in the SCODA gel when the concentration of collagen was increased 10-fold. SCODA is capable of purifying collagen-contaminated samples but only at lower concentrations. Perhaps not all the DNA is bound by collagen when the inhibitor is at a lower molar ratio to the DNA and can be separated from the bound DNA with SCODA. Future work will focus on methods to dissociate the collagen-DNA complex prior to SCODA purification.

Silica-membrane columns have proven to be an effective DNA extraction and purification tool (11, 14). SCODA was very effective and exceeded those purification levels with the QIAquick® method. Additionally, the Aurora has the benefit of accommodating high-volume samples (up to 5 mL) and solid-support sample input (e.g. buccal swabs, FTA punches, etc.) in a semi-automated, minimal-step approach. The initial results from this study demonstrated that SCODA technology potentially provides an additional platform for the extraction of nucleic acids. Future work will be on validating the system for routine and challenged forensic samples.



ESM Figure 1. Real-time images of collagen interaction with DNA in SCODA gel (stained with final concentration 1X SYBR Gold); (a) positive control - 400 ng DNA, (b) 400 ng DNA and 15 µg collagen, (c) 400 ng DNA and 150 µg collagen

ACKNOWLEDGMENTS

This project was supported by Award No. 2009-DN-BX-K188, awarded by the National Institute of Justice, Office of Justice Programs, U.S. Department of Justice. The opinions, findings, and conclusions or recommendations expressed in this publication are those of the authors and do not necessarily reflect those of the Department of Justice.

We especially would like to thank Jason Maydan and Boreal Genomics, as well as Bobby LaRue, for their support and contribution to this project.

CONFLICT OF INTEREST

The authors SS, PM, JLK, and BB declare they have no conflict of interest.

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.

REFERENCES

1. Zahra N, Hadi S, Smith JA, Iyengar A, Goodwin W. Development of internal amplification controls for DNA profiling with the AmpFℓSTR® SGM Plus® kit. Electrophoresis 2011 6; 32(11):1371-1378.

2. Akane A, Matsubara K, Nakamura J, Takahasi S, Kimura K. Identification of the heme compound copurified with deoxyribonucleic acid (DNA) from bloodstains, a major inhibitor of polymerase chain reaction (PCR) amplification. J Forensic Sci 1994 3;39:362-372.

3. de Franchis R, Cross NC, Foulkes NS, Cox TM. A potent inhibitor of taq polymerase copurifies with human genomic DNA. Nucleic Acids Res. 1988 11;16(21):10355.

4. Eckhart L, Bach J, Ban J, Tschachler E. Melanin binds reversibly to thermostable DNA polymerase and inhibits its activity. Biochem Biophys Res Commun 2000 5;271:726-730.

5. Kim S, Labbe RG, Ryu S. Inhibitory effects of collagen on the PCR for detection of *Clostridium perfringens*. Appl Environ Microbiol 2000 3;66:1213-1215.

6. Funes-Huacca ME, Opel K, Thompson R, McCord BR. A comparison of the effects of PCR inhibition in quantitative PCR and forensic STR analysis. Electrophoresis. 2011;32(9):1084-9.

7. Watson RJ, Blackwell B. Purification and characterization of a common soil component which inhibits the polymerase chain reaction. Can J Microbiol 2000 7;46(7):633-642.

8. Seo SB, Lee HY, Zhang AH, Kim HY, Shin DH, Lee SD. Effects of humic acid on DNA quantification with Quantifiler® Human DNA Quantification kit and short tandem repeat amplification efficiency. Int J Legal Med 2011 11;126:961-968.

9. Alaeddini R. Forensic implications of PCR inhibition-a review. Forensic Sci Int-Gen 2012 5;6:297-305.

10. Kreader CA. Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. Appl Environ Microbiol. 1996 3;62(3):1102-6.

11. Yang DY, Eng B, Waye JS, Dudar JC, Saunders SR. Improved DNA extraction from ancient bones using silica-based spin columns. Am J Phys Anthropol 1998 4;105(4):539-543.

12. Courts C, Madea B. Full STR profile of a 67-year-old bone found in a fresh water lake. J Forensic Sci 2011 10;56:S172-S175.

13. Loreille OM, Parr RL, McGregor KA, Fitzpatrick CM, Lyon C, Yang DY, Speller CF, Grimm MR, Grimm MJ, Irwin JA, Robinson EM. Integrated DNA and fingerprint analyses in the identification of 60-year-old mummified human remains discovered in an Alaskan glacier. J Forensic Sci 2010 5;55:813-818.

14. Lee HY, Park MJ, Kim NY, Sim JE, Yang WI, Shin K. Simple and highly effective DNA extraction methods from old skeletal remains using silica columns. Forensic Science International: Genetics. 2010 10;4(5):275-80.

15. Kemp BM, Monroe C, Smith DG. Repeat silica extraction: A simple technique for the removal of PCR inhibitors from DNA extracts. Journal of Archaeological Science. 2006 12;33(12):1680-9.

16. Marziali A, Pel J, Bizzotto D, Whitehead LA. Novel electrophoresis mechanism based on synchronous alternating drag perturbation. Electrophoresis 2005 1;26(1):82-90.

17. Pel J, Broemeling D, Mai L, Poon HL, Tropini G, Warren RL, Holt RA, Marziali A. Nonlinear electrophoretic response yields a unique parameter for separation of biomolecules. Proc Natl Acad Sci USA 2009 7;106:14796-14801.

18. So A, Pel J, Rajan S, Marziali A. Efficient genomic DNA extraction from low target concentration bacterial cultures using SCODA DNA extraction technology. Cold Spring Harb Protoc 2010 10.

19. Broemeling DJ, Pel J, Gunn DC, Mai L, Thompson JD, Poon H, Marziali A. An instrument for automated purification of nucleic acids from contaminated forensic samples. JALA 2008 2;13:40-48.

20. Engel K, Pinnell L, Cheng J, Charles TC, Neufeld JD. Nonlinear electrophoresis for purification of soil DNA for metagenomics. J Microbiol Meth 2012 1;88:35-40.

21. Green RL, Roinestad IC, Boland C, Hennessy, LK. Developmental validation of the Quantifiler™ real-time PCR kits for the quantification of human nuclear DNA samples. J Forensic Sci 2005 7;50:809-825.

22. King C, Debruyne R, Kuch M, Schwarz C, Poinar H. A quantitative approach to detect and overcome PCR inhibition in ancient DNA extracts. BioTechniques. 2009 11;47(5):941-9.

23. Davis CP, King JL, Budowle B, Eisenberg AJ, Turnbough MA. Extraction platform evaluations: a comparison of Automate Express™, EZ1® Advanced XL, and Maxwell® 16 Bench-top DNA extraction systems. Legal Medicine 2012 1;14:36-39.

24. Wang DY, Chang CW, Lagacé RE, Calandro LM, Hennessy LK. Developmental validation of the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit: an established multiplex assay with improved performance. J Forensic Sci 2012 11;57:453-465.

25. Opel KL, Chung D, McCord BR. A study of PCR inhibition mechanisms using real time PCR. J Forensic Sci 2010 1;55(1):25-33.

26. Kaya M, Toyama Y, Kubota K, Nodasaka Y, Ochiai M, Nomizu M, Nishi N. Effect of DNA structure on the formation of collagen-DNA complex. Int J Biol Macromol 2005 5;35:39-46.

27. Svintradze DV, Mrevlishvili GM, Metreveli N, Jariashvili K, Namicheishvili L, Skopinska J, Sionkowska A. Collagen-DNA complex. Biomacromolecules 2008 1;9:21-28.

SECTION 3

Improving PCR of LCN Samples

"To raise new questions, new possibilities, to regard old problems from a new angle, requires creative imagination and marks real advance in science." ~ Albert Einstein

CHAPTER 5

Utility of Amplification Enhancers in Low Copy Number DNA Analysis

Submitted to International Journal of Legal Medicine
January 8, 2014

Pamela L. Marshall
Jonathan L. King
Bruce Budowle

ABSTRACT

One parameter that impacts the robustness and reliability of forensic DNA analyses is the amount of template DNA used in the polymerase chain reaction (PCR). With short tandem repeat (STR) typing, low copy number (LCN) DNA samples can present exaggerated stochastic effects during the PCR that result in heterozygote peak height imbalance, allele drop out, and increased stutter. Despite these effects, there has been little progress toward decreasing the formation of stutter products and heterozygote peak imbalance effects during PCR. In an attempt to develop a more robust system that is less refractory to stochastic effects, the PCR additives, betaine, DMSO, PEG and PCRboost®, were investigated on low quantity DNA samples. The effects of the additives were assessed by evaluating STR typing results. Of the four additives, the only positive effects were observed with betaine treatment. Betaine, at a final concentration of 1.25 mol/L, was found to improve the robustness of the amplification, specifically by decreasing stutter in a dual locus system. In contrast, the addition of 1.25 mol/L betaine to commercial STR amplification kits did not affect stutter ratios. However, the addition of betaine did lead to increased yield of PCR products in all commercial kits tested. The results support that betaine can improve amplification efficiency of LCN DNA samples.

KEYWORDS PCR enhancer · Betaine · Stochastic effects · STR typing · LCN DNA

INTRODUCTION

The examination of samples with low quantities of template DNA commonly referred to as "low copy number" (LCN) or low template DNA analysis has a major limitation: stochastic effects during the PCR are exacerbated, causing heterozygote peak height imbalance, allele drop out, and increased stutter (i.e., artifacts due to slippage during the PCR). All these phenomena can complicate interpretation of LCN profiles. A potential approach to improve robustness of amplification of low template DNA is to modify the PCR by use of additives which effectively concentrate the target and enzyme (i.e. volume excluders), alleviate the paused extension of primer, stabilize the enzyme, and/or reduce instability of the template strand. Robustness of amplification can be measured by reduced stutter values, better heterozygote balance, and increased PCR product yield. A variety of PCR additives and enhancing agents have been the focus of efforts to improve amplification and stringency (1-15). The most successful of the additives tested have been dimethyl sulfoxide (DMSO), glycerol, polyethylene glycol (PEG), betaine and formamide. Weissersteiner *et al.* (16) first introduced the glycine betaine as a powerful PCR additive to counteract effects of NaCl and other high salt inhibition of *Taq* polymerase. Since then, betaine has been used as a PCR facilitator, not only as a single compound, but also in combination with other additives (1-9, 11-13, 17-26). Betaine is believed to facilitate PCR via strand separation, lowering melting temperature (T_m), and acting as an isostabilizing agent, equalizing the contribution of GC- and AT-base pairings to the stability of the DNA duplex (13, 21). Furthermore, certain DNA sequences can cause the DNA polymerase to pause, a phenomenon that can be counteracted by betaine. It has been suggested that betaine disrupts the contorted DNA helix without perturbing the polymerase-DNA interaction (22). In fact, betaine has been used to enhance formation of long PCR products, in diagnostic PCR, on

GC rich template, and in low temperature PCRs (22). Another advantage of using betaine is that it acts as an osmoprotectant, increasing the resistance of the polymerase to denaturation (1). Betaine also allows the PCR to overcome some low level of contaminants that can co-purify with DNA, allowing for PCR of low quality DNA samples (22).

Other potential PCR enhancers include PEG and DMSO. PEG is an additive which effectively concentrates the target and enzyme, acting as a volume excluder or molecular crowder (27-31). DMSO is thought to assist in amplification by reducing secondary structure, facilitating strand separation by disrupting base pairing, which is particularly useful for GC rich templates (11, 18). Recently, PCRboost® (Biomatrica, San Diego, CA), a novel additive, became commercially available. Previous studies have shown that PCRboost® has the ability to enhance yield as much as 5-fold, specificity, and consistency of the PCR (32).

There has been little research, however, on whether these additives can overcome some of the negative stochastic effects of LCN typing. Although these additives may have beneficial effects on some amplification systems, it is impossible to predict which agents will be useful in a particular context and therefore, need to be tested. This paper investigated the effects on amplification of low quantity DNA samples and STR products in the presence of betaine, DMSO, PEG and PCRboost®.

MATERIALS AND METHODS

Buccal Swabs: Buccal swabs of 100 individuals were obtained and stored at room temperature until extraction. All samples were collected with informed consent and were anonymized to ensure the privacy of the contributing subjects in accordance with University of North Texas Health Science Center IRB.

DNA Extraction: AutoMate Express™ Forensic DNA Extraction System (Life Technologies, Carlsbad, CA) was performed according to manufacturer instructions. The DNA from the buccal swabs was extracted using the PrepFiler Express™ Forensic DNA Extraction Kit. Cell lysis using the PrepFiler Express™ Kit was performed by adding 500 µL of the PrepFiler lysis solution to the biological sample in a LySep™ column assembly. The PrepFiler lysis solution was prepared by mixing 500 µL of PrepFiler lysis buffer and 5 µL of 1.0 M freshly prepared dithiothreitol (DTT). The lysis mixture was incubated at 70°C for 40 min with shaking at 750 rpm using an Eppendorf Thermomixer. Following lysis, the LySep™ column assembly was centrifuged for 2 min at 10,000 × *g* to transfer the lysate to the sample tube. The lysate in the sample tube was processed on the Automate Express Forensic DNA extraction instrument using the PrepFiler Express™ instrument protocol. The elution volume was 50 µL. The DNA extracts obtained were stored at 4°C and −20°C for short- and long-term storage, respectively.

PCR Additives: Each additive was placed in the PCR at a final concentration as follows:

- Betaine (Sigma, St. Louis, MO): 0, 0.5 mol/L, 1.25 mol/L, and 2 mol/L.
- Dimethyl Sulfoxide (Sigma): 0, 1%, 5%, and 10%.
- Mixtures of betaine and DMSO: 1.25 mol/L betaine and 5% DMSO.
- Polyethylene Glycol (PEG 8000) (Promega, Madison, WI): 0, 1%, 2.5%, and 5%.
- PCRboost® according to manufacturer's instructions (7.5µl added to replace final water volumes in the amplification reaction mix) (33).

Primers: D18S51 and D21S11 primer information was provided kindly by Life Technologies (Life Technologies, Carlsbad, CA) and primers were synthesized by Eurofins MWG Operon (Huntsville, AL). The forward primer for D18S51 was fluorescently labeled with FAM. The forward primer for D21S11 was fluorescently labeled with JOE. The reverse primers were not

labeled. Primer concentrations were optimized to obtain comparable signal of D18S51 and D21S11 products, resulting in a final concentration of 0.25 µM for each primer.

DNA Quantification

The quantity of extracted DNA was determined using a reduced volume protocol of the Applied Biosystems® Quantifiler™ Human DNA Quantification Kit (Life Technologies, Foster City, CA) on an Applied Biosystems® 7500 Real-Time PCR System (Life Technologies). Negative (no template DNA) and reagent blank controls were included on each assay plate. Samples then were normalized to 25 and 100 pg/µL (amounts routinely considered low copy number).

DNA Amplification

Amplifications for commercially available STR kits were performed according to the manufacturer's instructions but with six additional PCR cycles. For the AmpFℓSTR® Identifiler® kit (Life Technologies), thermal cycling was performed on a GeneAmp® PCR System 9700 (Life Technologies) as follows: initial denaturation at 95°C for 11 minutes; 34 cycles of 94°C for 1 minute, 59°C for 1 minute, and 72°C for 1 minute; hold at 60°C for 60 minutes; and an indefinite hold at 4°C. For the AmpFℓSTR® Identifiler® Plus kit (Life Technologies), **t**hermal cycling was performed on a GeneAmp® PCR System 9700 (Life Technologies) as follows: initial denaturation at 95°C for 11 minutes; 34 cycles of 94°C for 20 seconds, 59°C for 3 minutes; hold at 60°C for 10 minutes; and an indefinite hold at 4°C. For the PowerPlex® ESI 17 Pro System (Promega Corportaion, Madison, WI), **t**hermal cycling was performed on a GeneAmp® PCR System 9700 as follows: initial denaturation at 96°C for 2 minutes; 36 cycles of 94°C for 30 seconds, 59°C for 2 minutes, and 72°C for 90 seconds; hold at

60°C for 45 minutes; and an indefinite hold at 4°C. Positive (9947A), negative (no template DNA), and reagent blank controls also were included on each assay plate.

Capillary Electrophoresis

Capillary electrophoresis was performed on an Applied Biosystems® 3130xl Genetic Analyzer (Life Technologies) using POP-4™ polymer (Life Technologies), and data were analyzed using Applied Biosystems® GeneMapper® ID v3.2 software (Life Technologies), according to the manufacturer's recommended protocol. For this study, the analytical threshold was set at 25 RFU to capture as many stutter peaks as possible.

Data Analysis:

PCR product yield (based on RFU), peak height ratio (PHR) for heterozygous loci, and proportion of stutter and variance of these ratios were evaluated and compared with controls using an in-house program designed using Microsoft® Excel. Intra-locus PHRs were calculated for a given locus by dividing the peak height of an allele with a lower RFU value by the peak height of an allele with a higher RFU value, and then multiplying this value by 100 to express the PHR as a percentage. Stutter percentages were calculated by dividing the peak height of the stutter allele (generally n-4 position) by the peak height of the true allele, and then multiplying this value by 100 to express the stutter as a percentage.

RESULTS AND DISCUSSION

DMSO and Betaine

To determine the potential for reduction of stochastic effects in LCN DNA samples, the effects of betaine and DMSO were evaluated separately and in combination. These reagents initially were tested with a limited sample size of five to establish concentration and reagent combination parameters which would then be explored in a larger evaluation study. A two locus multiplex (D18S51 and D21S11) was developed to test the impact of the additives. These loci were selected because they tend to display higher amounts of stutter than other loci (34). Preliminary testing was carried out using 0.5 mol/L, 1.25 mol/L, and 2 mol/L of betaine and 1%, 5%, and 10% of DMSO to determine the best concentrations for further investigation. The concentration of 1.25 mol/L betaine and 5% DMSO were found to have the most positive effects on stutter and thus were pursued with further testing (data not shown). Higher concentrations of betaine (2 mol/L) and DMSO (10%) had no observable differences on STR typing results. In fact, in high amounts, DMSO can reduce *Taq* polymerase activity by up to 50% (21).

DNA template amounts of 25 pg or 100 pg were amplified in reactions with and without additives: Control – no PCR additive, 1.25 mol/L betaine, 5% DMSO, a mixture of 1.25 mol/L betaine and 5% DMSO. The 1.25 mol/L betaine treatment (based on error bar distribution) significantly reduced stutter by approximately 50% or more at both loci in the 25 pg samples and by 25% in 100 pg samples (**Figures 1a** and **1c**, respectively). 5% DMSO treatment reduced stutter by 50% and 16% of the mean stutter value at the loci D18S51 and D21S11, respectively, in the 25 pg samples but showed no effect in reducing stutter at the D18S51 locus in the 100 pg samples (**Figures 1a** and **1c**, respectively). In fact, treatment with DMSO increased stutter at the D21S11 locus in the 100 pg samples (**Figure 1c**). A mixture of 1.25 mol/L betaine and 5%

DMSO did not provide any observable difference than that observed with the betaine treatment alone, suggesting no benefit. While treatment with betaine reduced stutter, no differences were observed on average allele peak height among the treatment groups in either 25 or 100 pg samples at the D18S51 locus but a reduction in signal was observed at the D21S11 locus (**Figures 1b** and **1d**).

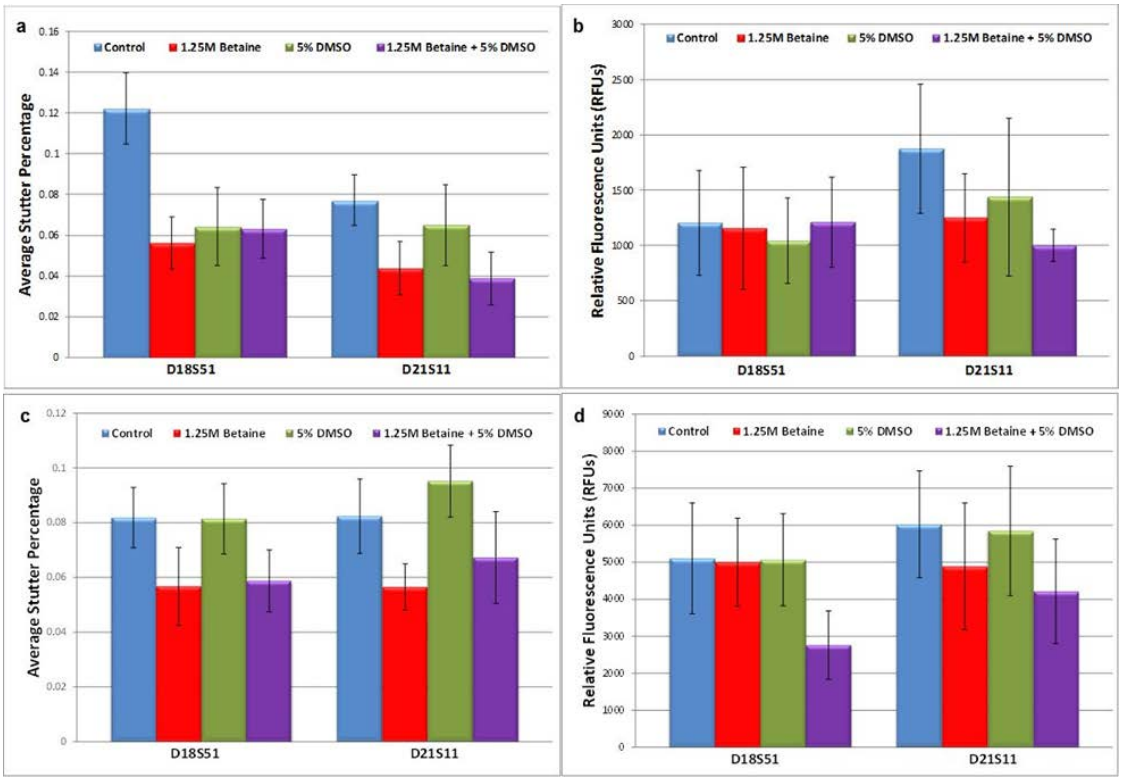


Figure 1. DNA from five different individuals at 25 pg or 100 pg total DNA were added to custom duplex reaction mixes containing: Control – no PCR enhancer, 1.25 mol/L betaine, 5% DMSO, or a mixture of 1.25 mol/L betaine and 5% DMSO. Samples were amplified in triplicate. Average stutter percentages and RFUs of alleles were calculated. 1a) Average stutter percentage – 25 pg; 1b) Average peak height – 25 pg; 1c) Average stutter percentage – 100 pg; and 1d) Average peak height – 100 pg

Based on these results, only betaine was selected for further study. DNA from 86 individuals at two known quantities (25 and 100 pg/µL) were amplified for the same two locus multiplex in the presence of 1.25 mol/L betaine and compared with a no betaine control.

Although not significant, reduction in stutter was observed for betaine treated 25 pg and 100 pg samples at both loci, with the effect of betaine greater at the D18S51 locus (**Table 1**).

TABLE 1 Average Stutter Percentage for Control and Betaine Treated Samples

25pg	D18S51 BETAINE STUTTER	D18S51 BETAINE TRUE ALLELE	D18S51 CONTROL STUTTER	D18S51 CONTROL TRUE ALLELE	D21S11 BETAINE STUTTER	D21S11 BETAINE TRUE ALLELE	D21S11 CONTROL STUTTER	D21S11 CONTROL TRUE ALLELE
AVERAGE RFUs	63	720	90	889	70	1024	89	1054
STDEV	48	534	70	637	64	864	71	833
STUTTER %	**0.06**		**0.1**		**0.06**		**0.08**	
STDEV	0.03		0.05		0.02		0.03	
100pg	**D18S51 BETAINE STUTTER**	**D18S51 BETAINE TRUE ALLELE**	**D18S51 CONTROL STUTTER**	**D18S51 CONTROL TRUE ALLELE**	**D21S11 BETAINE STUTTER**	**D21S11 BETAINE TRUE ALLELE**	**D21S11 CONTROL STUTTER**	**D21S11 CONTROL TRUE ALLELE**
AVERAGE RFUs	116	1735	177	1757	152	2347	214	2421
STDEV	87	1395	127	1249	133	1721	169	1788
STUTTER %	**0.05**		**0.09**		**0.07**		**0.08**	
STDEV	0.02		0.05		0.09		0.03	

The effect of betaine on two commercially-available forensic identification kits, AmpFℓSTR® Identifiler® and AmpFℓSTR® Identifiler® Plus (Life Technologies) was performed on a limited sample size of ten individuals. Amplification reaction mixes were prepared for control (no betaine) and a final concentration of 1.25 mol/L betaine test groups on either 25 pg or 100 pg total template DNA. The effect of betaine treatment on peak height of 25 and 100 pg samples for Identifiler® and Identifiler® Plus amplifications are shown in **Figures 2a-d**. A trend of higher RFU values was observed for most loci with both 25 pg and 100 pg total DNA samples following betaine treatment using the Identifiler® kit (**Figures 2a** and **2b**, respectively) and

Identifiler® Plus amplification kit (**Figures 2c** and **2d**, respectively) compared with controls. The effect of betaine treatment on RFU values appeared to be greater for several loci (D5S818, TH01, D13S317, D21S11, and D3S1358) in both the Identifiler® and Identifiler® Plus amplification kits.

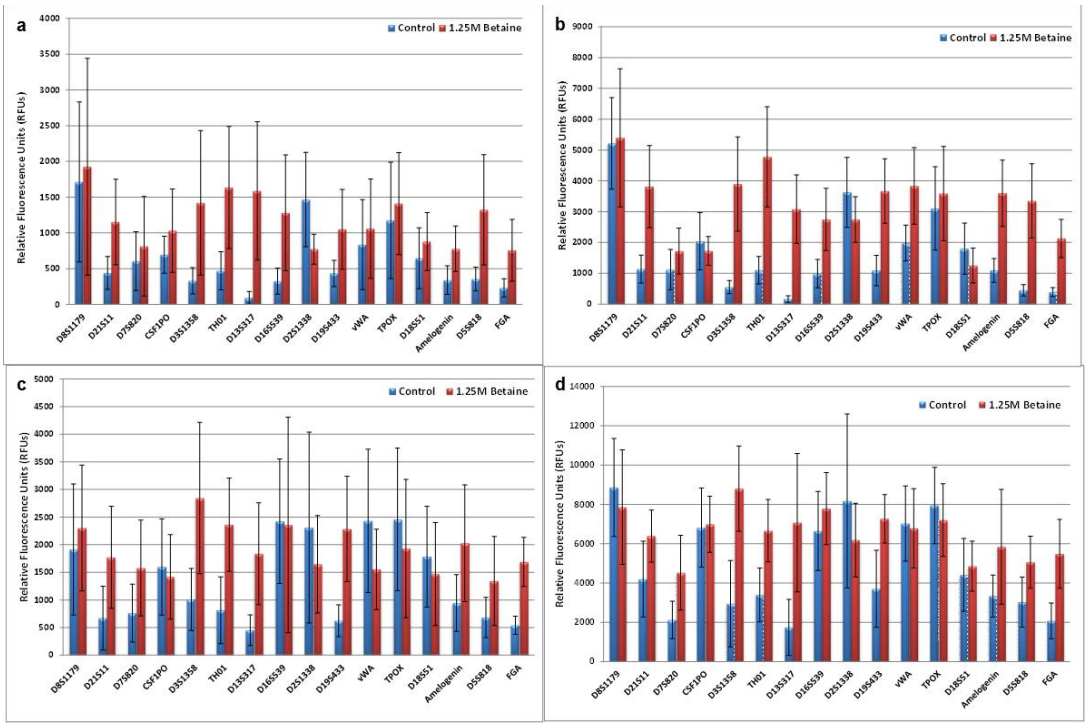


Figure 2. DNA from ten different individuals at 25 pg and 100 pg total DNA were added to Identifiler® or Identifiler® Plus amplification mix containing: Control – no PCR enhancer or 1.25 mol/L betaine. Samples were amplified in triplicate. Average RFUs were calculated. 2a) Identifiler® - 25 pg; 2b) Identifiler® - 100 pg; 2c) Identifiler® Plus – 25 pg; and 2d) Identifiler® Plus – 100 pg

No reduction on stutter was observed following betaine treatment for either the 25 pg or 100 pg total DNA samples using the Identifiler® and Identifiler® Plus amplification kits (data not shown). This observation is not consistent with our previous preliminary data on the duplex. It may be that the Identifiler® and Identifiler® Plus kits contain some additives (proprietary information not available to us) that already maximize the benefits of stutter reduction.

Regarding allele recovery, the total number of observed alleles (combined for ten individuals) was compared with the total number of actual alleles (combined for ten individuals). At 25 pg total input DNA, betaine treatment yielded 83% allele recovery while approximately 82% of alleles were recovered in the control group using the Identifiler® amplification kit (data not shown). With Identifiler® Plus, betaine treatment showed a total allele recovery of 82% compared with 75% in the control group at 25 pg template DNA. At 100pg total input DNA, betaine treatment yielded 100% allele recovery using both the Identifiler® and Identifiler® Plus amplification kits, while the control group had an average percentage of total allele recovery of 99% for both amplifications (data not shown).

These increased RFU values with betaine treatment also were evaluated when only allele peaks greater than an arbitrarily chosen 750 RFUs were counted, as these would be better indicators of increased PCR product yield. For the Identifiler® amplification, the number of allele peaks greater than 750 RFU more than doubled following betaine treatment. While the effect was not as great with the Identifiler® Plus amplification, the same trend was observed. Following betaine treatment of 25 pg samples, the number of allele peaks greater than 750 RFU increased from 97 in the control to 160, and for 100 pg samples, increased from 190 to 278.

The data described above suggested that betaine treatment enhances PCR product yield based on increased number of allele peak heights and increased number of complete profiles observed when compared with no treatment. A larger study then was performed using 25 pg and 100 pg DNA from buccal samples from 81 individuals using a different commercial kit, i.e., PowerPlex® ESI 17 Pro System. Betaine treated samples displayed increased RFU values for the 25 pg and 100 pg samples (**Figures 3a** and **3b**). Betaine treatment of 25 pg samples yielded higher total alleles recovered and hence, more completes profiles, compared with no treatment.

For the 100 pg samples, betaine treatment yielded 73 (of 81) complete profiles, while the control group only yielded 23 complete profiles.

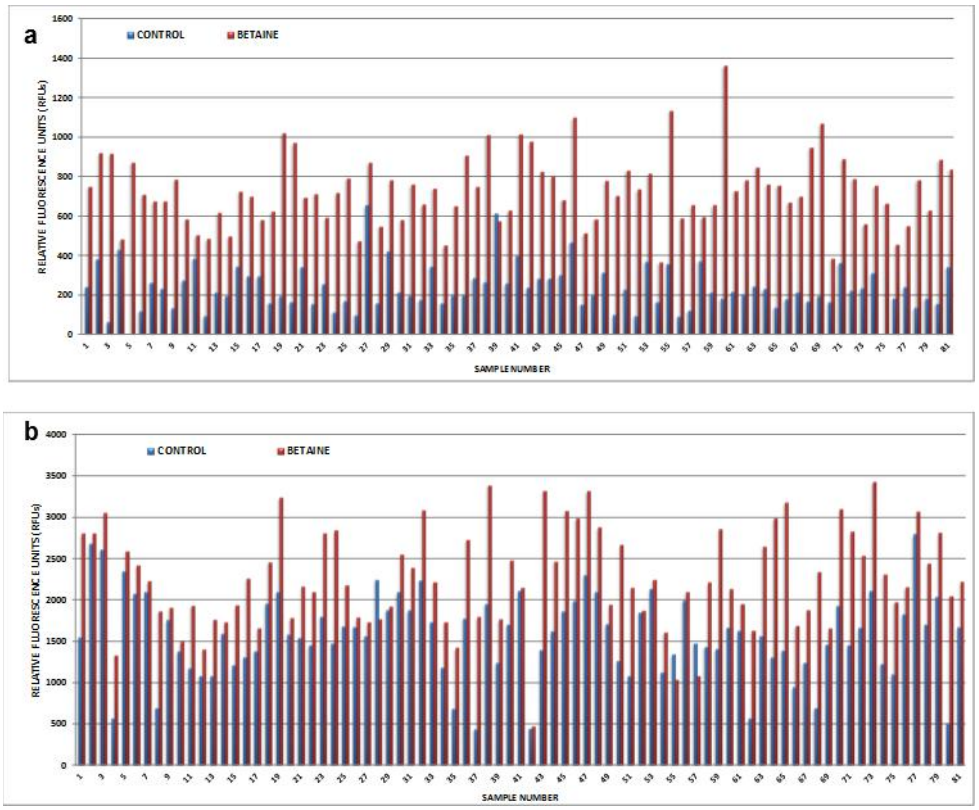


Figure 3. DNA from 81 individuals at 25 pg and 100 pg were added to PowerPlex® ESI 17 Pro System amplification mix containing: Control – no PCR enhancer or 1.25 mol/L betaine, and amplified. Samples were amplified in triplicate. Average RFUs were calculated. 3a) 25 pg and 3b) 100 pg

PEG and PCRboost®

The effectiveness of PEG and PCRboost® on the PCR of LCN DNA samples with the D18S51 and D21S11 duplex also was tested. There were no improvements following treatment with either PEG or PCRboost® on the amplification of LCN DNA samples. For the 25 pg samples, PEG and especially PCRboost® treatment reduced peak height values (**Figure 4a**). For the 100 pg samples, 1% PEG and PCRboost® treatment yielded slightly higher average peak

height values, while no changes were observed at higher concentrations of PEG (**Figure 4c**). No positive improvements were observed in PHR values at either the 25 pg or 100 pg samples (**Figure 4b** and **4d**, respectively). No reductions in stutter were observed with either PEG or PCRboost® treatment compared with controls (data not shown). In fact, in low concentrations, PEG slightly increased the stutter at the D18S51 locus in samples at the lower DNA quantity of 25 pg.

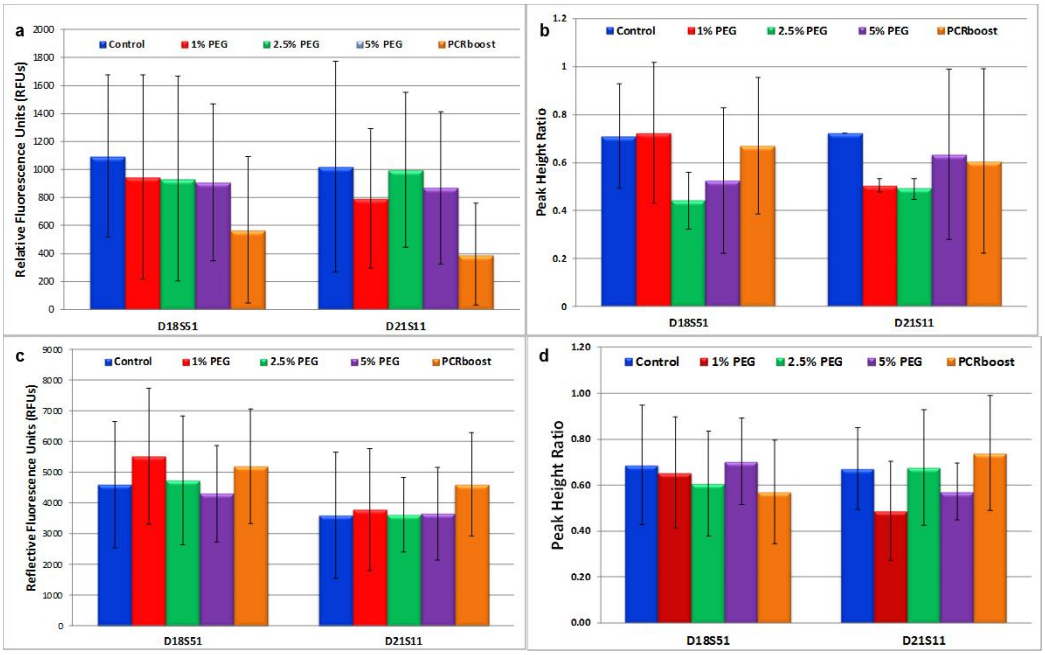


Figure 4. DNA from five different individuals at 25 pg or 100 pg total DNA were added to custom duplex reaction mixes containing: Control – no PCR enhancer, 1% PEG, 2.5% PEG, 5% PEG, and PCRboost®, and amplified. Samples were amplified in triplicate. Average RFUs of alleles and average PHRs were calculated. 4a) Average RFUs – 25 pg; 4b) Average PHRs – 25 pg; 4c) Average RFUs – 100 pg; and 4d) Average PHRs – 100 pg

The effect of PEG and PCRboost® on a commercially-available forensic identification kit, AmpFℓSTR® Identifiler® Plus **(**Life Technologies), was performed on a limited sample size of ten individuals. The effect of PEG and PCRboost® treatment on average allele peak height of 25 pg and 100 pg samples is shown in **Figures 5** and **6**, respectively. No improvements in peak

height were observed with either PEG or PCRboost® treatment. In fact, PCRboost® treatment decreased peak heights on average compared with the control, notably at some loci (**Figure 6**). Additionally, stutter was not reduced and in some instances, increased, for 25 pg and 100 pg samples (data not shown). The results indicated that in our hands neither PEG nor PCRboost® improved the amplification of LCN DNA samples.

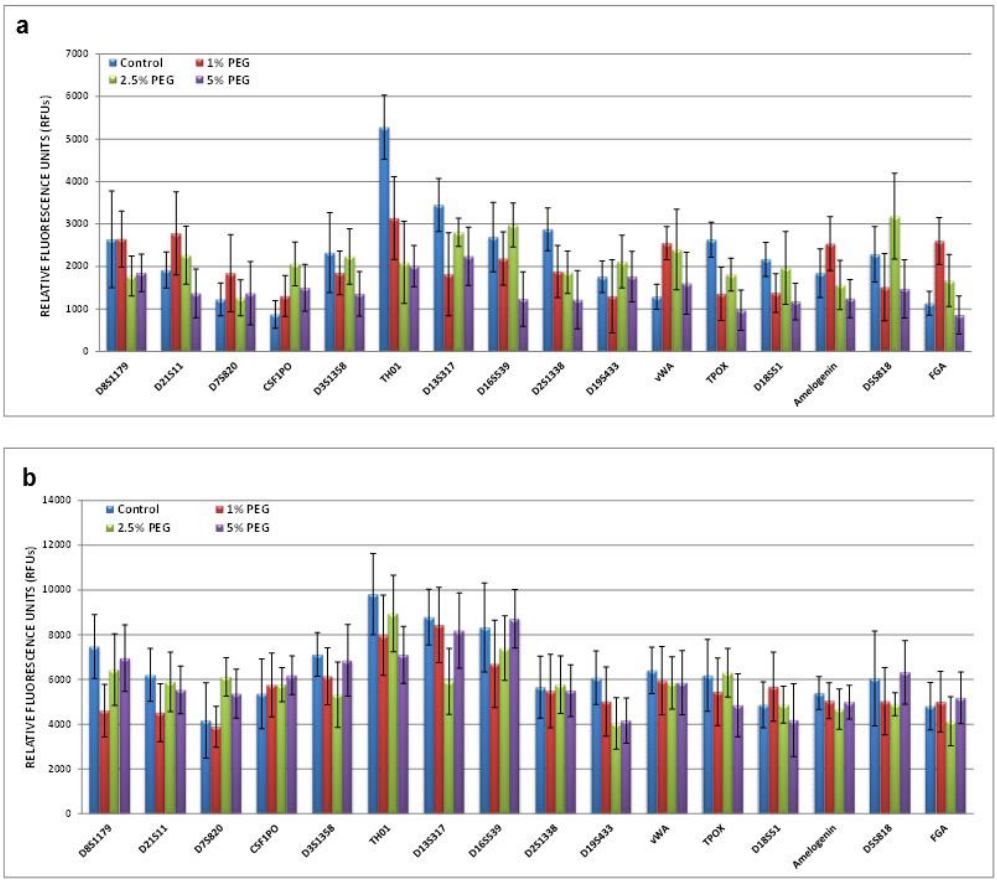


Figure 5. DNA from five different individuals at 25 pg or 100 pg total DNA were added to Identifiler® Plus amplification mix containing: Control – no PCR enhancer, 1% PEG, 2.5% PEG, and 5% PEG and amplified. Samples were amplified in triplicate. Average RFUs of alleles were calculated. 5A) Average Peak Height – 25 pg and 5B) Average Peak Height – 100 pg

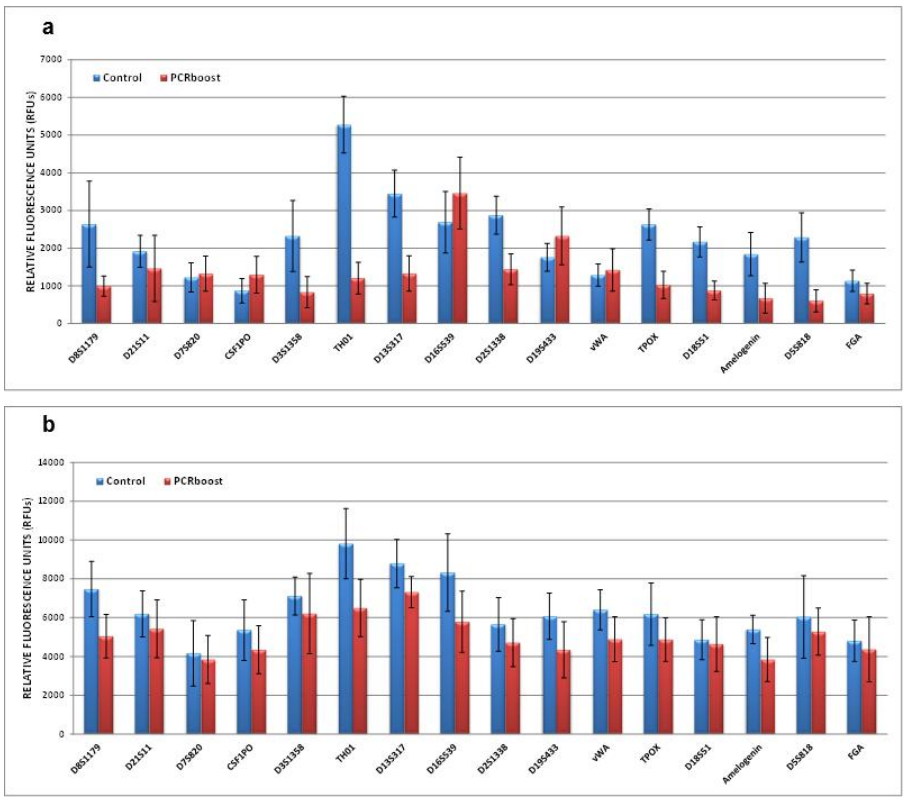


Figure 6. DNA from five different individuals at 25 pg or 100 pg total DNA were added to Identifiler® Plus amplification mix containing: Control – no PCR enhancer or PCRboost® and amplified. Samples were amplified in triplicate. Average RFUs of alleles were calculated. 6A) Average Peak Height – 25 pg and 6B) Average Peak Height – 100 pg

CONCLUSIONS

This study showed that addition of betaine can increase the yield of PCR products in LCN samples in the two locus multiplex and all commercial kits tested. Contrary to previous studies, the other additives had no impact on PCR product yield and in fact PCRboost® had a negative impact (27, 33). While betaine treatment showed an initial promise with decreasing stutter with the duplex, this effect was not observed in the commercial kits. In fact, significant reductions in stutter were not observed with any PCR additive tested. Seo *et al* (35) demonstrated some reduction in stutter peaks by lowering the annealing/extension temperature to

56 °C. To date, this is the only method that has shown any potential in the reduction of stutter peaks while maintaining number of detected alleles and peak heights. Addition of betaine may be able to reduce the number of PCR cycles used; however, overall imbalance in peak height ratio will likely still persist. Our data are consistent with other studies that it is quite difficult to reduce stutter or improve peak height imbalance (36-39). However, similar to other approaches, betaine treatment, increased PCR product yield, resulting in reduced allele dropout and better representation of the true DNA profile (37, 40-43). Therefore, betaine may be another consideration for enhancing allele detection of the PCR of LCN DNA samples.

SPECIAL ACKNOWLEDGMENTS

This project was supported in part by Award No. 2012-IJ-CX-0016, awarded by the National Institute of Justice, Office of Justice Programs, U.S. Department of Justice. The opinions, findings, and conclusions or recommendations expressed in this publication are those of the authors and do not necessarily reflect those of the Department of Justice.

CONFLICT OF INTEREST

The authors PLM, JLK, and BB declare that they have no conflict of interest.

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.

REFERENCES

1. Chakrabarti R, Schutt CE. The enhancement of PCR amplification by low molecular weight amides. Nucleic Acids Res. 2001 6;29(11):2377-81.

2. Fazekas A, Steeves R, Newmaster S. Improving sequencing quality from PCR products containing long mononucleotide repeats. BioTechniques. 2010 4;48(4):277-85.

3. Haqqi T, Zhao X, Panciu A, Yadav SP. Sequencing in the presence of betaine: Improvement in sequencing of the localized repeat sequence regions. J Biomol Tech. 2002 12;13(4):265-71.

4. Henke W, Herdel K, Jung K, Schnorr D, Loening SA. Betaine improves the PCR amplification of GC-rich DNA sequences. Nucleic Acids Res. 1997 10;25(19):3957-8.

5. Horakova H, Polakovicova I, Shaik GM, Eitler J, Bugajev V, Draberova L, Draber P. 1,2-propanediol-trehalose mixture as a potent quantitative real-time PCR enhancer. BMC Biotechnol. 2011 4;11:41.

6. Hube F, Reverdiau P, Iochmann S, Gruel Y. Improved PCR method for amplification of GC-rich DNA sequences. Mol Biotechnol. 2005 9;31(1):81-4.

7. Jensen MA, Fukushima M, Davis RW. DMSO and betaine greatly improve amplification of GC-rich constructs in de novo synthesis. PLoS One. 2010 6;5(6):e11024.

8. Juen A, Traugott M. Amplification facilitators and multiplex PCR: Tools to overcome PCR-inhibition in DNA-gut-content analysis of soil-living invertebrates. Soil Biol Biochem. 2006 7;38(7):1872-9.

9. Mamedov TG, Pienaar E, Whitney SE, TerMaat JR, Carvill G, Goliath R, Subramanian A, Viljoen HJ. A fundamental study of the PCR amplification of GC-rich DNA templates. Computational Biology and Chemistry. 2008 12;32(6):452-7.

10. Nagai M, Yoshida A, Sato N. Additive effects of bovine serum albumin, dithiothreitol, and glycerol on PCR. Biochem Mol Biol Int. 1998 1;44(1):157-63.

11. Musso M, Bocciardi R, Parodi S, Ravazzolo R, Ceccherini I. Betaine, dimethyl sulfoxide, and 7-deaza-dGTP, a powerful mixture for amplification of GC-rich DNA sequences. J Mol Diagn. 2006 11;8(5):544-50.

12. Ralser M, Querfurth R, Warnatz H, Lehrach H, Yaspo M, Krobitsch S. An efficient and economic enhancer mix for PCR. Biochem Biophys Res Commun. 2006 9;347(3):747-51.

13. Rees WA, Yager TD, Korte J, von Hippel PH. Betaine can eliminate the base pair composition dependence of DNA melting. Biochemistry. 1993 1;32(1):137-44.

14. Sarkar G, Kapelner S, Sommer SS. Formamide can dramatically improve the specificity of PCR. Nucleic Acids Res. 1990 12;18(24):7465.

15. Spiess AN, Mueller N, Ivell R. Trehalose is a potent PCR enhancer: Lowering of DNA melting temperature and thermal stabilization of taq polymerase by the disaccharide trehalose. Clin Chem. 2004 7;50(7):1256-9.

16. Weissensteiner T, Lanchbury JS. Strategy for controlling preferential amplification and avoiding false negatives in PCR typing. BioTechniques. 1996 12;21(6):1102-8.

17. Chakrabarti R, Schutt CE. Novel sulfoxides facilitate GC-rich template amplification. BioTechniques. 2002 4;32(4):866, 868, 870,2, 874.

18. Kang J, Soog Lee M, Gorenstein DG. The enhancement of PCR amplification of a random sequence DNA library by DMSO and betaine: Application to in vitro combinatorial selection of aptamers. J Biochem Biophys Methods. 2005 8;64(2):147-51.

19. Koumoto K, Ochiai H, Sugimoto N. Structural effect of synthetic zwitterionic cosolutes on the stability of DNA duplexes. Tetrahedron. 2008 1;64(1):168-74.

20. Kovarova M, Draber P. New specificity and yield enhancer of polymerase chain reactions. Nucleic Acids Res. 2000 7;28(13):E70.

21. Kurz M. Compatible solute influence on nucleic acids: Many questions but few answers. Saline Systems. 2008 6;4:6.

22. Paul N. H. Optimizing multiplex and LA-PCR with betaine. Trends Biochem Sci. 1997 6;22(6):225-6.

23. Sahdev S, Saini S, Tiwari P, Saxena S, Singh Saini K. Amplification of GC-rich genes by following a combination strategy of primer design, enhancers and modified PCR cycle conditions. Mol Cell Probes. 2007 8;21(4):303-7.

24. Spiess A, Ivell R. A highly efficient method for long-chain cDNA synthesis using trehalose and betaine. Anal Biochem. 2002 2;301(2):168-74.

25. Vasudevamurthy MK, Lever M, George PM, Morison KR. Betaine structure and the presence of hydroxyl groups alters the effects on DNA melting temperatures. Biopolymers. 2009;91(1):85-94.

26. Zhang Z, Yang X, Meng L, Liu F, Shen C, Yang W. Enhanced amplification of GC-rich DNA with two organic reagents. BioTechniques. 2009 9;47(3):775-9.

27. Ballantyne KN, van Oorschot RAH, John Mitchell R, Koukoulas I. Molecular crowding increases the amplification success of multiple displacement amplification and short tandem repeat genotyping. Anal Biochem. 2006 8;355(2):298-303.

28. Budowle B, van Daal A. Extracting evidence from forensic DNA analyses: Future molecular biology directions. BioTechniques. 2009 4;46(5):339,40, 342-50.

29. Minton AP. The influence of macromolecular crowding and macromolecular confinement on biochemical reactions in physiological media. J Biol Chem. 2001 4;276(14):10577-80.

30. Zimmerman SB, Harrison B. Macromolecular crowding increases binding of DNA polymerase to DNA: An adaptive effect. Proc Natl Acad Sci U S A. 1987 4;84(7):1871-5.

31. Sasaki Y, Miyoshi D, Sugimoto N. Effect of molecular crowding on DNA polymerase activity. Biotechnology Journal. 2006;1(4):440-6.

32. Le L, Clabaugh K, Chang A, Meinenger M, Coble MD, Loreille O, Scheible M, Demara R, de Rozieres S, Latterich M, Muller R, Lee SB. Recovering DNA profiles from low quantity and low quality forensic samples. Presentation—19th international symposium on human identification; 2008.

33. Biomatrica | PCRboost product information [Internet]; cited 12/5/2012]. Available from: http://www.biomatrica.com/pcrboost.php.

34. Leclair B, Fregeau CJ, Bowen KL, Fourney RM. Systematic analysis of stutter percentages and allele peak height and peak area ratios at heterozygous STR loci for forensic casework and database samples. J Forensic Sci. 2004 9;49(5):968-80.

35. Seo SB, Ge J, King JL, Budowle B. Reduction of stutter ratios in short tandem repeat loci typing of low copy number DNA samples. Forensic Science International: Genetics. 2014 1;8(1):213-8.

36. Kloosterman AD, Kersbergen P. Efficacy and limits of genotyping low copy number (LCN) DNA samples by multiplex PCR of STR loci. J Soc Biol. 2003;197(4):351-9.

37. Budowle B, Hobson DL, Smerick JB, Smith JAL. Low Copy Number - Consideration and Caution. Madison, WI: Promega Corporation; 2001. Report No.: 1-26.

38. Weiler NE, Matai AS, Sijen T. Extended PCR conditions to reduce drop-out frequencies in low template STR typing including unequal mixtures. Forensic Science International: Genetics. 2012;6(1):102-7.

39. Michel S, De Bast A, Vandenbroere I, Froment O. Interpretation of low-copy-number DNA profile after post-PCR purification. Forensic Science International: Genetics Supplement Series. 2009;2(1):542-3.

40. Westen AA, Nagel JH, Benschop CC, Weiler NE, de Jong BJ, Sijen T. Higher capillary electrophoresis injection settings as an efficient approach to increase the sensitivity of STR typing. J Forensic Sci. 2009 5;54(3):591-8.

41. Smith PJ, Ballantyne J. Simplified low-copy-number DNA analysis by post-PCR purification. J Forensic Sci. 2007 7;52(4):820-9.

42. 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 12;123(2-3):215-23.

43. Leclair B, Sgueglia JB, Wojtowicz PC, Juston AC, Fregeau CJ, Fourney RM. STR DNA typing: Increased sensitivity and efficient sample consumption using reduced PCR reaction volumes. J Forensic Sci. 2003 9;48(5):1001-13.

SECTION 4

Recommendations for Interpretation Guidelines

"Insanity is doing the same thing over and over again and expecting different results." ~ Albert Einstein

CHAPTER 6

An evaluation of the transfer of saliva-derived DNA

International Journal of Legal Medicine, November 2012, 126(6), 851-861

David H. Warshauer
Pamela L. Marshall
Shamika Kelley
Jonathan L. King
Bruce Budowle

ABSTRACT

Studies of DNA transfer have focused largely on the transfer of sloughed off epithelial cells from individuals' hands. This research examines primary, secondary, and tertiary transfer events involving DNA originating from saliva, a commonly encountered body fluid. More routine human behaviors were simulated to evaluate transfer, and the effects of drying time, moisture, and surface composition were investigated. The results agree with previous findings which indicate that the presence of moisture, as well as a smooth non-porous surface as the primary substrate, increases the efficiency of transfer. Previous transfer studies have found that the last individual to come into contact with an item is usually the major contributor to the resulting DNA mixture, unless conditions are simulated in which a "good shedder" serves as the primary depositor and a "poor shedder" serves as the secondary depositor. The results of this study indicate that when saliva is the source of the transferred DNA, the primary depositor is often the major contributor. These findings suggest that shedder status is less relevant with regard to touch DNA samples in a forensic setting, and emphasize the need for caution when analyzing such samples.

Keywords Primary Transfer · Secondary Transfer · Tertiary Transfer · Saliva · STR typing · Shedder · Moisture

INTRODUCTION

Validated forensic DNA analysis techniques are capable of providing reliable genetic information from biological evidence that can be used to associate or exclude individuals as potential contributors of samples collected at crime scenes (1-4). These methods offer both high discrimination power and a high sensitivity of detection. In 1997, van Oorschot and Jones (5) found that objects handled by single individuals yielded profiles consistent with those of the handlers, while objects handled by multiple individuals produced a DNA mixture. They also suggested that a handshake between two individuals for as little as one minute was sufficient to transfer DNA between the individuals. Since then, "touch DNA" analysis, the examination of DNA transferred through contact, has become a subject of interest in the field of forensic genetics. Subsequent studies investigated primary transfer, i.e., events wherein DNA is directly transferred from an individual to an object or another individual (6-8). Barbaro *et al*. (9) analyzed DNA originating from residual sweat and epithelial cells left on pens and compared it with DNA obtained from semen and saliva stains; the results allowed for correct source attribution. Other studies (10-12) have found that certain individuals, termed "good shedders," appear to have a greater propensity for depositing DNA when touching an object, as measured by complete genetic profiles; others, described as "poor shedders," do not leave behind as much DNA. Djuric *et al*. (13) also noted that transfer from an individual to another individual follows a pattern similar to that of transfer from an individual to an object, and that the DNA obtained is often a mixture consistent with the profiles of both individuals. Touch DNA analysis has since been employed for the investigation of a wide variety of commonly touched sample types, including bullet casings, documents, and even bedding (14-17). The principles associated with this form of DNA analysis have direct bearing on the interpretation of forensic profiles and the

relevance of such information even for single-source samples. Therefore, it is necessary to understand the potential impact of DNA transfer on a given sample.

While the aforementioned studies mainly addressed primary transfer, other studies have investigated secondary transfer, a variation of DNA transfer in which the original source individual does not make direct contact with the final recipient individual or object. Instead, DNA is transferred through an intermediary vector. Secondary transfer was noted by van Oorschot and Jones (5) in their original study, where they mentioned that the genetic profiles of handlers of an item were sometimes observed in profiles obtained from the hands of subsequent holders of the item. Additional studies confirmed these findings and documented the occurrence of secondary DNA transfer (18-21). Studies of secondary transfer involving human vectors often indicated that, under normal conditions, the majority of detectable DNA on the final object generally originates from the vector (18,22,23). Profiles originating from the primary individual have only been observed as the dominant profiles in secondary transfer studies conducted under arranged conditions in which the primary individual was a good shedder and the vector was a poor shedder (10,18). Furthermore, most secondary transfer studies have been limited in that they have focused mainly on DNA deposited through skin epithelial cells sloughed off during contact with individuals' hands.

Skin cells are expected to slough off and transfer through contact. However, skin cell transfer studies are somewhat contrived and likely do not approximate real-world activities. Skin cells may not be the primary source of transfer DNA in a number of scenarios, and other sources richer in DNA may be transferred routinely. Saliva, for example, contains substantial amounts of DNA, well above what may be considered trace levels (24-26). However, there have been very few studies of DNA transfer with regard to saliva, even though it is a body fluid commonly

encountered and transferred between individuals and/or objects. For instance, it is not uncommon for a person to lick his or her thumb while turning the pages of a book, or for an individual to hold a pen in his or her mouth while studying or reading. In the latter example, the deposition of saliva-derived DNA on the pen is a primary transfer event. If the pen is later handed to a second person, the transmission of the first individual's DNA to the surface of the second individual's hand constitutes a secondary transfer event.

The study described herein was conducted under the hypothesis that saliva, which is rich in epithelial cells, may be a more prevalent source of genetic material during transfer events than the epithelial cells deposited from a hand. Thus, the transfer of saliva-derived DNA could often result in higher levels of detectable genetic material than have been observed with previous hand contact transfer studies. Furthermore, the genetic profile of the primary individual, i.e., the source, may be more prevalent in such cases of secondary transfer due to the presence of saliva-derived epithelial cells.

MATERIALS AND METHODS

Subjects: Following University of North Texas Health Science Center IRB approval, four individuals (two male and two female) were used for this study. One male subject was paired with one female subject in a manner that allowed for the fewest shared alleles between the genetic profiles of the individuals within each pair to obtain maximum value of mixture data interpretation.

Extraction, Quantification, Amplification, Capillary Electrophoresis, and Data Analysis

DNA was collected from each donor using the Fitzco® CEP Swab™ Cell Collection System (Fitzco Inc., Spring Park, MN) and extracted from the swabs using the Qiagen® QIAamp® DNA Mini (Qiagen Inc., Valencia, CA) extraction procedure for buccal swabs. The quantity of extracted DNA was determined using the Applied Biosystems® Quantifiler™ Human DNA Quantification Kit (Life Technologies, Carlsbad, CA) on an Applied Biosystems® 7500 Real-Time PCR System (Life Technologies), according to the manufacturer's protocol. Amplification was performed using the Applied Biosystems® AmpFlSTR® Identifiler® Plus PCR Amplification Kit (Life Technologies) on an Applied Biosystems® GeneAmp® PCR System 9700 thermal cycler (Life Technologies), according to the manufacturer's recommendations. Capillary electrophoresis was then performed on an Applied Biosystems® 3130*xl* Genetic Analyzer (Life Technologies) using POP-4™ polymer (Life Technologies), and analyzed using Applied Biosystems® GeneMapper® ID v3.2 software (Life Technologies), according to the manufacturer's recommended protocol. In cases where the standard 28 cycles of PCR did not yield interpretable results, samples were re-amplified in duplicate using 34 cycles. Alleles were only called if they appeared in both replicates and had peak heights $\geq$ 50 RFU.

Experimental Design

This study was divided into three sets of experiments to examine the different types of DNA transfer: primary, secondary, and tertiary. At the beginning of all trials, subjects washed their hands with soap and water and dried them. In trials where thumbs were moistened with saliva, the subjects extended their tongues and ran their thumbs down their tongues once. Deposition of saliva on pen surfaces was accomplished by having the subjects hold the back (non-capped) ends

of pens (that had been exposed previously to UV irradiation) in their mouths for 30 seconds. For some experiments, DNA was transferred via contact with plastic conical tubes. In these tests, subjects were required to grip UV-treated 50 mL conical tubes with moderate pressure for 15 seconds. In certain trials, the presence of sweat was simulated by spraying the subjects' thumbs or palms once with DNAse/RNAse-free distilled water from an atomizer. In all trials, DNA collection was performed using the double swab technique (27). Unless otherwise noted, drying times of 5 and 30 minutes were employed. Tests were conducted in duplicate for each of these drying times. Specific descriptions of the individual experiment conditions can be found in **Table 1**.

Data analysis

DNA quantification values were used to determine the amount of DNA loss due to transfer steps, as well as to gauge the general efficiency of the PCR reaction. The efficiencies of the transfer events themselves were determined by calculating the percentages of alleles in each individual's profile that were observed following transfer. The proportions of DNA contribution by each individual in dual-subject trials were obtained by comparing the peak heights of alleles at loci that displayed at least one allele unique to one of the individuals in the test pair to the total contribution of those alleles. A locus containing only one unique allele from individual A, for example, was counted as having 100% contribution from individual A. A locus containing a 750 RFU allele peak unique to individual A and a 250 RFU allele peak unique to individual B was counted as having 75% contribution from individual A and 25% contribution from individual B. Contribution percentages were then averaged for each individual across all loci to obtain the final values.

Trial No.	Transfer Type	Transfer Steps	Procedure
1	Primary	M→BTh	Thumbs were licked, and swabbed after each drying period; 16 samples (4 per subject)
2	Primary	M→GTh	Similar to Trial 1, except that subjects wore gloves before washing; 16 samples (4 per subject)
3	(Reference)	Pa	Bare palms were swabbed after washing, to collect DNA; 16 samples (4 per subject)
4	Primary	M→Pe	Saliva was deposited on pens, which were swabbed after each drying period; 16 samples (4 per subject)
5	Secondary	M→BTh→Tu	Similar to Trial 1, except that subjects grasped sterilized plastic conical tubes after each drying time; tubes then were swabbed; 16 samples (4 per subject)
6	Secondary	M→Pe→Pa	Similar to Trial 4, except that subjects were required to pass the pens to their designated partners after each drying time; pens were gripped like tubes; the partners' palms then were swabbed; 16 samples (8 per pair)
7	Secondary	M→GTh→Tu	Similar to Trial 2, except that subjects gripped plastic conical tubes after each drying time; 16 samples (4 per subject)
8	Secondary	M→Pe→MstPa	Similar to Trial 6, except that recipient subjects' palms were moistened before they grasped the plastic tubes, to simulate sweat; palms then were swabbed; 16 samples (8 per pair)
9	Secondary	M→MstBTh→Tu	Similar to Trial 5, except that subjects' thumbs were moistened after each drying time, in order to imitate sweat; 16 samples (4 per subject)
10	Tertiary	M→BTh→Tu→Pa	Similar to Trial 5, except that after grasping the plastic tubes, subjects passed them to their partners; partners grasped the tubes, and their palms then were swabbed; roles of each pair then were reversed; 5-minute drying time only; 8 samples (4 per pair)
11	Tertiary	M→BTh→Tu→MstPa	Similar to Trial 10, except that recipient partners' palms were moistened prior to gripping the tubes, to imitate sweat; roles of each pair then were reversed; 5-minute drying time only; 8 samples (4 per pair)
12	Tertiary	M→Pe→Pa→Tu	Similar to Trial 6, except that recipient partners each grasped plastic tubes after gripping the pens; tubes then were swabbed; roles of each pair then were reversed; 5-minute drying time only; 8 samples (4 per pair)
13	Tertiary	M→MstBTh→Tu→Pa	Similar to Trial 9, except that initial subjects' thumbs were moistened prior to grasping the tubes, to simulate sweat; recipient partners' palms then were swabbed; roles of each pair then were reversed; 5-minute drying time only; 8 samples (4 per pair)
14	Tertiary	M→Pe→MstPa→Tu	Similar to Trial 12, except that recipient subjects' palms were moistened prior to them grasping the pens, in order to imitate sweat; tubes then were swabbed; roles of each pair then were reversed; 5-minute drying time only; 8 samples (4 per pair)
15	Tertiary	M→GTh→Tu→Pa	Similar to Trial 10, except that each initial subject wore a latex glove after washing; after gripping the tubes, recipient partners' palms were swabbed; roles of each pair then were reversed; 5-minute drying time only; 8 samples (4 per pair)
16	Tertiary	M→Gth→Tu→MstPa	Similar Trial 15, except that recipient subjects' palms were moistened prior to grasping the tubes, to simulate sweat; recipient partners' palms then were swabbed; roles of each pair then were reversed; 5-minute drying time only; 8 samples (4 per pair)

Table 1. Transfer Experiments. For each trial, the type of transfer, shorthand notation, and procedural notes are listed. The shorthand used in this table is: *M*: mouth; *BTh*: bare thumb; *GTh*: gloved thumb; *Pa*: palm; *Pe*: pen; *Tu*: tube; *MstPa*: moistened palm; *MstBTh*: moistened bare thumb. Trial 3 was used as a reference point for the amount of DNA on a bare palm, and thus did not represent a transfer event.

RESULTS AND DISCUSSION

A number of studies have focused on the transfer of DNA between clean hands and/or objects. The study herein contributes to our understanding of primary and secondary transfer when another source relatively richer in DNA is involved in the transfer. Saliva is a common biological material that is routinely transferred among individuals. To address the potential impact of saliva on the interpretation of DNA typing results associated with transfer, primary and secondary transfer experiments were conducted that approximated more typical human behavior, but still in a controlled manner (e.g., male and female subjects were required to lick their thumbs or hold pens in their mouths to simulate common habits). An additional set of experiments addressed tertiary transfer events, where DNA deposited from an individual to an object or individual is then transferred to another object or individual. The genetic data collected from the experiments were analyzed to assess the amount of DNA transferred, the relative decrease in the levels of genetic material that occurred as the number of vectors increased, and the proportions of DNA that were contributed by the primary and vector individuals.

Primary Transfer Trial Results

The quantity of saliva-derived DNA obtained from the licked bare thumbs of the subjects was compared with that obtained from the subjects' gloved thumbs to roughly estimate DNA transferred in the single saliva deposition event. The DNA yield from the bare palms of the subjects also was quantified to develop a general baseline of native DNA levels for the individuals involved in the studies.

DNA quantification results for the primary transfer experiments, such as those shown for subject 001 (**Figure 1**), revealed that, in many instances, slightly more DNA was yielded from gloved thumbs than bare thumbs. It is likely that this observation was due to the smoother, less porous surface of the glove allowing for more efficient collection of DNA via the swabbing technique than from the rougher, ridged thumb surface. The findings are consistent with those described by Goray *et al.* (19), who demonstrated that smooth, non-porous surfaces, such as plastic, yielded higher quantities of recovered transferred DNA than rougher, porous surfaces, such as cotton and wool. The effects of a smoother surface composition, in addition to the greater surface area, also explain why DNA was sometimes obtained in larger quantities from pens held in the mouths of subjects as opposed to the subjects' thumbs (**Figure 1**). In addition, substantial variation in DNA yield is observed from one replicate to another in each trial, indicating, as expected, that deposited DNA varies widely from one instance to the next of deposition by licking. The variation in DNA deposition and the effects of the different surface areas on the DNA yield made the estimation of saliva-derived DNA quantity in a single saliva deposition event problematic. Thus, quantifying loss through transfer was difficult, and inferences made from this part of the study must be recognized as providing trends and general conclusions.

Amplification at 28 cycles of the DNA from the primary transfer trial samples yielded full genetic profiles in almost every case (**Table 2**). The notable exception to these results was the group of Trial 3 samples (i.e., swabbing of bare palms), which generally yielded no genetic profiles. The DNA from the Trial 3 samples was amplified using 34 PCR cycles and reanalyzed. Even with increased sensitivity of detection, these samples only yielded genetic profiles showing

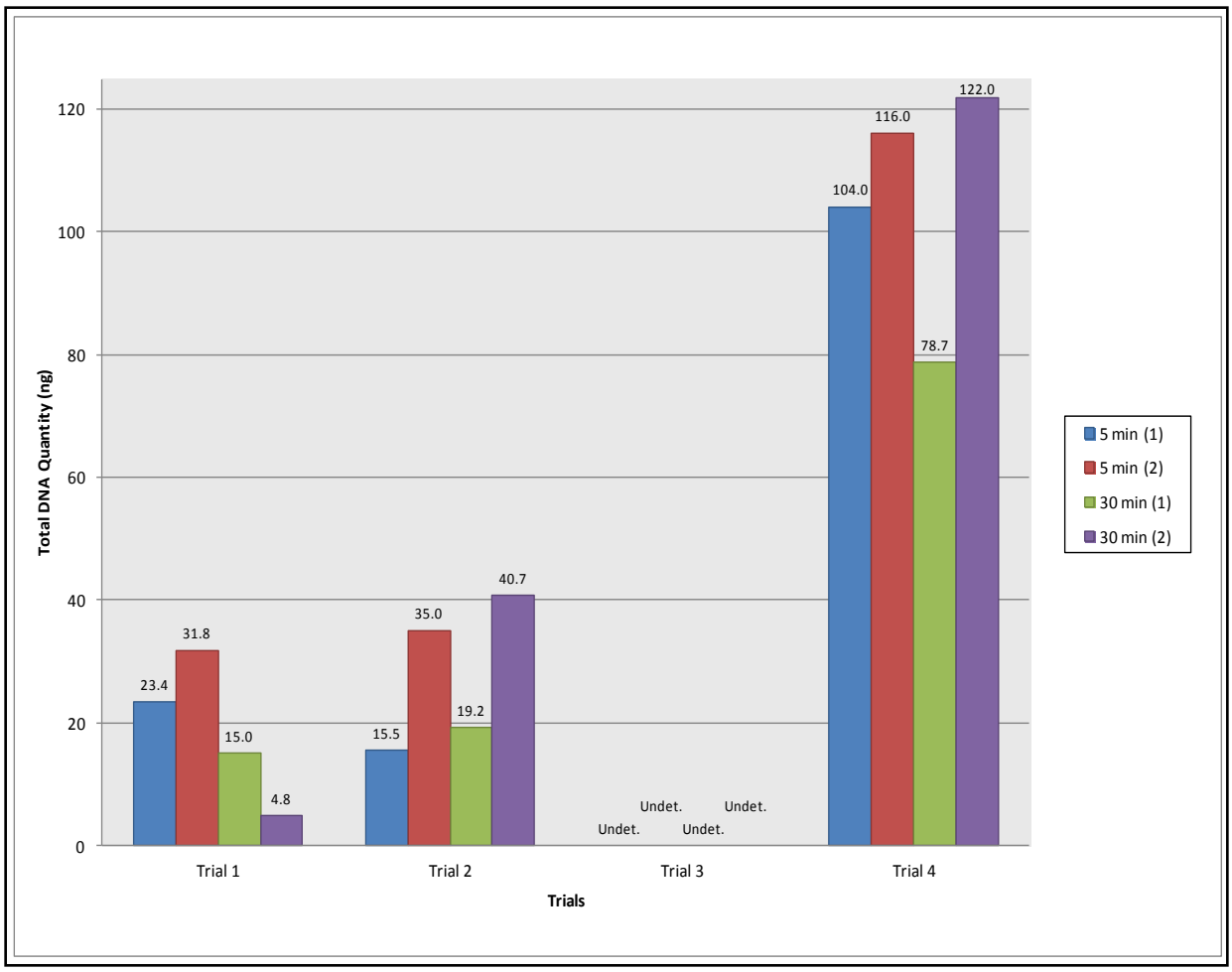


Figure 1. Primary Transfer Trial DNA Quantities – Subject 001. The total quantity of collected DNA is shown for each of the primary transfer experiments involving subject 001. Drying time replicates are displayed without averaging to illustrate the degree of variation from one saliva deposition event to the next. Times reflect duration of drying prior to transfer event. Trial 1 was the primary transfer of saliva to bare thumbs, while Trial 2 represented the primary transfer of saliva to gloved thumbs. Trial 3 was the swabbing of bare palms; Trial 4 was the primary transfer of saliva to pen surfaces.

up to 22.2% of the expected alleles, with one exception that showed 53.8% of the alleles (data not shown). These results indicated that the sloughed off epithelial cells on the subjects' palms were not sources of abundant DNA.

		Trial 1	Trial 2	Trial 3	Trial 4
Subject 001	5 min (1)	100.0%	100.0%	0.0%	100.0%
	5 min (2)	100.0%	100.0%	0.0%	100.0%
	30 min (1)	100.0%	100.0%	0.0%	100.0%
	30 min (2)	100.0%	100.0%	0.0%	100.0%
Subject 002	5 min (1)	100.0%	100.0%	11.5%	100.0%
	5 min (2)	100.0%	100.0%	0.0%	100.0%
	30 min (1)	100.0%	100.0%	0.0%	100.0%
	30 min (2)	100.0%	100.0%	0.0%	100.0%
Subject 003	5 min (1)	100.0%	100.0%	0.0%	100.0%
	5 min (2)	100.0%	100.0%	0.0%	100.0%
	30 min (1)	100.0%	100.0%	0.0%	100.0%
	30 min (2)	100.0%	100.0%	0.0%	100.0%
Subject 004	5 min (1)	100.0%	100.0%	0.0%	96.7%
	5 min (2)	100.0%	100.0%	0.0%	100.0%
	30 min (1)	100.0%	100.0%	0.0%	30.0%
	30 min (2)	100.0%	100.0%	3.3%	56.7%

Table 2. Primary Transfer Trial Profile Completion Percentages (28 PCR Cycles). The percentages of observed alleles of each subject's full genetic profile following 28 cycles of PCR are listed for each drying time replicate in each experiment. Times reflect duration of drying prior to transfer event. Trial 1 was the primary transfer of saliva to bare thumbs, while Trial 2 represented the primary transfer of saliva to gloved thumbs; Trial 3 was the swabbing of bare palms; Trial 4 was the primary transfer of saliva to pen surfaces.

Secondary Transfer Trial Results

The quantities of recovered DNA were compared with those assessed in the primary transfer experiments to provide a rough estimate of the proportion of DNA lost during the steps of the transfer process. The percentages of obtainable profiles for Trials 5 and 9 (transfer of saliva on bare thumbs to plastic tubes, and transfer of saliva on moistened bare thumbs to plastic tubes, respectively), as well as Trials 6 and 8 (transfer of saliva on pen surfaces to palms, and transfer of saliva on pen surfaces to moistened palms, respectively), were compared to assess the effects of simulated sweat (i.e., moistened hands) on secondary DNA transfer. In trials that involved

two subjects (Trials 6 and 8), the DNA profiles were compared with the subjects' reference profiles to assess the relative ratios of primary and secondary contributor DNA, based on the peak height ratios observed in the electropherograms.

Quantification results from the secondary transfer trials wherein moisture was absent indicated that a single transfer event can lead to dramatically reduced yield of DNA from a dry source, which is consistent with the findings of Goray *et al.* (19). The Trial 6 samples, however, yielded sufficient interpretable DNA quantity values to provide a rough estimate of the decrease in recoverable DNA (**Table 3**). DNA quantity estimates for each subject's samples in this trial were averaged, and these averages were compared. The mean percentage of DNA loss due to a single transfer step was 81.2%, indicating that DNA loss was substantial. Further estimation of DNA loss due to transfer events was not possible because most of the quantity estimates for the various secondary transfer trial samples (i.e., non-moistened transfers) corresponding to previously quantified primary transfer trial samples were undeterminable (Table 3).

Amplification of 10 µL of extract at 28 PCR cycles was insufficient to yield full genetic profiles from samples from Trials 5-8 (**Table 4**). In fact, 90.6% of the single-subject samples from these trials amplified at 28 PCR cycles yielded profiles that contained less than half of the expected alleles. Of these, 65.5% failed to show even a single allele. The samples were re-amplified at 34 PCR cycles, and more complete genetic profiles were thus obtained (Table 4). Samples from Trials 8 and 9 were amplified only at 34 cycles, forgoing the 28-cycle amplification, as the previous trials had demonstrated that the recoverable DNA from such samples could be considered "low-copy DNA," and required more amplification cycles to yield detectable results (28,29). Based on the results, subsequent tertiary trial samples were amplified only at 34 cycles, as well.

		Trial 5	Trial 6	Trial 7	Trial 8	Trial 9
Subject 001	5 min (1)	-	-	-	0.75	1.33
	5 min (2)	-	1.66	-	1.66	0.29
	30 min (1)	-	3.73	-	4.86	-
	30 min (2)	0.42	-	-	2.90	0.51
Subject 002	5 min (1)	-	1.38	-	3.32	0.50
	5 min (2)	-	5.34	-	3.58	0.54
	30 min (1)	-	-	-	3.53	0.27
	30 min (2)	-	3.86	-	0.85	0.76
Subject 003	5 min (1)	-	2.96	0.86	4.82	3.11
	5 min (2)	0.40	5.06	-	1.65	2.94
	30 min (1)	0.78	0.43	-	1.35	3.85
	30 min (2)	2.55	2.44	-	-	2.16
Subject 004	5 min (1)	-	0.84	-	-	0.27
	5 min (2)	1.15	5.14	-	-	0.54
	30 min (1)	1.58	-	-	-	-
	30 min (2)	0.85	0.60	-	-	0.58

Table 3. Secondary Transfer Trial DNA Quantities. The total quantities of DNA (ng) collected for each of the replicates in each secondary transfer trial are listed. Values of "-" represent no detectable DNA by the quantification assay. Times reflect duration of drying prior to transfer event. Trial 5 was the transfer of saliva on bare thumbs to plastic tubes; Trial 6 represented the transfer of saliva on pen surfaces to palms; Trial 7 was the transfer of saliva on gloved thumbs to plastic tubes; Trial 8 was the transfer of saliva on pen surfaces to moistened palms; Trial 9 represented the transfer of saliva on moistened bare thumbs to plastic tubes.

		Trial 5		Trial 7	
		28 Cycles	34 Cycles	28 Cycles	34 Cycles
Subject 001	5 min (1)	0.0%	0.0%	0.0%	0.0%
	5 min (2)	0.0%	0.0%	0.0%	0.0%
	30 min (1)	0.0%	**14.8%**	0.0%	0.0%
	30 min (2)	3.7%	**37.0%**	0.0%	**3.7%**
Subject 002	5 min (1)	0.0%	**38.5%**	0.0%	0.0%
	5 min (2)	7.7%	**42.3%**	0.0%	0.0%
	30 min (1)	3.8%	0.0%	0.0%	**11.5%**
	30 min (2)	3.8%	**7.7%**	0.0%	0.0%
Subject 003	5 min (1)	31.0%	**86.2%**	3.4%	0.0%
	5 min (2)	41.4%	**93.1%**	0.0%	**3.4%**
	30 min (1)	31.0%	**65.5%**	0.0%	**17.2%**
	30 min (2)	100.0%	89.7%	0.0%	**3.4%**
Subject 004	5 min (1)	43.3%	**66.7%**	0.0%	0.0%
	5 min (2)	30.0%	**56.7%**	0.0%	0.0%
	30 min (1)	56.7%	**96.7%**	0.0%	0.0%
	30 min (2)	60.0%	**93.3%**	0.0%	0.0%

Table 4. Secondary Transfer Trial Profile Completion Percentages – Trials 5 and 7. The percentages of alleles of each subject's genetic profile detected via capillary electrophoresis following 28 and 34 cycles of PCR are listed.

In the cases of Trials 6 and 8, both of which involved two subjects, the peak heights of the observed alleles were compared to attempt to determine the percentage of each subject's contribution to the DNA mixture. Any allele unique to one of the subjects in the pair was considered for these calculations. The percentages of major and minor allele contribution were averaged for each replicate in each trial. In all but one of the replicates in Trial 6 that yielded unique alleles, the original depositor (the subject that held the pen in his/her mouth) was definitively shown to be the major contributor of the DNA (**Figure 2**). Similarly, the original depositor was shown to be the major DNA contributor in all but two replicates of Trial 8 (data not shown). These results are not surprising, since the DNA quantity estimates from the Trial 3 samples indicated that very little DNA was present on subjects' clean palms. Thus, the transfer of DNA-rich saliva to a pen and then onto a subject's palm would likely result in a DNA mixture that is predominantly from the saliva.

The effects of simulated sweat on the transfer process were investigated in Trials 8 and 9. To do so, the percentages of obtainable profiles for Trials 5 and 9 were compared. In all but two of the replicates in these trials, the samples from Trial 9 yielded profiles that displayed more of the expected alleles than those yielded by the Trial 5 samples (data not shown). Of these 14 samples, 10 yielded profiles that displayed an additional 25% or more of the expected alleles from the depositing subject. These results suggest that moist surfaces facilitate DNA transfer more efficiently than dry ones, which is consistent with the results obtained by Goray *et al.* (19). A comparison of the percentages of obtainable profiles for Trials 6 and 8 was not as informative, because the samples from both trials yielded fairly complete genetic profiles from the primary contributors. The percentages of obtainable profiles from the secondary contributor (the receiving subject) were not significantly different. These results also are consistent with the

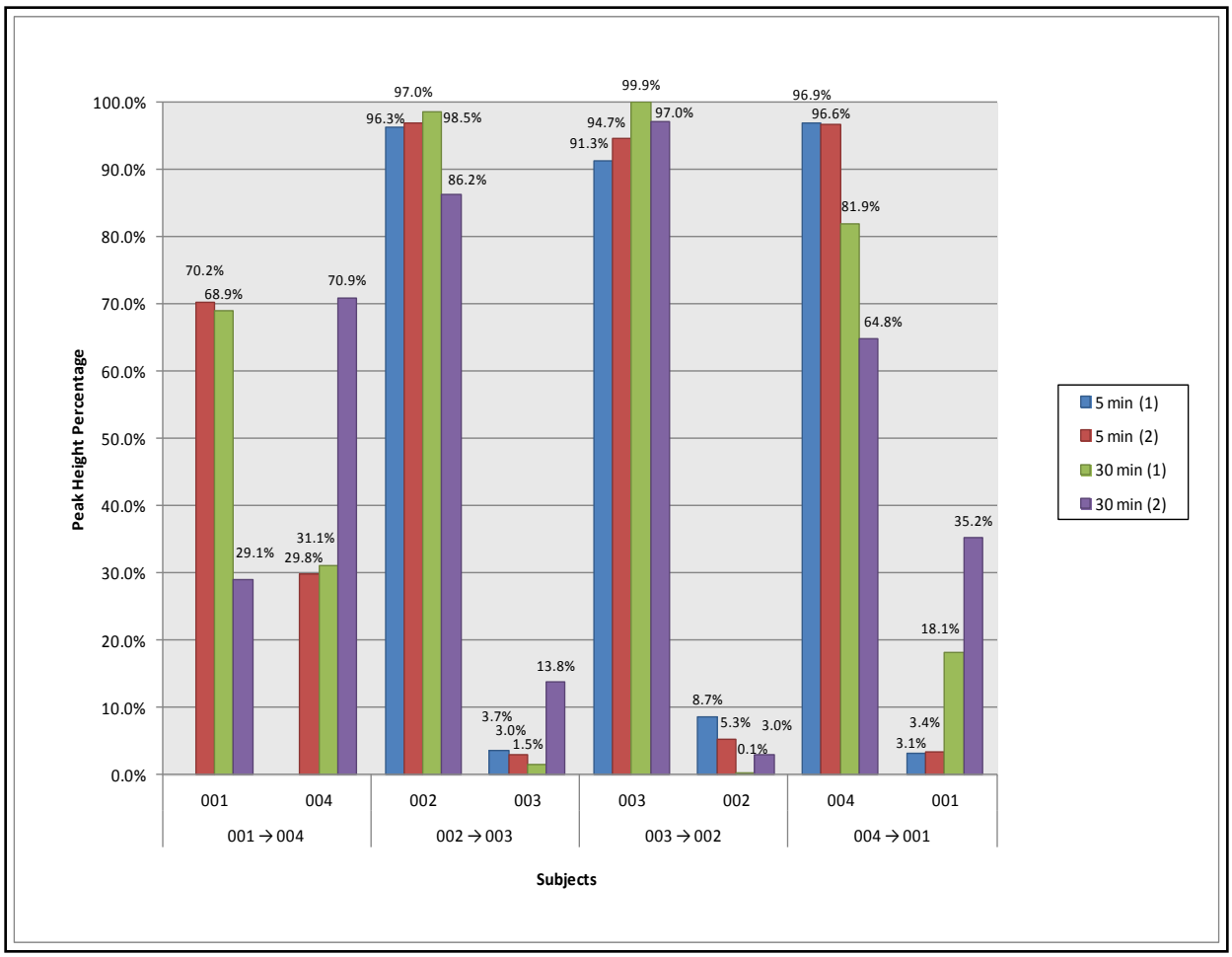


Figure 2. Major/Minor Contributor Percentages (Peak Height) – Trial 6 (34 PCR Cycles). The calculated percentages of contribution to the DNA mixture by each individual's unique alleles, based on peak height data, are displayed for each replicate. Times reflect duration of drying prior to transfer event. The order of transfer for each pair of subjects in the study is shown on the X axis. Trial 6 represented the secondary transfer of saliva on pen surfaces to palms.

results obtained in previous trials that indicated that there was not a relatively substantial quantity of DNA on a clean palm initially.

Tertiary Transfer Trial Results

DNA typing data obtained from this third set of experiments were used to evaluate the effects of tertiary transfer of DNA. The majority of the quantities of recovered DNA were not sufficient to be detected by the quantification assay. Thus, it was not feasible to estimate the amount of DNA

lost during the third step of the transfer process. The fact that the quantities were mostly undetectable did indicate that a very large portion of the recoverable DNA is lost during the tertiary transfer process, which is consistent with the previous findings of over 80% DNA loss in a single transfer event. Each tertiary transfer trial had one or more corresponding versions involving the use of simulated sweat. The percentages of obtainable profiles for these trials were compared to assess the effects of moisture on tertiary DNA transfer.

Overall, the percentages of subject profiles that were observed following the tertiary transfer events were much lower than those recovered after the secondary transfer events. In fact, 87.5% of the profiles observed after tertiary transfer displayed less than half of the expected alleles (data not shown). These findings were consistent with the concept that tertiary transfer substantially diminishes the amount of recoverable DNA.

The recovered profile percentages for tertiary transfer trials involving subject 003 (**Figure 3**), for example, demonstrate the effects of imitated sweat (moisture) on the tertiary transfer process. When the percentages of primary subjects' profiles yielded by Trial 10 (transfer of saliva on bare thumbs to plastic tubes, and then to palms) were compared with those yielded by Trial 11 (transfer of saliva on bare thumbs to plastic tubes, and then to moistened palms), it was evident that greater portions of the primary subjects' DNA profiles were transferred when the recipient partners' palms were moistened. This observation was consistent with the secondary transfer results and the findings of Goray *et al*. (19). It should be noted that higher proportions of the primary subjects' DNA were transferred to the recipients' palms when the primary subjects' thumbs were moistened prior to gripping the tubes. A comparison of the profile percentages for Trials 12 and 14 (transfer of saliva on pen surfaces to palms, and then to plastic tubes, and transfer of saliva on pen surfaces to moistened palms, and then to plastic tubes,

respectively), and Trials 15 and 16 (transfer of saliva on gloved thumbs to plastic tubes, and then to palms, and transfer of saliva on gloved thumbs to plastic tubes, and then to moistened palms, respectively), showed the same general trend of more efficient transfer when moisture was present.

As with the dual-subject secondary transfer trial results, the peak heights of the observed alleles in these tertiary transfer trials were compared to attempt to determine the percentage of each subject's contribution to the DNA mixture. Generally, the secondary depositors (the subjects whose palms were swabbed) were shown to be the major contributors of the DNA mixtures in trials where moisture was absent. This was the case in all of the replicates in Trials 10 and 15 based on unique alleles. These results agree with previously published observations of DNA transfer (18,22,23), and differ from the results of the secondary transfer portion of this study, where the primary contributor of the DNA was shown to contribute the majority of the DNA in the resulting mixture. However, these findings should be expected, as the additional transfer step involved in the tertiary transfer process likely diminished the amount of DNA deposited by the initial contributor. Two applicable replicates of Trial 11, though, revealed that the primary contributor's DNA was the major component of the mixture (**Table 5**). These results were consistent with our earlier findings; that is, the presence of moisture at a subsequent transfer step increases the likelihood of transferring DNA deposited by a primary contributor during tertiary transfer. The results of other trials involving the presence of moisture generally indicated that the primary depositor was the major contributor of DNA, as well. For instance, all of the replicates in Trial 14 indicated that the primary contributors' DNA made up the majority of the mixtures. These results are consistent with the earlier findings of this study, indicating that greater smooth surface area increases efficiency of transfer. Similar results were obtained

from Trial 13, where all but 2 applicable replicates showed that the primary contributors were the major sources of the DNA in the mixtures. The results of Trial 16, however, showed that the secondary depositor was the predominant contributor. It should be noted that the results of Trial 12, wherein moisture was absent, still revealed the primary depositor to be the main contributor. This may be due to the characteristics of the pens' surfaces and variation in the amount of DNA deposited, as noted above. Lastly, the number of unique alleles observed in the tertiary study was a small percentage of the total possible alleles between the pairs of individuals and therefore the number of alleles displayed in Table 5 should not be misconstrued. The data were only the unique alleles and not the total alleles observed. Moreover, the method used in the study to increase sensitivity of detection employed only additional PCR cycles. Sensitivity can be enhanced further, for example, by reduced PCR volumes and post-PCR clean-up. With increased sensitivity of detection methods, more alleles will likely be detected, but still following the trends observed in this study.

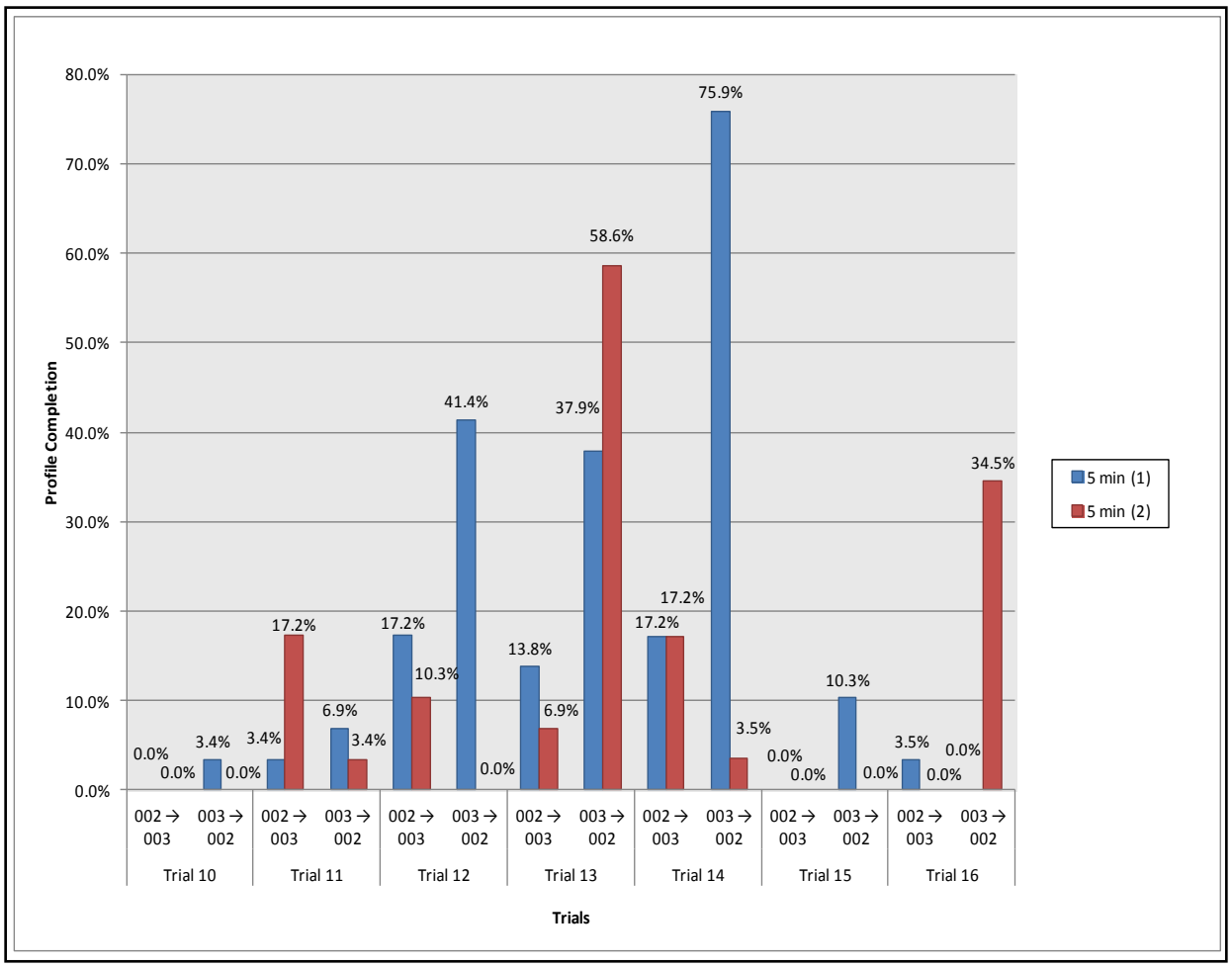


Figure 3. Tertiary Transfer Trial Profile Percentage – Subject 003. The percentages of alleles in subject 003's genetic profile are displayed for each replicate in each trial. The order of transfer used in each trial is shown on the X axis. Times reflect duration of drying prior to transfer event. Trial 10 was the transfer of saliva on bare thumbs to plastic tubes and then to palms; Trial 11 represented the transfer of saliva on bare thumbs to plastic tubes and then to moistened palms; Trial 12 was the transfer of saliva on pen surfaces to palms and then to plastic tubes; Trial 13 was the transfer of saliva on moistened bare thumbs to plastic tubes and then to palms; Trial 14 was the transfer of saliva on pen surfaces to moistened palms and then to plastic tubes; Trial 15 was the transfer of saliva on gloved thumbs to plastic tubes and then to palms; Trial 16 represented the transfer of saliva on gloved thumbs to plastic tubes and then to moistened palms.

		Primary Depositor						Secondary Depositor					
		5 min (1)			5 min (2)			5 min (1)			5 min (2)		
Trial 10	001 → 004	0.0%	(-)	[-]	0.0%	(-)	[-]	100.0%	(1)	[468 RFU]	100.0%	(2)	[513 RFU]
	002 → 003	-	-	-	-	-	-	-	-	-	-	-	-
	003 → 002	-	-	-	0.0%	(-)	[-]	-	-	-	100.0%	(1)	[213 RFU]
	004 → 001	-	-	-	0.0%	(-)	[-]	-	-	-	100.0%	(5)	[456 RFU]
Trial 11	001 → 004	10.9%	(1)	[344 RFU]	50.0%	(1)	[334 RFU]	89.1%	(5)	[572 RFU]	50.0%	(1)	[435 RFU]
	002 → 003	0.0%	(-)	[-]	0.0%	(-)	[-]	100.0%	(1)	[982 RFU]	100	(4)	[764 RFU]
	003 → 002	**100.0%**	**(1)**	**[413 RFU]**	-	-	-	0.0%	(-)	[-]	-	-	-
	004 → 001	0.0%	(-)	[-]	0.0%	(-)	[-]	100.0%	(7)	[1441 RFU]	100.0%	(9)	[999 RFU]
Trial 12	001 → 004	**100.0%**	**(2)**	**[676 RFU]**	**100.0%**	**(9)**	**[1099 RFU]**	0.0%	(-)	[-]	0.0%	(-)	[-]
	002 → 003	**100.0%**	**(9)**	**[990 RFU]**	**100.0%**	**(2)**	**[630 RFU]**	0.0%	(-)	[-]	0.0%	(-)	[-]
	003 → 002	**100.0%**	**(6)**	**[1002 RFU]**	0.0%	(-)	[-]	0.0%	(-)	[-]	100.0%	(1)	[462 RFU]
	004 → 001	**100.0%**	**(7)**	**[947 RFU]**	**100.0%**	**(8)**	**[3371 RFU]**	0.0%	(-)	[-]	0.0%	(-)	[-]
Trial 13	001 → 004	**100.0%**	**(2)**	**[1241 RFU]**	**100.0%**	**(3)**	**[828 RFU]**	0.0%	(-)	[-]	0.0%	(-)	[-]
	002 → 003	**81.3%**	**(2)**	**[857 RFU]**	33.3%	(1)	[869 RFU]	18.7%	(1)	[289 RFU]	66.6%	(2)	[471 RFU]
	003 → 002	**87.5%**	**(7)**	**[620 RFU]**	**100.0%**	**(8)**	**[1037 RFU]**	12.5%	(1)	[650 RFU]	0.0%	(-)	[-]
	004 → 001	-	-	-	0.0%	(-)	[-]	-	-	-	100.0%	(8)	[596 RFU]
Trial 14	001 → 004	**100.0%**	**(6)**	**[742 RFU]**	**100.0%**	**(7)**	**[3169 RFU]**	0.0%	(-)	[-]	0.0%	(-)	[-]
	002 → 003	**100.0%**	**(6)**	**[900 RFU]**	**100.0%**	**(9)**	**[902 RFU]**	0.0%	(-)	[-]	0.0%	(-)	[-]
	003 → 002	**100.0%**	**(13)**	**[1370 RFU]**	-	-	-	0.0%	(-)	[-]	-	-	-
	004 → 001	**100.0%**	**(1)**	**[356 RFU]**	**100.0%**	**(2)**	**[523 RFU]**	0.0%	(-)	[-]	0.0%	(-)	[-]
Trial 15	001 → 004	-	-	-	-	-	-	-	-	-	-	-	-
	002 → 003	-	-	-	-	-	-	-	-	-	-	-	-
	003 → 002	0.0%	(-)	[-]	-	-	-	100.0%	(1)	[289 RFU]	-	-	-
	004 → 001	0.0%	(-)	[-]	0.0%	(-)	[-]	100.0%	(7)	[1348 RFU]	100.0%	(7)	[1012 RFU]
Trial 16	001 → 004	-	-	-	-	-	-	-	-	-	-	-	-
	002 → 003	0.0%	(-)	[-]	-	-	-	100.0%	(1)	[662 RFU]	-	-	-
	003 → 002	-	-	-	0.0%	(-)	[-]	-	-	-	100.0%	(11)	[2265 RFU]
	004 → 001	0.0%	(-)	[-]	-	-	-	100.0%	(2)	[660 RFU]	-	-	-

Table 5. Major/Minor Contributor Percentages (Peak Height) – Trials 10-16 (34 PCR Cycles). The calculated percentages of contribution to the DNA mixture by each individual's unique alleles, based on peak height data, are listed for each replicate in each trial. The numbers of unique alleles observed are listed in parentheses. The average peak heights are listed in brackets. The orders of transfer for each pair of subjects in the trials are indicated. Instances in which the primary depositor was shown to be the major contributor of DNA to the mixture are displayed in bold. Times reflect duration of drying prior to transfer event. Trial 10 was the transfer of saliva on bare thumbs to plastic tubes and then to palms; Trial 11 represented the transfer of saliva on bare thumbs to plastic tubes and then to moistened palms; Trial 12 was the transfer of saliva on pen surfaces to palms and then to plastic tubes; Trial 13 was the transfer of saliva on moistened bare thumbs to plastic tubes and then to palms; Trial 14 was the transfer of saliva on pen surfaces to moistened palms and then to plastic tubes; Trial 15 was the transfer of saliva on gloved thumbs to plastic tubes and then to palms; Trial 16 represented the transfer of saliva on gloved thumbs to plastic tubes, and then to moistened palms.

CONCLUSIONS

Previous studies on DNA transfer events have focused primarily on the transfer of DNA found in sloughed off epithelial cells from individuals' palms. In these cases, the major contributor to the resulting DNA mixture often was shown to be the last person to come in contact with the tested object. The data herein support that there is notable loss of DNA with each transfer event. In addition, the results of this study indicate that when saliva is the original source of the transferred DNA, the initial contributor's genetic material can comprise the majority of the resulting mixture. The presence of moisture during the transfer event, as well as the texture and surface area of the object(s) to which the DNA was transferred were factors contributing to this phenomenon. Also, the results of the tertiary transfer trials indicate that the presence of moisture during the initial deposition of DNA from the primary source plays a more substantial role in the transfer of this DNA than moisture present during subsequent transfer steps. Given that saliva is a likely source of transferred DNA, all individuals can essentially be considered "good shedders." This concept, coupled with the inherent uncertainty as to the means of DNA deposition in forensic samples, negates the relevance of shedder status consideration in low-copy number forensic analysis. Caution should be exercised when inferring that the major component of a touched DNA sample was derived from the last person to come in contact with the item. This study involved only four individuals and yet constituted a substantial amount of work. While the trends are likely to hold with an increased number of individuals being studied, additional studies are advocated. Studies of DNA transfer events using other commonly encountered sources of genetic material also would benefit the field of forensic genetics.

ACKNOWLEDGMENTS

This project was supported by Award No. 2009-DN-BX-K188, awarded by the National Institute of Justice, Office of Justice Programs, U.S. Department of Justice. The opinions, findings, and conclusions or recommendations expressed in this publication are those of the authors and do not necessarily reflect those of the Department of Justice.

We would like to sincerely thank Carey Davis and Sarah Schmedes for their assistance during this study. We are also grateful to the volunteers who generously provided samples for this research.

REFERENCES

1. Gill P, Jeffreys AJ, Werrett DJ. Forensic application of DNA "fingerprints". Nature 1985 12;318:577-579.

2. Sweet D, Lorente JA, Valenzuela A. PCR-based DNA typing of saliva stains recovered from human skin. J Forensic Sci 1997 5;42:447-451.

3. Higuchi R, von Beroldingen CH, Sensabaugh GF, Erlich HA. DNA typing from single hairs. Nature 1988 4;332:543-546.

4. Hochmeister MN, Budowle B, Borer UV, Eggmann U, Comey CT, Dirnhofer R. Typing of deoxyribonucleic acid (DNA) extracted from compact bone from human remains. J Forensic Sci 1991 11;36(6):1649-1661.

5. van Oorschot RAH, Jones MK. DNA fingerprints from fingerprints. Nature 1997 10;387:767.

6. 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 Congr Ser 2006 4;1288:651-3.

7. Staiti N, Romano C, Trapani C, Ginestra E, Leo B, Schiavone S. Analysis of LCN DNA from synthetic ropes: a practical approach used in real homicide investigation. Forensic Sci Int Genet Suppl 2008 8;1(1):446-447.

8. Tokutomi T, Takada Y, Kanetake J, Mukaida M. Identification using DNA from skin contact: case reports. Leg Med (Tokyo) 2009 4;11:S576-S577.

9. Barbaro A, Cormaci P, Barbaro A. LCN DNA typing from touched objects. Int Congr Ser 2006 4;1288:553-555.

10. Lowe A, Murray C, Whitaker J, Tully G, Gill P. The propensity of individuals to deposit DNA and secondary transfer of low level DNA from individuals to inert surfaces. Forensic Sci Int 2002 9;129:25-34.

11. Phipps M, Petricevic S. The tendency of individuals to transfer DNA to handled items. Forensic Sci Int 2007 5;168:162-168.

12. Lowe A, Murray C, Richardson P, Wivell R, Gill P, Tully G, Whitaker J. Use of low copy number DNA in forensic inference. Int Congr Ser 2003 1;1239:799-801.

13. Djuric M, Varljen T, Stanojevic A, Stojkovic O. DNA typing from handled items. Forensic Sci Int Genet Suppl 2008 8;1:411-412.

14. Horsman-Hall KM, Orihuela Y, Karczynski SL, Davis AL, Ban JD, Greenspoon SA. Development of STR profiles from firearms and fired cartridge cases. Forensic Sci Int Genet 2009 9;3:242-250.

15. Dieltjes P, Mieremet R, Zuniga S, Kraaijenbrink T, Pijpe J, de Knijff P. A sensitive method to extract DNA from biological traces present on ammunition for the purpose of genetic profiling. Int J Legal Med 2010 7;125:597-602.

16. Sewell J, Quinones I, Ames C, Multaney B, Curtis S, Seeboruth H, Moore S, Daniel B. Recovery of DNA and fingerprints from touched documents. Forensic Sci Int Genet 2008 9;2:281-285.

17. Petricevic SF, Bright JA, Cockerton SL. DNA profiling of trace DNA recovered from bedding. Forensic Sci Int 2006 5;159:21-26.

18. Farmen RK, Jaghø R, Cortez P, Frøyland ES. Assessment of individual shedder status and implication for secondary transfer. Forensic Sci Int Genet Suppl 2008 8;1:415-417.

19. Goray M, Eken E, Mitchell RJ, van Ooorschot RAH. Secondary DNA transfer of biological substances under varying test conditions. Forensic Sci Int Genet 2010 2;4(2):62-67.

20. Rutty GN. An investigation into the transference and survivability of human DNA following simulated manual strangulation with consideration of the problem of third party contamination. Int J Legal Med 2002 6;116:170-173.

21. Wiegand P, Heimbold C, Klein R, Immel U, Stiller D, Klintschar M. Transfer of biological stains from different surfaces. Int J Leg Med 2011 9;125:727-731.

22. 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 5;47(3):442-450.

23. Ladd C, Adamowicz MS, Bourke MT, Scherczinger CA, Lee HC. A systematic analysis of secondary DNA transfer. J Forensic Sci 1999 11;44:1270-1272.

24. Walsh DJ, Corey AC, Cotton RW, Forman L, Herrin GL, Word CJ, Garner DD. Isolation of deoxyribonucleic acid (DNA) from saliva and forensic science samples containing saliva. J Forensic Sci 1992 3;37:387-395.

25. Quinque D, Kittler R, Kayser M, Stoneking M, Nasidze I. Evaluation of saliva as a source of human DNA for population and association studies. Anal Biochem 2006 6;353:272-277.

26. Hochmeister MN, Budowle B, Jung J, Borer UV, Comey CT, Dirnhofer R. PCR-based typing of DNA extracted from cigarette butts. Int J Legal Med 1991;104:229-233.

27. Pang BCM, Cheung BKK. Double swab technique for collecting touched evidence. Leg Med 2007 7;9(4):181-184.

28. Budowle B, Eisenberg AJ, van Daal A. Validity of low copy number typing and applications to forensic science. Croat Med J 2009 4;50(3):207-217.

29. Balding DJ, Buckleton J. Interpreting low template DNA profiles. Forensic Science International: Genetics. 2009 12;4(1):1–10.

CHAPTER 7

Recommendations for a Statistical Approach for a Robust Probability Model for LCN DNA Profile Interpretation

DNA Interpretation: Steele and Balding (1) stated "because there is no clear distinction between LTDNA and standard DNA profiling, any method of analysis for LTDNA profiles should return the same results as would a standard analysis when presented with profiles obtained using optimal DNA template." For LCN DNA analysis, however, the recovered DNA profiles often are difficult to interpret due to greater complexity and are especially problematic for mixed profiles (2-5). One of the most important tasks for a DNA analyst is the interpretation of DNA profile results. The main challenges are due to stochastic effects of the PCR:

1. Heterozygote imbalance – allele pairs are more prone to imbalance, and the variance in the distribution of the observed ratio of peak heights is increased, which means that standard interpretation guidelines (applicable for single source profiles generated from greater than 100 pg of template DNA) would no longer apply.
2. Allele dropout – Sampling and amplification stochastic effects with low level template can result in the failure of one or both alleles to amplify or produce a peak that does not exceed a defined threshold level.
3. Increased stutter peaks – stutter peaks are often relatively larger than those observed with higher template quantities.
4. Contamination (i.e., allele dropin) – peaks which cannot be identified as artifacts, but do not reside in the sample. Their origin can be postulated to derive from extraneous DNA present in the laboratory, in the sample matrix or introduced during collection and transfer of evidence.

If true DNA alleles can dropout and spurious alleles can dropin, one should ask how can such evidence ever be correctly interpreted? For some, the answer to this question is that probabilistic models can approximate the events in a particular case and such analyses may be informative. In some cases, computer software has been developed for in-house use or made available on the internet (6-11). These programs are based on two general approaches for the interpretation of LCN profiles: a biological model and a statistical model.

Biological Model: The biological model exploits replicate analyses; that is, the sample is divided into two or more aliquots and "independently" analyzed with a LCN process. A "consensus" profile containing "true" alleles then is determined by observation of alleles presenting in two or more aliquots (e.g., a specific allele is observed at least twice in three aliquots). The consensus profiling method was introduced to overcome the exaggerated stochastic effects associated with low copy number DNA typing (12), but more so to reduce allele dropin effects. The theory behind replicate analyses is that if contamination occurs rarely and randomly, then observing an allele multiple times increases confidence that it is an allele truly from the sample. The biological method is particularly useful for the elimination of non-repeating spurious alleles that appear in a profile as a result of allele dropin and it is relatively easy to apply (13-16). In addition, simple mathematical logic can be used to account for alleles observed in multiple replicates to make the interpretation process easier. Various studies have described the use of the biological model in LCN analysis (13, 14, 16-18).

The biological model is not without criticism. Some scientists have suggested that splitting an already low level sample into multiple aliquots increases the stochastic effects generated in LCN typing. As a result, differences are likely to be seen in replicates of the "same"

sample (19). Additionally, by creating a consensus profile, valuable information may be lost. In fact, Forster *et al.* (15) reported the loss of approximately one-third of the alleles obtained for trace DNA quantities amplified under two different conditions (condition 'C': 28 cycles, 2 µL DNA extract, Post-PCR clean up with MinElute, 30 s injection time, and 4kV; condition 'D': 34 cycles, 1 µL DNA extract, no Post-PCR clean up, 10 s injection time, and 3kV). Critics of the biological model instead have advocated concentrating LCN DNA samples and analysis in a single sample rather than diluting and splitting for replicate analyses (19).

Advocates of the biological model maintain that a loss of reproducibility is the normal result of LCN DNA profiling (20, 21). Gill and Buckleton (21) stated, without any data to support their contention, that replicate analyses are preferable to concentrating a sample as this would not usually increase the overall quantity of DNA above the 100 pg stochastic threshold, and stochastic effects still are expected to occur in non-replicated samples. Pfeifer *et al.* (22) advocated replication to overcome interpretation problems inherent in LCN DNA profiles. Yet, little empirical evidence exists that shows that splitting a LCN DNA extract and creating a consensus profile produces a more accurate STR profile than a concentrated single analysis of a LCN DNA sample or vice versa. In an effort to address this gap, Grisedale and van Daal (12) investigated the quality of consensus profiles compared with profiles obtained using the entire low template extract for amplification. They concluded that consensus profiling may not produce the most informative DNA profile for LCN DNA samples. Their study did not quiet advocates of replicate analysis. Kokshoorn and Blankers (23) were quick to respond to the study, stating that replicate analyses and consensus profiling of LCN DNA samples was best in terms of reliability and objectivity. In their response, Grisedale and van Daal (24) agreed that while the consensus approach does have benefits to LCN DNA typing, particularly in eliminating non-

repeating spurious alleles from the final profile, with the development of statistical models that can accommodate stochastic effects and allele dropin, it may be beneficial to perform a single amplification with three times the amount of template, due to loss of profile information with the consensus approach.

There are other limitations to the biological model to consider. Not all of the information, or alleles, presented in each profile are considered and indeed some information is ignored. In addition, since a stutter peak under LCN conditions can sometimes be greater in height than stutter defined by standard STR typing, the impact of stutter needs to be considered. In fact, in recent years, several groups have sought to include the probability of stutter in their models or to develop stutter peak height models (17, 25-27). Gill *et al.* (17) compensated for stutter by treating stutter alleles as real alleles and including them in the LR calculation. Balding and Buckleton (26) proposed weighted averages of the LR numerator and denominator, similar to an earlier Gill *et al.* (28) approach. Bright *et al.* (29) and Kelly *et al.* (25) have described a series of models that may be used for the calculation of expected values for allele and stutter peak heights and the stutter ratio (ratio of allelic peak height to stutter peak height).

The New York City OCME's FST includes stutter as well but, only as a definition of dropin (7). FST's dropin definition includes stutter as well as extraneous peaks that are not in a stutter position and is modelled as the author's state, "it is never possible to distinguish with certainty whether an extraneous allele in stutter position is actually stutter or not."

Alternatives to the original biological model have been suggested, such as generating a composite profile that includes all alleles seen in the replicate profiles (30) or pooling the sample aliquots post-PCR for a single capillary electrophoresis (CE) injection (14). Yet, it is clear that a

more sophisticated model is needed to address the confidence of consensus/replicate calls and the probabilities of dropin, dropout, and stutter.

Statistical Model: The statistical model incorporates uncertain events (i.e., stochastic effects and contamination) to assess the probability of a LCN profile. The Bayesian approach provides a posterior probability regarding the source, when data in hand are considered with a prior probability of the knowledge of the source, and adds to the information obtained with the likelihood ratio. The Bayesian method uses all of the information in the profile and provides an assessment of the strength of evidence based on what has actually been observed (the electropherograms themselves) rather than what has been deduced (and which alleles are thought to be associated with the donor) (31). The Bayesian approach often is used to apply model parameters, enable predictions about unknown variables, and to perform model selection (32, 33).

LIKELIHOOD RATIO (LR):

When a crime has been committed, the evidence in the case is denoted with E. This evidence may be a blood stain on the jacket of the suspect or a blood stained fingerprint left behind at the crime scene. An explanation is needed to describe the evidence. Two explanations are of interest, a hypothesis (H) from the prosecutor, H_P and one hypothesis from the defense, H_D. Judges, jurors, and other parties are interested in the answer to the question: Which hypothesis (H_P or H_D) is more supported, given the evidence? The likelihood ratio (LR) is a measure of the value of evidence regarding the two hypotheses. It indicates the extent to which the evidence is in favor of one hypothesis over the other.

So,

$$LR = \frac{P(E \mid H_P)}{P(E \mid H_D)}$$

If H_P is the hypothesis that the suspect is the contributor to the evidentiary profile and H_D is the hypothesis that the suspect is not the contributor, we can summarize the results as follows:

- If the LR is greater than 1, the evidence is in favor of H_P, it is reported as X times more likely to find the evidence E when H_P is true, than when H_D is true.
- If the LR is less than 1, the evidence is in favor of H_D, and it is reported as X times more likely to find the evidence E when H_D is true, than when H_P is true.
- If the LR is 1, the evidence favors neither H_P nor H_D; it is reported as equally likely.

In the Bayesian approach, with the use of prior probability, the LR is transformed to obtain the relative odds of one hypothesis against another given the DNA data of the evidence (and that from known persons tested). The Bayes' formula can be described as the following formula:

$$\frac{P(H_P \mid E)}{P(H_D \mid E)} = \frac{P(E \mid H_P)}{P(E \mid H_D)} \times \frac{P(H_P)}{P(H_D)}$$

In other words, the Bayes' formula is the LR multiplied by the prior odds.

Random Man Not Excluded: The major alternative to the LR approach is to report the combined inclusion probability, often referred to as random man not excluded (RMNE) probability. This approach simplifies the DNA profile evidence to the observation that an individual of interest, Q, cannot be excluded from having contributed DNA to the evidentiary

profile. Thus, the RMNE uses only the evidentiary profile. The RMNE does not require the number of contributors to be specified and it is perceived to be easier to explain in court.

However, Balding and Buckleton (26) rejected the RMNE approach as inappropriate for addressing allele dropout and stated, it "can potentially lead to serious misrepresentation of the evidence," the New York City OCME continued to use RMNE for several years. The modest value of the RMNE has to be interpreted with the caution that not all included persons together explain the whole evidentiary sample. Moreover, allele dropout and dropin make the concept of inclusion difficult to define. With the LR, more information is used so that the statistical weight becomes stronger but it requires more assumptions, such as the number of contributors.

With the LCN statistical model, the modeling should incorporate each of the events that may occur during the process at all loci no matter what the profile of the suspect is. The probabilities of allele dropout, allele dropin, stutter, and contamination and all possible genotypes should be considered. The most difficult, and most critical, aspects of the model to satisfy are measuring the features or parameters of the stochastic events during PCR and determining whether these parameters are independent. These parameters are related to the starting amount and quality of DNA, the protocol, instrument, commercial kits, and circumstantial factors such as mixtures, but are mostly dependent on the results obtained.

It is known in some cases that these parameters are not independent from locus to locus or allele to allele. A recent study by Puch-Solis *et al*. (34) showed that both mean peak heights and stutter percentages depend on marker and fragment length, and may to some degree, be influenced by dye channel. There are additional considerations for mixtures, in that each of the component contributors may be below the LCN DNA quantitative threshold of, for example, 200 pg. Thus, the amount of DNA from each contributor is unknown. Thus, all components of the

mixture may be subject to increased stochastic effects. In fact, each contributor to the profile likely will have a different dropout rate. If these parameters are not properly considered, a scientist could misinterpret the evidence leading to an improper weighting of the evidence, which could then potentially misinform a fact finder.

A number of statistical methods have become available for LCN DNA STR profile analysis. **Table 1** summarizes the key features of available software programs, and the programs are briefly discussed below.

LoComatioN: Gill *et al*. (6, 17) addressed the difficulties in interpretation of DNA profiles recovered from LCN typing using a LR approach. Gill *et al.* (6) defines the words dropin and contamination as one entity, and states that dropin can be distinguished as one or two additional alleles. Yet, some dropin events may reflect slippage during the PCR process (stutter), but may also be due to contamination of the sample before the PCR process. Locomation uses a 'Q' designation for the dropout of an unknown allele. Say for example that at the TH01 locus, the crime stain profile has an allele of 11 and the suspect's profile is 9, 11. For the prosecution's hypothesis to be true, there would have been dropout of the suspect's allele 9. The Q designation would be used, where $P(Q) = 1 - p_{11}$. Gill *et al.* (35) stated that in the absence of degradation, it would be reasonable to assume that the probability of allele dropout, P(D), is independent of the locus (i.e. constant across all loci). This statement demonstrates a lack of understanding of the effect of inhibition in addition to sampling effects of low copy DNA. LoComatioN employs a probability of contamination, P(C), based on laboratory records of contamination in negative controls and previous work by Gill and Kirkham (18). The use of negative controls to estimate P(C) is discussed in more detail in a later section.

Table 1. Summary of key features of software programs available for low copy number DNA STR profile analysis.

Software Program	Main Contributors	Description:	Model	Open Source	Model Parameter Estimates	Reference(s)
Forensim	Haned and Gill	R package for forensic DNA profile simulation and analysis that includes functions to compute LTDNA LRs in a manner similar to LoComatioN.	Discrete (Particular Values not Range of Values)	Yes -- http://forensim.r-forge.r-project.org	Requires user-supplied estimates for the model parameters (dropout, dropin, and Fst)	11
LoComatioN	Forensic Science Service	Probabilistic model that evaluates LRs, conditioned on the probability of allelic dropout and dropin.	Discrete/Semi-continuous	Not made available commercially or as freeware	Requires user-supplied estimates for the model parameters (dropout, dropin, and Fst)	6
Forensic Statistical Tool (FST)	New York City OCME	Model allows for allelic dropout and drop-in. FST can be used for single source samples and for mixtures of DNA from two or three contributors, with or without known contributors.	Discrete	Intend to make it more widely available (currently not transportable outside OCME)	Uses empirical estimates generated from in-house training data	7
likeLTD	Balding and Buckleton	R package that maximizes likelihoods over the model parameters	Discrete	Yes -- https://sites.google.com/site/baldingstatisticalgenetics/software/likeltd-r-forensic-dna-r-code	Maximizes likelihoods over the model parameters (avoids need to specify prior distributions)	26, 38
TrueAllele	Perlin	Models background noise, allowing it to use peak heights at all allelic positions without considering dropout	Continuous	No	Prior distribution is assumed for all unknowns	8, 9
LCstat	Benschop	Enables the automated analysis of Gene-Mapper allele data for generating consensus profiles.	N/A	Yes -- http://www.liacs.nl/~hmeiland/projects/lcstat/	N/A	16,37
Lab Retriever	Lohmueller, Rudin, and Inman	Probabilistic Approach that employs LRs incorporating the P(Do)	Discrete	Yes -- http://scieg.org/lab_retriever.html	Uses empirical validation data from a government laboratory	36
STRmix	Bright, Taylor, Buckleton	Uses standard statistical methods and a MCMC engine, modelling the height of both allelic and stutter peaks. Model parameters include degradation and amplification efficiency for each locus.	Continuous	For Purchase: http://strmix.esr.cri.nz/	Uses empirical estimates	25, 27, 29

Using these various calculations to determine the probabilities of contamination, stutter, and allelic dropout, Gill *et al*. were the first to describe a statistical method to apply to the evaluation of LCN DNA profiles, LoComatioN. However, even 14 years later, this method and software are not implemented. Gill and Buckleton (21) explained this delay was due to lack of business interest and stated, "It is of course disappointing that nearly a decade later, vendors still have not developed commercial solutions based on our statistical thinking." However, it is not due to a lack of business interest, but the fact that the software may not translate into practical casework conditions.

TrueAllele®: The TrueAllele system is based on linear mixture analysis (LMA), a mathematical method for resolving DNA mixtures. A TrueAllele analysis module first processes the signal data (removes artifacts, classifies peaks, etc.) TrueAllele then interprets the data using a probability model which incorporates many different variables, including genotypes of contributors, DNA quantities, amplification artifacts, and the uncertainties of these variables. TrueAllele then uses Markov Chain Monte Carlo (MCMC) statistical sampling. TrueAllele was designed to take into account the possibility of the phenomena of allelic dropout, allelic dropin, and stutter. Yet, whether the system accurately estimates the probability of these phenomena occurring in a particular case is uncertain. As with any type of computer modeling, the accuracy of TrueAllele depends, in part, on the accuracy of the underlying assumptions (36). Equally important is the question: are the results of TrueAllele reproducible? In other words, does the system produce the same LR each time it is run on the same profile? The answer is no. Because there are random elements in the way the system does its modeling, such as the random choice of which hypotheses to consider, in which order, no two computer runs will be exactly the same

(37). Also, TrueAllele may delete inconsistent information without reducing the weight of the evidence.

likeLTD: A more advanced approach compared with that introduced by Gill *et al*. was described by Balding and Buckleton (26, 38) and is freely available at https://sites.google.com/site/baldingstatisticalgenetics/software/likeltd-r-forensic-dna-r-code. The model is used for interpreting low template DNA (LTD) mixtures where peaks are classified as present, in stutter position, or masking. The set of masking alleles is defined as every peak above a designated threshold that either corresponds to an allele of a known contributor, or is in a stutter position to that allele. Alleles are defined as present, absent, or uncertain; dropout is modelled using in general the approach of Tvedebrink *et al.* (39, 40). However, this model has two important limitations: it does not use peak height information, only the presence or absence of peaks, and assumes a dropout rate for a given allele that is the same over all loci. Peak heights yield more information for interpretation – that is to say, one can use peak height values to infer a probability of dropout. An estimate of dropout without taking into account peak height values could seriously misrepresent the data. It is unclear if this software is being used by any crime laboratories for casework.

Forensim: In 2011, Haned and Gill (11) introduced Forensim, a freely available software (http://forensim.r-forge.r-project.org) that enables the calculation of LRs for complex STR profiles and incorporates allele dropin and dropout rates for multiple contributors and multiple replicates. For this method a software package was written in R. However, a downside of the method is that it does not use the peak heights and that it needs an expert opinion on the allele dropout probability. The problem of the model not using peak height data was discussed in the previous model. The need of expert opinion raises two issues. The first, that the expert is

properly trained to interpret DNA profiles. The second, that the expert is unbiased in his/her approach to interpret DNA profiles. In essence, Forensim depends on an analyst's subjective judgment of the profile which creates room for expert disagreement.

LabRetriever: Lohmueller, *et al.* released "LabRetriever", a freely available software tool (http://scieg.org/lab_retriever.html) that can be used to calculate LRs and incorporates a probability of dropout P(D) (41). It is based on the propositions of Balding and Buckleton. The P(D) is calculated using average peak height of all visible peaks or "those in the relevant component" of a mixed sample. LabRetriever uses a universal $P(D_o)$ derived from NIST data.

When testing this software using casework data with known allele dropout, I applied the average peak height to the excel workbook (downloaded from http://scieg.org/lab_retriever.html). The average peak height value for the evidentiary sample was 688 RFU. **Figure 1** shows the screen images from the excel calculation. The result I obtained was a P(D) of zero, even though these data presented with allele dropout (Figure 1).

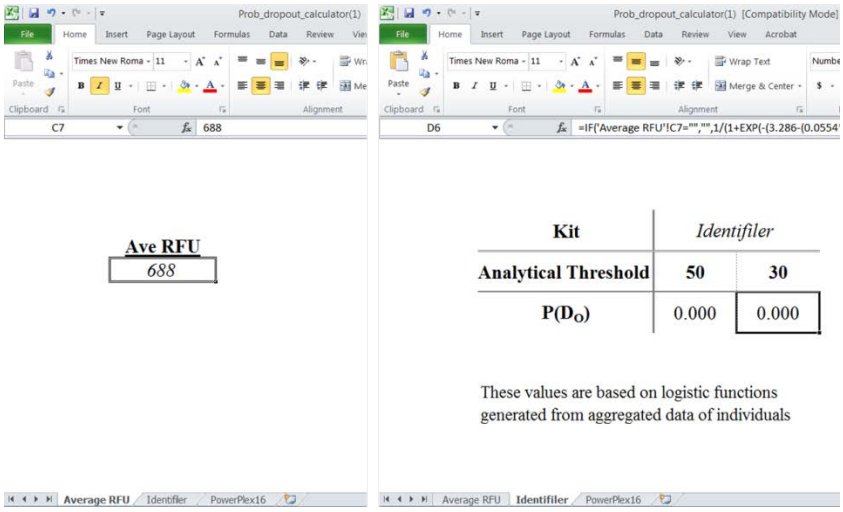


Figure 1. Probability dropout calculator downloaded from LabRetriever software program. Average RFUs (688) for evidentiary sample analyzed using the Identifiler kit were plugged into the calculator and a $P(D_o)$ was calculated as zero.

Table 2. Detected Alleles for Victim, Suspect, and Evidence and Likelihood Ratios for Challenged Sample Data Loaded into LabRetriever software program.

Locus	Detected	Unattributed	Assumed	Suspected	Likelihood Ratio			
	Blank LabRet datafile | Evidence		Blank LabRet datafile | Victim	Blank LabRet datafile | Suspect	Locus	AFRICAN _AMERICAN	CAUCASIAN	HISPANIC
D8	10 14 15	10	14 15	13	D8	1.99E-04	2.15E-04	1.93E-04
D21	29 32	32	28 29	29 33 2	D21	2.96E-04	2.99E-04	2.90E-04
D7	8 10 11 12	10 12	8 11	11 12	D7	9.38E-02	4.61E-02	4.74E-02
CSF	10		10 11	11	CSF	1.49E-01	2.06E-01	1.81E-01
D3	15 16	15	16	17 18	D3	1.57E-06	1.77E-06	1.65E-06
TH0	7 9		7 9	6 7	TH0	3.03E-02	1.01E-01	5.27E-02
D13	8 11 12 13	8 11	12 13	10 11	D13	3.66E-04	2.31E-04	3.13E-04
D16	8 10 11	8 10	11	8 10	D16	8.34E+01	2.74E+02	1.15E+02
D2	17 19 23 24	23 24	17 19	17 24	D2	7.68E-02	5.54E-02	1.01E-01
D19	12 13 15 2	13	12 15 2	13 2 14	D19	2.06E-06	2.35E-06	2.48E-06
vWA	15 16 18 19	18 19	15 16	17 18	vWA	5.39E-04	3.49E-04	4.89E-04
TPO	8 11		8 11	8 10	TPO	2.82E-02	1.66E-02	1.81E-02
D18	12 14 15	14	12 15	14 15	D18	2.13E+01	9.35E+00	8.73E+00
D5	9 11 12	9	11 12	9 12	D5	1.70E+01	1.09E+01	1.31E+01
FGA	22 23 25 26	22 26	23 25	24 26	FGA	7.92E-04	1.94E-03	1.08E-03
					total	8.23E-31	1.03E-30	4.13E-31
P(DI)	P(DO)	IBD Probabilities						
0.01	0	1	0	0				

Table 2 LabRetriever software run information. The table shows the detected alleles for victim, suspect, and evidence and the generated LRs for three reference population groups. Several different average RFU values were inserted into the probability calculator and an average RFU of less than 300 was needed in order to invoke any P(D). While LabRetriever software is using peak height value information, it is clear from this example that it is not being used in the right way. While LabRetriever uses a universal $P(D_o)$ derived from NIST data, for this software program to work laboratory specific values are necessary.

LCstat: Benschop *et al*. (16, 42) created this 'low copy statistics' program, which enables automated analysis of GeneMapper allele data for generating consensus profiles. However, this program does not take into account the peak height of the alleles. Without peak

height values, one cannot indirectly infer a probability of dropout. As previously stated, models which do not take into account peak height values could seriously misrepresent the data. Testing of the software cannot be carried out as the website provided in the manuscripts (http://www.liacs.nl/~hmeiland/projects/lcstat/) cannot be found. Hugo Meiland, the developer of LCstat, responded to a query regarding the missing link. He is changing the software server location and will update the link location accordingly.

Forensic Statistic Tool (FST): FST was created by the Office of the Chief Medical Examiner (OCME), New York City, and uses empirically estimated dropout rates that varied the number of PCR cycles, STR locus, number of contributors, and mixture ratios ("either unequal or approximately equal") (7, 14). The calibration data to estimate dropout rates used 2,000 amplifications of 700 DNA samples with between one and three contributors and between 6.25-500 pg of DNA template for single source and 25-500pg for two and three person mixtures.

A major criticism of the FST method is that the dropout rates function on the total amount of DNA. Unlike peak height values, the use of quantity of DNA as an estimate has never been considered for dropout estimation and is not reliable. Quantification methods are not senstitive enough at low levels of DNA and a quantity of 6 pg cannot be trusted. Additionally, the quantity of DNA does not provide any information on the DNA within the sample (i.e. allelic makeup). In FST, the dropout rates also function on the number of contributors (unknown in some casework samples) and the dropin rates are a function of the number of PCR cycles. Dropin is not just a result of increased PCR cycle number but can be caused by contamination (of sample from collection to the laboratory or in lab consummables).

Mitchell *et al.* (7) attempted to develop a degradation model for the software. In the study, UV light was used to degrade known samples and dropout rates were calculated.

Unfortunately, while the idea of integrating the effect of degradation had merit, Mitchell *et al.* (7) chose to ignore the study, as "use of the degradation module resulted in LRs closer to 1.0 for both true contributors and non-contributors."

There is a lack of transparency of the FST validation study for the values used for the parameters of the software and no concordance or similar study was carried out by a different laboratory. If the data are not available and if the software is not available, it cannot be tested by other laboratories and thus, will not be a generally accepted software program. One must ask, "Why is there a reluctance to share the data?"

The allele dropout and dropin rates employed by the program are specific to OCME's protocols, kits and equipment (this is true of all models which have been developed). To the OCME's credit, they realize that the application to data generated in another laboratory would require assessment and perhaps adjustment of these rates, as would alteration of OCME's current protocols, kits or equipment. Also, FST is not publicly available and cannot be tested independently but Mitchell *et al.* (7) stated that they intend to make it more widely available. But to date, it is not available.

STRmix™: STRmix™ was developed by New Zealand Crown Research Institute (ESR) and Forensic Science South Australia (FSSA). The software combines biological modelling and mathematical processes and has been written and tested by practitioners in forensic DNA interpretation (25, 27, 29). Using standard and well-established statistical methods, STRmix™ is a fully continuous model and its mathematics are accessible to DNA analysts, so results may be understood and explained.

The software analyzes a DNA profile and determines which combination of genotypes best explain the data, using standard statistical methods and a Markov Chain Monte Carlo (MCMC) engine, modelling the height of both allelic and stutter peaks. The underlying models are described in Bright *et al.* (27, 29). The model parameters of STRmix include degradation and amplification efficiency for each locus and can incorporate an F_{ST} adjustment into the LR. The software can also include multiple replicates of a sample from consensus testing. STRmix™ has been used for routine casework interpretation at ESR and FSSA since August 2012 and is now the Australasian standard for DNA interpretation. Although not open source, it is available for purchase (http://strmix.esr.cri.nz/). STRmix™ provides a good framework for many of the current problems with LCN DNA interpretation, but still needs to be subjected to peer-review.

INHERENT PROBLEMS WITH SOFTWARE MODELS: Gill and Buckleton (21) have argued that 'nothing else is required, other than to educate scientists, judges and lawyers', in the 'uses and practicalities' of LCN DNA statistical models. With this statement, Gill and Buckleton lay the blame on their own field of forensics for the failures of LCN testing and analysis. This criticism of others in the field comes even while Gill and Buckleton did not apply what they themselves had advocated. In fact, Lawless (37) stated, "this argument implies that ignorance and a lack of interest in the kind of techniques they advocate is responsible for the apparent lack of consistency between theory and practice". Underlying issues still exist with the current statistical methodologies, and addressing these issues is a better way. The various issues that need to be considered are the following:

Estimation of Dropout and Dropin: With any DNA profile, if allele dropout and/or dropin are possible (this includes any partial DNA profile), it is not possible to think only in terms of a match or non-match. The various possibilities can only be properly evaluated in

probabilistic terms by means of a LR and its inherent principles. While the LR approach is widely accepted in statistics and generally is a valid concept for the interpretation of DNA evidence, the stochastic events of LCN typing must be modeled.

To compute a LR, an estimate is made as to how probable the observed results in the evidentiary sample would be if the defendant was, and was not, a contributor. Because LR focuses on the probability of obtaining the results observed under contrasting hypotheses, it avoids the difficulty of estimating the size of the "included" group. In order to compute an accurate LR, one must know the probability that allelic dropout and/or dropin may occur (19). Without knowledge of the dropout probability, one cannot know the probability of obtaining the observed results if the defendant (H_P) or an unknown individual (H_D) was a contributor, which means one cannot accurately compute a LR.

Estimates of the probability of allele dropout or dropin may be little more than guesses but several studies have attempted to measure the uncertainty of dropin and dropout events (11, 40, 43-45). Logistic regression models were originally proposed to estimate the dropout probability (43, 45). Gill *et al*. (45) suggested that the probability of dropout could be derived from a measure of the quality of the DNA profiles based on their observed peak heights. Tvedebrink *et al*. (40, 43) illustrated how the model parameters can be estimated from experimental data, using average peak height as a covariate. Haned *et al.* (11, 44) proposed a simulation approach to evaluate the efficiency of the logistic model. These observations have provided information regarding the use of a statistical model(s) in LCN typing. However, because of multiple factors that lead to dropout and dropin, case specific estimates are difficult to obtain. While LR computations can accommodate dropout and dropin, Ge and Budowle (47) have suggested invoking these rates with ranges of values (continuous model).

Indeed, some of the models to date suffered from built-in bias by not selecting *a priori* the loci that may have experienced allele dropout or by not assuming that allele dropout and dropin can occur at any locus. These approaches used the reference profile of the suspect (or victim) to determine which loci demonstrate allele dropout or dropin, a biased approach that should not be tolerated. However, there are forensic laboratories which continue to use some of these practices in the interpretation of LCN samples.

One recommendation to avoid this bias would be the incorporation of allele dropout and dropin rates for all loci to the various components of a sample (either single source or mixtures). The incorporation of dropout and dropin rates has been advocated in recent years in several studies (6, 14, 21). Further recommendations would be the accommodation of all features or parameters of stochastic effects (i.e., dropout, stutter, peak height imbalance) be contained in a single framework, and the incorporation of the rationale that stochastic events across loci and amplicon sizes are **NOT** independent (e.g., if dropout occurs at one locus, it is more likely to occur at other loci).

LACK OF CONSENSUS ON CONSENSUS: Replicate analyses and allele redundancy have been the cornerstones of "reliability" for LCN typing (13). While presumably, the more replicates that show the same allele(s) the more reliable is the allele call(s) than those with less redundancy, the number of replicates requisite for such analyses has not been adequately defined. Benschop *et al.* (16) obtained the best results using the n/2 consensus approach, which includes alleles detected in at least half of the replicates, when at least four replicates are performed. Within this n/2 method, the authors stated that the most appropriate number of replicates for considering a profile to be a true one is four. However, Grisedale and van Daal (46) advocated that splitting aliquots reduced the amount of genetic typing results that can be

recovered and there may be a balance between confidence of allele calls and gaining the most information possible. While Pfeifer (22) recommended replicate analyses, she also stated, "generally recommending the consensus interpretation thus seems not to be justified: a more differentiated approach appears to be worthwhile, e.g. the amount of dropouts, the number of replicates, choice and combination of kits and even a marker specific procedure might be taken into account."

ACCURATE QUANTIFICATION OF DNA: The uncertainty at the heart of LCN DNA analysis is the small quantities of DNA that are analyzed. It is very difficult to accurately quantify DNA at these levels. If there are differences concerning the starting quantity of DNA, there will be problems with the quantities used in validation studies and any inferences made regarding quantity with unknown evidence samples. The minute quantities associated with LCN DNA, in the picogram range, mean that accurate and consistent assumption of quantification of samples are very difficult to attain. For example, FST was calibrated with data (quantified with an Alu-based real time PCR system) to estimate dropout rates using as little as 6.25 pg of DNA template for single source samples. Not only is the sensitivity of current quantification methods not reliable at this low level of DNA, but using such low levels of DNA leads to sampling effects within the DNA itself.

It is well known that when processing a small number of starting templates during the PCR, exaggerated stochastic sampling effects will occur. What has been less well defined, however, is that variation in quantification of template DNA, pipetting volume inaccuracies, and Poisson distribution effects can impact the amount of template DNA placed in a PCR. A question that has been posed by Ge and Budowle (47) is, how likely is it to obtain for example 33 pg in each aliquot when a 100 pg sample is split three ways? Additionally, how likely is it

that the DNA of each allele is split equally three ways? It is not just quantity of the DNA that is a concern, but the makeup of the DNA in a sample that is requisite for models.

A recent study by Ge and Budowle expanded on this issue (47). Ge and Budowle stated that the probability is high that each replicate will have equal or comparatively equal amounts of DNA and the potential is relatively equal to obtain the true genotype for each replicate with high amounts of input DNA. For low copy DNA analysis, however, the distributions of the probabilities of obtaining true genotype and dropout and dropin events will vary among replicates with LCN analysis. Consequently, the amount of DNA in each replicate may not (and in fact is more likely not to) be equal (47).

STOCHASTIC THRESHOLDS: Interpretation of DNA evidence depends upon the ability of the analyst to properly compare the DNA profile obtained from an item of evidence and the reference DNA profile of a victim or suspect. Typically, minimum amounts (0.5 – 1 ng) of DNA template are recommended for a PCR, so that stochastic effects can be reduced to manageable levels. However, since variation in the quantification of template DNA, pipetting volume inaccuracies, degradation and inhibition can impact the amount of template DNA placed in a PCR, a stochastic interpretation threshold is used instead for STR typing (19). A minimum peak height established by in-house laboratory validation studies, serves as a stochastic control. Those peaks below this threshold are not interpreted or are interpreted with extreme caution and for limited purposes.

With LCN DNA, the height of allelic peaks have no relationship with a stochastic threshold as typically all data are subjected to increased stochastic effects, unlike conventional STR typing. Since LCN typing inherently refers to the interpretation of results that would normally be below the stochastic interpretation threshold, there is no minimum peak height

criterion for interpretation that is similar to that of standard STR typing. In fact, the stochastic threshold was established and validated with typing analyses using conditions very different than those for LCN typing. Grisedale and van Daal (46) recently stated, "with the increased sensitivity of LTDNA analyses, one must accept that most, if not all, results experience substantial stochastic effects and fall below a stochastic threshold." While efforts have been made to establish a threshold for LCN typing (45, 48), the same authors cautioned that suitable interpretation guidelines also are required.

INTERPRETER BIAS*:* The interpretative issues surrounding LCN DNA profiles have been a source of controversy in forensic scientific circles since LCN techniques were first described. One issue is bias on behalf of the analyst interpreting the DNA profile. For example, invoking dropout explanations in order to include a suspect when it may have been that the alleles that allegedly dropped out were never there in the first place. All too often, interpretation is influenced by investigative narratives which are used to justify decisions concerning ambiguous data. For example, Perlin (49) described seven cases where, on the same data, human review gave appreciably different results than his advocated assessment using TrueAllele software. However, Thompson *et al.* (50) raised a concern with the use of TrueAllele software and described a recent case where TrueAllele was run on results of three separate amplifications (treating them as samples of the same underlying set of alleles), and produced three different LRs, the lowest 24,000 and the highest six billion. The prosecution expert elected not to report the LR of 24,000 and instead reported the LR of six billion, which was based on one of the three amplifications. For an unbiased approach, the expert should have provided all three LRs. Such bias is problematic and could lead to a misrepresentation or overweighting of the data.

THE DIFFERENCE BETWEEN WHAT IS RECOMMENDED AND WHAT IS PRACTICED: Contrary to the representations published in the literature, the exact manner in which LCN DNA profiling is used in casework is unclear at best: Budowle and van Daal (19) stated 'The forensic science community does not know what the practices of LCN DNA laboratories are and whether they are valid and reliable' (19). They added that there is 'a difference between what they recommended and what is practiced'. For example, we know that the Forensic Science Service (FSS) did not follow the often cited Gill *et al.* article (13) (written by FSS employees) for statistical assessment of LCN DNA evidence more than a decade after its publication. Additional criticisms were a lack of transparency of LCN methodologies and validation studies performed in individual laboratories. Without transparency, laboratories pursuing LCN analysis cannot learn from one another and share information. Also, it is unclear if the validations studies and interpretation guidelines comport. A laboratory's unwillingness to divulge their methods makes it impossible for another laboratory even to test that method. Methods can only be 'generally accepted' by the forensic community with independent testing, validation, and peer-review.

RECOMMENDATIONS: Recently, the International Society for Forensic Genetics published recommendations based on discrete models for forensic analysis of single source DNA profiles (51). They consider a single locus (DNA marker) and at that locus allow for artifacts, such as allele dropin and dropout, including the probability of their occurrence in the LR computations. Under assumptions of independence assumptions invoked in their model, they state that it readily extends to multiple loci and DNA mixtures. However, it is clear from the recommendations that it is nothing more than support for existing statistical models of some of the authors themselves.

Furthermore, it is troubling to see some statements in the recommendations, such as, “negative controls can be used to estimate the probability of drop-in within casework samples.” Yet in the same paragraph, “drop-in and contamination may have occurred even if the negative control is ‘clean’ and does not show any allele at all. To recap, the drop-in event is relatively rare and is measured by reference to negative controls.”

While negative controls are useful and do provide information regarding an individual laboratory’s contamination and the recommendation of a dropin rate for that part of the process may be appropriate, negative controls vary by lot, kit, instrument, etc. In essence, if a negative control is used for estimating dropin rate, it would have to be a new estimation with every change of lot, kit, instrument, etc. Additionally, a study of negative controls does not yield any information about possible contamination of a sample. In fact, the negative control is not a true representation of the DNA sample as it may have true contamination (which occurred sometime between deposition and getting to the laboratory) or have been placed inside a plastic tube which was not sterile.

While the ISFG does include a website link to various software tools (http://www.isfg.org/Software), there is only one paragraph that is truly a “recommendations” section of the article. These recommendations are not new. That is, many LCN DNA researchers have voiced the same recommendations since the inception of LCN DNA typing.

The recommendations are as follows:

"1. The introduction of software solutions to interpret DNA profiles must be accompanied by a validation process ensuring conformity with existing standard laboratory procedures.

2. Validation studies should be carried out to characterize drop-out and drop-in probabilities bearing in mind that these will differ between processes (some guidance is given in the appendices).

3. Open-source is strongly encouraged since this solution offers unrestricted peer review and best assurance that methods are fit for purpose.

4. Internal laboratory policies are necessary in order to address the quality of the data that will be required to attempt a comparative interpretation.

5. Strict anti-contamination procedures must be established to minimize the introduction of any additional levels of uncertainty. Software tools used for casework implementation must be evaluated with known samples and each laboratory will have to establish reporting guidelines and testimony training to properly present the results to courts."

MY RECOMMENDATIONS: While I support the above ISFG recommendations, it is clear that statistical interpretations, and supporting data for probabilities, need to be defined better and developed to convey the uncertainty associated with LCN DNA analyses. Balding and Buckleton (26) have provided, in my opinion, a good initial interpretation strategy that can be built upon. The parameters from their study are described below.

- Dropout Probabilities: P(1−D), P(D), and P(D^2) represent the probabilities of no dropout, partial dropout, and complete dropout, respectively and D_2 represents complete dropout of a homozygote at a locus. For all of these probabilities, the notation of a line above, such as P($\overline{D}$), represents the absence of dropout. Balding and Buckleton (26) assume dropout is independent across two alleles of a heterozygote and also across loci but note that the parameters are readily extended to allow D to vary over alleles. The authors also note that the value of D at a locus could depend on the peak height, and could increase with allele length.
- Dropin Probability: P(C) represents probability of dropin. Balding and Buckleton (26) make the assumption that at most, one dropin occurs per locus, but note that this restriction can be relaxed.
- Stutter: Balding and Buckleton (26) proposed weighted averages of LR numerator and denominator for stutter.
- Masking of Alleles: In the presence of masking, Balding and Buckleton (26) noted that the 2p rule has a substantial effect, overstating the LR. For the 2p rule, the allele frequency estimate is doubled for a single reported allele even when dropout is possible. The authors assign probabilities for masking of alleles, P(M).
- Use of F_{ST} to allow for remote shared ancestry between the suspect and other possible contributors to the evidentiary profile.
- LR: All of these parameters are incorporated into a LR. All possibilities are addressed in a single LR.

The framework set in place by Balding and Buckleton is a good starting point. Two things need to be further addressed in this method, the assumption of independence and the lack of the use of peak height information. Additionally, more empirical data needs to be made available to estimate the rates of dropout, dropin, contamination, and stutter for LCN DNA, in order to test recommended models. I would make the recommendation that multiple dropins be interpreted as an additional unknown contributor. Lastly, the estimate of dropout rates can be quite variable for a given amount of LCN DNA and therefore, it may be better to provide a range of LRs to accommodate such variation.

More sophisticated interpretation guidelines are needed that address the issues raised in this chapter. Factors that impact the reliability of allele designation and statistical weight assessment should be incorporated into a guideline for the human identity testing community so that the degree of confidence can be properly conveyed and those making identifications will be properly informed.

REFERENCES

1. Steele CD, Balding DJ. Statistical evaluation of forensic DNA profile evidence. Annu.Rev.Stat.Appl. 2014;1:20.1,20.24.

2. Cowell RG, Lauritzen SL, Mortera J. Probabilistic expert systems for handling artifacts in complex DNA mixtures. Forensic Science International: Genetics. 2011 6;5(3):202-9.

3. Weusten J, Herbergs J. A stochastic model of the processes in PCR based amplification of STR DNA in forensic applications. Forensic Science International: Genetics. 2012;6(1):17-25.

4. Michel S, De Bast A, Vandenbroere I, Froment O. Interpretation of low-copy-number DNA profile after post-PCR purification. Forensic Science International: Genetics Supplement Series. 2009;2(1):542-3.

5. Leclair B, Fregeau CJ, Bowen KL, Fourney RM. Systematic analysis of stutter percentages and allele peak height and peak area ratios at heterozygous STR loci for forensic casework and database samples. J Forensic Sci. 2004 9;49(5):968-80.

6. Gill P, Kirkham A, Curran J. LoComatioN: A software tool for the analysis of low copy number DNA profiles. Forensic Sci Int. 2007 3;166(2-3):128-38.

7. Mitchell AA, Tamariz J, O'Connell K, Ducasse N, Budimlija Z, Prinz M, Caragine T. Validation of a DNA mixture statistics tool incorporating allelic drop-out and drop-in. Forensic Science International: Genetics. 2012;6(6):749-61.

8. Perlin MW, Legler MM, Spencer CE, Smith JL, Allan WP, Belrose JL, Duceman BW. Validating TrueAllele® DNA mixture interpretation*,†. J Forensic Sci. 2011;56(6):1430-47.

9. Perlin MW, Szabady B. Linear mixture analysis: A mathematical approach to resolving mixed DNA samples. J Forensic Sci. 2001;46(6):1372-8.

10. Kelly H, Bright J, Buckleton JS, Curran JM. A comparison of statistical models for the analysis of complex forensic DNA profiles. Science & Justice. 2014 1;54(1):66-70.

11. Hinda H. Forensim: An open-source initiative for the evaluation of statistical methods in forensic genetics. Forensic Science International: Genetics. 2011 8;5(4):265-8.

12. Grisedale KS, van Daal A. Comparison of STR profiling from low template DNA extracts with and without the consensus profiling method. Investig Genet. 2012 7;3(1):14.

13. 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 7;112(1):17-40.

14. Caragine T, Mikulasovich R, Tamariz J, Bajda E, Sebestyen J, Baum H, Prinz M. Validation of testing and interpretation protocols for low template DNA samples using AmpFlSTR identifiler. Croat Med J. 2009 6;50(3):250-67.

15. Forster L, Thomson J, Kutranov S. Direct comparison of post-28-cycle PCR purification and modified capillary electrophoresis methods with the 34-cycle "low copy number" (LCN) method for analysis of trace forensic DNA samples. Forensic Sci Int Genet. 2008 9;2(4):318-28.

16. Benschop CCG, van der Beek CP, Meiland HC, van Gorp AGM, Westen AA, Sijen T. Low template STR typing: Effect of replicate number and consensus method on genotyping reliability and DNA database search results. Forensic Science International: Genetics. 2011 8;5(4):316-28.

17. Gill P, Curran J, Neumann C, Kirkham A, Clayton T, Whitaker J, Lambert J. Interpretation of complex DNA profiles using empirical models and a method to measure their robustness. Forensic Sci Int Genet. 2008 3;2(2):91-103.

18. Gill P, Kirkham A. Development of a simulation model to assess the impact of contamination in casework using STRs. J Forensic Sci. 2004 5;49(3):485-91.

19. Budowle B, Eisenberg AJ, van Daal A. Validity of low copy number typing and applications to forensic science. Croat Med J. 2009 6;50(3):207-17.

20. Buckleton J. Validation issues around DNA typing of low level DNA. Forensic Sci Int Genet. 2009 9;3(4):255-60.

21. Gill P, Buckleton J. A universal strategy to interpret DNA profiles that does not require a definition of low-copy-number. Forensic Science International: Genetics. 2010 7;4(4):221-7.

22. Pfeifer CM, Klein-Unseld R, Klintschar M, Wiegand P. Comparison of different interpretation strategies for low template DNA mixtures. Forensic Science International: Genetics. 2012;6(6):716-22.

23. Kokshoorn B, Blankers BJ. Response to grisedale and van daal: Comparison of STR profiling from low template DNA extracts with and without the consensus profiling method. Investig Genet. 2013 1;4(1):1,2223-4-1.

24. Grisedale K, van Daal A. Comment on kokshoorn, B, and blankers, BJ 'response to grisedale, KS and van daal, A: Comparison of STR profiling from low template DNA extracts with and without the consensus profiling method'. Investig Genet. 2013 1;4(1):2.

25. Kelly H, Bright J, Buckleton JS, Curran JM. Identifying and modelling the drivers of stutter in forensic DNA profiles. Australian Journal of Forensic Sciences. 2013:1-10.

26. Balding DJ, Buckleton J. Interpreting low template DNA profiles. Forensic Science International: Genetics. 2009;4(1):1-10.

27. Bright J, Taylor D, Curran JM, Buckleton JS. Developing allelic and stutter peak height models for a continuous method of DNA interpretation. Forensic Science International: Genetics. 2013;7(2):296-304.

28. Gill P, Sparkes B, Buckleton J. Interpretation of simple mixtures of when artefacts such as stutters are present-with special reference to multiplex STRs used by the forensic science service. Forensic Sci Int. 1998;95(3):213-24.

29. Bright J, Taylor D, Curran JM, Buckleton JS. Developing allelic and stutter peak height models for a continuous method of DNA interpretation. Forensic Science International: Genetics. 2013;7(2):296-304.

30. Bright J, Gill P, Buckleton J. Composite profiles in DNA analysis. Forensic Science International: Genetics. 2012;6(3):317-21.

31. Cowen S, Debenham P, Dixon A, Kutranov S, Thomson J, Way K. An investigation of the robustness of the consensus method of interpreting low-template DNA profiles. Forensic Science International: Genetics. 2011 11;5(5):400-6.

32. Taroni F, Biedermann A, Garbolino P, Aitken CGG. A general approach to bayesian networks for the interpretation of evidence. Forensic Sci Int. 2004 1;139(1):5-16.

33. Biedermann A, Taroni F. Bayesian networks and probabilistic reasoning about scientific evidence when there is a lack of data. Forensic Sci Int. 2006 3;157(2–3):163-7.

34. Puch-Solis R, Rodgers L, Mazumder A, Pope S, Evett I, Curran J, Balding D. Evaluating forensic DNA profiles using peak heights, allowing for multiple donors, allelic dropout and stutters. Forensic Science International: Genetics. 2013;7(5):555-63.

35. Gill P, Curran J, Elliot K. A graphical simulation model of the entire DNA process associated with the analysis of short tandem repeat loci. Nucleic Acids Res. 2005 1;33(2):632-43.

36. Curran JM, Walsh SJ, Buckleton JS. Empirical support for the reliability of DNA evidence interpretation in australia and new zealand. Australian Journal of Forensic Sciences. 2008;40(2):99-108.

37. Lawless CJ. The low template DNA profiling controversy: Biolegality and boundary work among forensic scientists. Soc Stud Sci. 2013;43(2):191-214.

38. Balding DJ. Evaluation of mixed-source, low-template DNA profiles in forensic science. Proc Natl Acad Sci U S A. 2013 7;110(30):12241-6.

39. Tvedebrink T, Eriksen PS, Mogensen HS, Morling N. Estimating the probability of allelic drop-out of STR alleles in forensic genetics. Forensic Science International: Genetics. 2009;3(4):222-6.

40. Tvedebrink T, Eriksen PS, Mogensen HS, Morling N. Statistical model for degraded DNA samples and adjusted probabilities for allelic drop-out. Forensic Science International: Genetics. 2012;6(1):97-101.

41. Lohmueller KE, Rudin N. Calculating the weight of evidence in low-template forensic DNA casework. J Forensic Sci. 2013;58:S243-9.

42. Benschop C, Haned H, Sijen T. Consensus and pool profiles to assist in the analysis and interpretation of complex low template DNA mixtures. Int J Legal Med. 2013;127(1):11-23.

43. Tvedebrink T, Asplund M, Eriksen PS, Mogensen HS, Morling N. Estimating drop-out probabilities of STR alleles accounting for stutters, detection threshold truncation and degradation. Forensic Science International: Genetics Supplement Series. 2013;4(1):e51-2.

44. Haned H, Egeland T, Pontier D, Pène L, Gill P. Estimating drop-out probabilities in forensic DNA samples: A simulation approach to evaluate different models. Forensic Science International: Genetics. 2011 11;5(5):525-31.

45. Gill P, Puch-Solis R, Curran J. The low-template-DNA (stochastic) threshold--its determination relative to risk analysis for national DNA databases. Forensic Sci Int Genet. 2009 3;3(2):104-11.

46. Grisedale KS, van Daal A. Comparison of STR profiling from low template DNA extracts with and without the consensus profiling method. Investigative Genet. 2012;3(1):14.

47. Ge J, Budowle B. Modeling one complete versus triplicate analyses in low template DNA typing. Int J Legal Med. 2013 10.

48. Puch-Solis R, Kirkham AJ, Gill P, Read J, Watson S, Drew D. Practical determination of the low template DNA threshold. Forensic Science International: Genetics. 2011 11;5(5):422-7.

49. Perlin MW. When good DNA goes bad. J Forensic Res S. 2013;11:2.

50. Thompson WC, Mueller LD, Krane D. Forensic DNA statistics: Still controversial in some cases.

51. Gill P, Gusmao L, Haned H, Mayr W, Morling N, Parson W, Prieto L, Prinz M, Schneider H, Schneider PM, Weir BS. Recommendations on the evaluation of STR typing results that may include drop-out and/or drop-in using probabilistic methods. Forensic Sci.Int.Genet. 2012;5(6):679-88.

SUMMARY

IMPROVED TOOLS FOR THE ROBUST ANALYSIS OF LOW COPY NUMBER AND CHALLENGED DNA SAMPLES

Low copy number (LCN) typing is a general technique used for analyzing low quantity DNA samples. Short tandem repeat (STR) testing on aged and extremely limited samples, such as "touch DNA" samples has increased over the past decade. These samples with low quantities of template DNA are typically subjected to exaggerated stochastic effects during the polymerase chain reaction (PCR), and these effects impact the reproducibility and reliability of DNA typing results. Current LCN methods are not analytically robust, and the confidence associated with a DNA profile and sample attribution is not well-defined.

The research described in this dissertation sought to develop and improve the analytical typing processes, creating a more robust system of LCN typing that is less refractory to stochastic effects, and given the more robust system, provide guidance on the statistical issues needed to assess the significance of a LCN typing result. In order to develop a more robust system, a number of research projects were undertaken. The results of these projects demonstrate that improvements can be made to enhance sample recovery, extraction efficiency, and conditions of the PCR and thus the stochastic effects can be reduced.

Success of DNA typing is related to the amount of target material recovered from an evidentiary item. Generally, the more DNA that is recovered, the better the chance is of obtaining a typing result that will be robust and reliable. One method of collecting stain materials is by swabbing. Recovery of DNA from a number of commercially-available swabs is not an efficient process. The X-Swab™ (Diomics Corporation, La Jolla, CA) is a unique bio-specimen collection material, which can be dissolved during certain extraction conditions. Therefore, more DNA may be collected from a substrate and be released from the swab matrix than other swabs. **Chapter 1** examined the ability to recover DNA from the X-Swab and success in STR typing in comparison with the Copan 4N6FLOQSwab™ (Brescia, Italy), a device which utilizes a

proprietary flocked-swab technology to maximize DNA collection and elution efficiency. DNA recovery was assessed by DNA quantification and by STR typing. Results demonstrated that the X-Swab material yielded greater DNA recovery, particularly of low quantity samples, compared with the 4N6FLOQSwab. Results also indicated that the X-Swab material itself enhances yield of PCR products.

Bones are frequently encountered in the identification of individuals in mass disasters and missing person investigations. Bone is a challenging tissue for DNA extraction and purification due to potential environmental and microbial DNA damage, possible DNA degradation, the presence of co-extracting inhibitors, and low levels of DNA. Additionally, areas of extensive mineralization within the bone may present a physical barrier to extraction reagents, thus preventing release of DNA (1-3). An ideal DNA isolation protocol should provide a maximum yield of DNA free from inhibitory compounds that may affect downstream applications and does not employ harmful reagents. **Chapter 2** described the DNA recovery, purity and overall extraction efficiency of a protocol employing a novel silica-based column, Hi-Flow® (Generon Ltd., Maidenhead, UK), compared with that of a standard organic DNA extraction methodology. The quantities of DNA recovered by each method were compared by real-time PCR and quality of DNA by short tandem repeat (STR) typing. Compared with a standard organic DNA isolation method, the results indicated that the Hi-Flow method provided equal or improved recovery and quality of DNA without the harmful effects of organic extraction. Moreover, larger extraction volumes (up to 20 mL) can be employed with the Hi-Flow method which enabled more bone sample to be extracted at one time.

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. **Chapter 3** described a study involving the use of pressure cycling technology (PCT). The effect of high pressure on inhibitors, and subsequently the PCR process, was assessed by measuring DNA quantity by quantitative PCR and evaluating STR typing results. The results support that PCT 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.

Various types of biological samples present challenges for extraction of DNA suitable for subsequent molecular analyses. Commonly used extraction methods, such as silica membrane columns and phenol-chloroform, while highly successful may still fail to provide a sufficiently pure DNA extract with some samples. Synchronous coefficient of drag alteration (SCODA), implemented in Boreal Genomics' Aurora Nucleic Acid Extraction System (Boreal Genomics, Vancouver, BC), is a new technology that offers the potential to remove inhibitors effectively while simultaneously concentrating DNA. In **Chapter 4**, SCODA was tested for its ability to remove various concentrations of forensically and medically relevant PCR inhibitors naturally found in tissue, hair, blood, plant, and soil samples. SCODA was used to purify and concentrate DNA from intentionally contaminated DNA samples containing known concentrations of hematin, humic acid, melanin, and tannic acid. SCODA methodology yielded overall higher efficiency of purification of highly contaminated samples compared

with the QIAquick® PCR Purification Kit (Qiagen, Valencia, CA). The Aurora provided an automated, minimal-step approach to successfully remove inhibitors and concentrate DNA from challenged samples.

One parameter that impacts the robustness and reliability of forensic DNA analyses is the amount of template DNA used in the PCR. With STR typing, low copy number (LCN) DNA samples can present exaggerated stochastic effects during the PCR that result in heterozygote peak height imbalance, allele drop out, and increased stutter. Despite these effects, there has been little progress toward decreasing the formation of stutter products and heterozygote peak imbalance effects during PCR. **Chapter 5** described an investigation of PCR additives, betaine, DMSO, PEG and PCRboost®, on low quantity DNA samples. The effects of the additives were assessed by evaluating STR typing results. Of the four additives, the only positive effects were observed with betaine treatment. The addition of betaine lead to increased yield of PCR products in all commercial kits tested. The results support that betaine can improve amplification efficiency of LCN DNA samples.

Interpretation of LCN DNA profiles may be improved with a better understanding of DNA transfer. The examination of DNA transferred through contact has become a major subject of interest in the field of forensic genetics. It has direct bearing on the interpretation related to the reconstruction of a case based on DNA profiles and the relevance of the information even for single source samples. Therefore, it is necessary to understand the potential impact of transfer DNA to a sample that may overwhelm the DNA on or in the sample or be interpreted as originating from that sample. Studies of DNA transfer have to date focused largely on the transfer of sloughed off epithelial cells from individual's hands. Previous transfer studies have found that the last individual to come into contact with an item is usually the major contributor to the

resulting DNA mixture, unless conditions are simulated in which a "good shedder" serves as the primary depositor and a poor shedder serves as the secondary depositor. **Chapter 6** examined primary, secondary, and tertiary transfer events involving DNA originating from saliva, a commonly encountered body fluid. More routine human behaviors were simulated to evaluate transfer, and the effects of drying time, moisture, and surface composition were investigated. The results agreed with previous findings which indicate that the presence of moisture, as well as a smooth nonporous surface as the primary substrate, increased the efficiency of transfer. The results of this study indicated that when saliva is the source of the transferred DNA, the primary depositor is often the major contributor. These findings suggest that shedder status is less relevant with regard to touch DNA samples in a forensic setting and emphasized the need for caution when analyzing such samples.

One of the most important tasks for a DNA analyst is the interpretation of DNA profile results. Steele and Balding (4) stated "because there is no clear distinction between LTDNA and standard DNA profiling, any method of analysis for LTDNA profiles should return the same results as would a standard analysis when presented with profiles obtained using optimal DNA template." For LCN DNA analysis, however, the recovered DNA profiles often are difficult to interpret due to greater complexity and are especially problematic for mixed profiles (5-8). The main challenges are due to stochastic effects of the PCR. **Chapter 7** explored the current statistical methods, their commonalities, and limitations. Recommendations were generated based on the existing problems that have yet to be addressed and built upon the framework set in place by Balding and Buckleton (9). Additionally, more empirical data will be necessary to estimate the rates of dropout, dropin, contamination, and stutter for LCN DNA, in order to test recommended models.

REFERENCES

1. Loreille OM, Diegoli TM, Irwin JA, Coble MD, Parsons TJ. High efficiency DNA extraction from bone by total demineralization. Forensic Sci Int Genet. 2007 6;1(2):191-5.

2. Hoff-Olsen P, Mevag B, Staalstrom E, Hovde B, Egeland T, Olaisen B. Extraction of DNA from decomposed human tissue. an evaluation of five extraction methods for short tandem repeat typing. Forensic Sci Int. 1999 11;105(3):171-83.

3. Pagan F, Lim C, Keglovic M, McNevin D. Comparison of DNA extraction methods for identification of human remains. Australian Journal of Forensic Sciences. 2012 06/01; 2014 1;44(2):117-27.

4. Steele CD, Balding DJ. Statistical evaluation of forensic DNA profile evidence. Annu.Rev.Stat.Appl. 2014;1:20.1,20.24.

5. Cowell RG, Lauritzen SL, Mortera J. Probabilistic expert systems for handling artifacts in complex DNA mixtures. Forensic Science International: Genetics. 2011 6;5(3):202-9.

6. Weusten J, Herbergs J. A stochastic model of the processes in PCR based amplification of STR DNA in forensic applications. Forensic Science International: Genetics. 2012;6(1):17-25.

7. Michel S, De Bast A, Vandenbroere I, Froment O. Interpretation of low-copy-number DNA profile after post-PCR purification. Forensic Science International: Genetics Supplement Series. 2009;2(1):542-3.

8. Leclair B, Fregeau CJ, Bowen KL, Fourney RM. Systematic analysis of stutter percentages and allele peak height and peak area ratios at heterozygous STR loci for forensic casework and database samples. J Forensic Sci. 2004 9;49(5):968-80.

9. Balding DJ, Buckleton J. Interpreting low template DNA profiles. Forensic Science International: Genetics. 2009;4(1):1-10.

CONCLUSIONS AND FUTURE DIRECTIONS

IMPROVED TOOLS FOR THE ROBUST ANALYSIS OF LOW COPY NUMBER AND CHALLENGED DNA SAMPLES

Low copy number (LCN) typing is a general technique used for analyzing low quantity DNA samples. These samples with low quantities of template DNA are typically subjected to exaggerated stochastic effects during the polymerase chain reaction (PCR), and these effects impact the reproducibility and reliability of DNA typing results. Current LCN methods are not analytically robust, and the confidence associated with a DNA profile and sample attribution is not well-defined. My research project sought to develop and improve the analytical typing processes so that samples which typically yield too little DNA can become suitable for standard methods of DNA analysis, thereby lessening the interpretation difficulties observed with LCN typing results. In order to improve LCN typing, several approaches were undertaken which include: 1) increasing DNA recovery using PCT, large volume silica columns, and SCODA; 2) methods for reducing PCR inhibition; 3) improvements to the robustness of the amplification through the use of PCR additives; and 4) improving DNA collection. Additionally, an assessment of the available software analysis tools for LCN DNA interpretation was performed.

PCT, silica columns, and SCODA were all investigated as potential tools for increasing DNA recovery and reducing PCR inhibition. PCT did not increase DNA yield but did reduce inhibitory effects of hematin and humic acid and thus, in effect, enhanced yield of contaminate samples. While PCT can improve the ability to type challenged or inhibited DNA samples, it is limited in the fact that it is not a high throughput instrument. However, PCT should be considered for particularly difficult casework samples. Results from the testing of silica column devices suggested equal or improved recovery and quality of DNA over other extraction methods. Moreover, larger extraction volumes (up to 20 mL) can be employed which enables more bone sample to be extracted at one time. The best extraction method with regards to increased DNA recovery and quality was SCODA. SCODA provides an automated, minimal-

step approach to successfully remove inhibitors and concentrate DNA from challenged samples; however, it too is limited in that it is not a high throughput instrument. Like PCT, SCODA should be considered for inhibited or challenged samples.

PCR additives were investigated for the purpose of reducing stochastic effects in LCN samples, specifically stutter. Of four additives tested, the only positive effects were observed with betaine treatment, and these effects were increased yield of PCR products, not stutter reduction. While the results support that betaine can improve amplification efficiency of LCN DNA samples, there are other methods (i.e. increased PCR cycle number) which improve DNA yield and are readily available.

A novel collection device, the Diomics' X-Swab, was examined for the purpose of improving DNA recovery. Results demonstrated that the X-Swab material yielded greater DNA recovery, particularly of low quantity samples, compared with a high-performing collection device. Results also indicated that the X-Swab material itself enhances yield of PCR products. The important outcome of the X-Swab study is that some samples that traditionally yield too little DNA for typing may become suitable for routine analysis. Because the investigation of the X-Swab was preliminary, future testing of the X-Swab should include an examination of the potential PCR enhancement with X-Swab polymer of challenged/degraded samples, such as bone, as well as a more thorough investigation of mock casework, stain, substrate, and touch samples. Furthermore, an investigation into the use of the X-Swab polymer as a storage medium recommended.

One of the most important tasks for a DNA analyst is the interpretation of DNA profile results. Recovered DNA profiles for LCN samples often are difficult to interpret due to greater complexity and are especially problematic for mixed profiles. There are two current approaches

which are employed to address these limitations, the biological model and the statistical model. Recommendations were generated based on the existing problems that have yet to be addressed by these models. In future, these statistical guidelines still need a lot of focus. More empirical data will be necessary to estimate the rates of dropout, dropin, contamination, and stutter for LCN DNA, in order to test recommended models. Factors that impact the reliability of allele designation and statistical weight assessment should be incorporated into a guideline for the human identity testing community so that the degree of confidence can be properly conveyed and those making identifications will be properly informed.

The work described in this dissertation 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.

REFERENCES

IMPROVED TOOLS FOR THE ROBUST ANALYSIS OF LOW COPY NUMBER AND CHALLENGED DNA SAMPLES

Akane A, Matsubara K, Nakamura H, Takahashi S, Kimura K. Identification of the heme compound copurified with deoxyribonucleic acid (DNA) from bloodstains, a major inhibitor of polymerase chain reaction (PCR) amplification. J Forensic Sci. 1994 3;39(2):362-72.

Alaeddini R. Forensic implications of PCR inhibition-a review. Forensic Sci Int-Gen 2012 5;6:297-305.

Alonso A, Martin P. A real-time PCR protocol to determine the number of amelogenin (X-Y) gene copies from forensic DNA samples. Methods Mol Biol. 2005;297:31-44.

Ambers A, Gill-King H, Dirkmaat D, Benjamin R, King J, Budowle B. Autosomal and Y-STR analysis of degraded DNA from the 120-year-old skeletal remains of ezekiel harper. Forensic Science International: Genetics. 2014 3;9(0):33-41.

Amory S, Huel R, Bilić A, Loreille O, Parsons TJ. Automatable full demineralization DNA extraction procedure from degraded skeletal remains. Forensic Science International: Genetics. 2012 5;6(3):398-406.

Anjos MJ, Andrade L, Carvalho M, Lopes V, Serra A, Oliveira C, Batista L, Balsa F, Brito P, Corte-Real F, Vide MC. Low copy number: Interpretation of evidence results. Int Congr Ser. 2006 4;1288:616-8.

Balding DJ, Buckleton J. Interpreting low template DNA profiles. Forensic Science International: Genetics. 2009 12;4(1):1–10.

Balding DJ. Evaluation of mixed-source, low-template DNA profiles in forensic science. Proc Natl Acad Sci U S A. 2013 7;110(30):12241-6.

Ballantyne KN, van Oorschot RAH, John Mitchell R, Koukoulas I. Molecular crowding increases the amplification success of multiple displacement amplification and short tandem repeat genotyping. Anal Biochem. 2006 8;355(2):298-303.

Balny C, Masson P, Heremans K. High pressure effects on biological macromolecules: From structural changes to alteration of cellular processes. Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology. 2002 3;1595(1–2):3-10.

Balny C. What lies in the future of high-pressure bioscience? Biochimica et Biophysica Acta (BBA) - Proteins & Proteomics. 2006 3;1764(3):632-9.

Barbaro A, Cormaci P, Barbaro A. LCN DNA typing from touched objects. Int Congr Ser 2006 4;1288:553-555.

Benschop C, Haned H, Sijen T. Consensus and pool profiles to assist in the analysis and interpretation of complex low template DNA mixtures. Int J Legal Med. 2013;127(1):11-23.

Benschop CCG, van der Beek CP, Meiland HC, van Gorp AGM, Westen AA, Sijen T. Low template STR typing: Effect of replicate number and consensus method on genotyping reliability and DNA database search results. Forensic Science International: Genetics. 2011 8;5(4):316-28.

Biedermann A, Taroni F. Bayesian networks and probabilistic reasoning about scientific evidence when there is a lack of data. Forensic Sci Int. 2006 3;157(2–3):163-7.

Biomatrica | PCRboost product information [Internet].; cited 12/5/2012]. Available from: http://www.biomatrica.com/pcrboost.php.

Bright J, Gill P, Buckleton J. Composite profiles in DNA analysis. Forensic Science International: Genetics. 2012;6(3):317-21.

Bright J, Taylor D, Curran JM, Buckleton JS. Developing allelic and stutter peak height models for a continuous method of DNA interpretation. Forensic Science International: Genetics. 2013;7(2):296-304.

Broemeling DJ, Pel J, Gunn DC, Mai L, Thompson JD, Poon H, Marziali A. An instrument for automated purification of nucleic acids from contaminated forensic samples. JALA 2008 2;13:40-48.

Brownlow RJ, Dagnall KE, Ames CE. A comparison of DNA collection and retrieval from two swab types (cotton and nylon flocked swab) when processed using three QIAGEN extraction methods. J Forensic Sci. 2012 5;57(3):713-7.

Buckleton J. Validation issues around DNA typing of low level DNA. Forensic Sci Int Genet. 2009 9;3(4):255-60.

Budowle B, Eisenberg AJ, van Daal A. Validity of low copy number typing and applications to forensic science. Croat Med J. 2009 6;50(3):207-17.

Budowle B, Hobson DL, Smerick JB, Smith JAL. Low Copy Number - Consideration and Caution. Madison, WI: Promega Corporation; 2001. Report No.: 1-26.

Budowle B, van Daal A. Extracting evidence from forensic DNA analyses: Future molecular biology directions. BioTechniques. 2009 4;46(5):339,40, 342-50.

Budowle B. DNA typing protocols : Molecular biology and forensic analysis. Natick, MA: Eaton Pub.; 2000.

Caragine T, Mikulasovich R, Tamariz J, Bajda E, Sebestyen J, Baum H, Prinz M. Validation of testing and interpretation protocols for low template DNA samples using AmpFlSTR identifiler. Croat Med J. 2009 6;50(3):250-67.

Chakrabarti R, Schutt CE. Novel sulfoxides facilitate GC-rich template amplification. BioTechniques. 2002 4;32(4):866, 868, 870,2, 874.

Chakrabarti R, Schutt CE. The enhancement of PCR amplification by low molecular weight amides. Nucleic Acids Res. 2001 6;29(11):2377-81.

Clement O, Whitney S, Muller-Cohn J, Muller R. Following nature's lead: Generating compounds for stabilizing biomolecules. Biopreservation and Biobanking. 2012;10(4):395-402.

Comey CT. DNA extraction strategies for amplified fragment length polymorphism analysis. J Forensic Sci. 1994;39:1254.

Considine KM, Kelly AL, Fitzgerald GF, Hill C, Sleator RD. High-pressure processing ? effects on microbial food safety and food quality. FEMS Microbiol Lett. 2008;281(1):1-9.

Courts C, Madea B. Full STR profile of a 67-year-old bone found in a fresh water lake. J Forensic Sci 2011 10;56:S172-S175.

Cowell RG, Lauritzen SL, Mortera J. Probabilistic expert systems for handling artifacts in complex DNA mixtures. Forensic Science International: Genetics. 2011 6;5(3):202-9.

Cowen S, Debenham P, Dixon A, Kutranov S, Thomson J, Way K. An investigation of the robustness of the consensus method of interpreting low-template DNA profiles. Forensic Science International: Genetics. 2011 11;5(5):400-6.

Curran JM, Walsh SJ, Buckleton JS. Empirical support for the reliability of DNA evidence interpretation in australia and new zealand. Australian Journal of Forensic Sciences. 2008;40(2):99-108.

Dadhania A, Nelson M, Caves G, Santiago R, Podini D. Evaluation of copan 4N6FLOQSwabs™ used for crime scene evidence collection. Forensic Science International: Genetics Supplement Series. 2013;4(1):e336-7.

Davis CP, King JL, Budowle B, Eisenberg AJ, Turnbough MA. Extraction platform evaluations: A comparison of AutoMate express, EZ1(R) advanced XL, and maxwell(R) 16 bench-top DNA extraction systems. Leg Med (Tokyo). 2012 1;14(1):36-9.

Davis CP, King JL, Budowle B, Eisenberg AJ, Turnbough MA. Extraction platform evaluations: a comparison of Automate Express™, EZ1® Advanced XL, and Maxwell® 16 Bench-top DNA extraction systems. Legal Medicine 2012 1;14:36-39.

Davoren J, Vanek D, Konjhodzic R, Crews J, Huffine E, Parsons TJ. Highly effective DNA extraction method for nuclear short tandem repeat testing of skeletal remains from mass graves. Croat Med J. 2007 8;48(4):478-85.

de Franchis R, Cross NC, Foulkes NS, Cox TM. A potent inhibitor of taq polymerase copurifies with human genomic DNA. Nucleic Acids Res. 1988 11;16(21):10355.

Delgado A, Kulisiewicz L, Rauh C, Benning R. Basic aspects of phase changes under high pressure. Ann N Y Acad Sci. 2010 2;1189:16-23.

Deng YJ, Li YZ, Yu XG, Li L, Wu DY, Zhou J, Man TY, Yang G, Yan JW, Cai DQ, Wang J, Yang HM, Li SB, Yu J. Preliminary DNA identification for the tsunami victims in thailand. Genomics Proteomics Bioinformatics. 2005 8;3(3):143-57.

Dieltjes P, Mieremet R, Zuniga S, Kraaijenbrink T, Pijpe J, de Knijff P. A sensitive method to extract DNA from biological traces present on ammunition for the purpose of genetic profiling. Int J Legal Med 2010 7;125:597-602.

Djuric M, Varljen T, Stanojevic A, Stojkovic O. DNA typing from handled items. Forensic Sci Int Genet Suppl 2008 8;1:411-412.

Dukes MJ, Williams AL, Massey CM, Wojtkiewicz PW. Technical note: Bone DNA extraction and purification using silica-coated paramagnetic beads. Am J Phys Anthropol. 2012 7;148(3):473-82.

Eckhart L, Bach J, Ban J, Tschachler E. Melanin binds reversibly to thermostable DNA polymerase and inhibits its activity. Biochem Biophys Res Commun 2000 5;271:726-730.

Eilert KD, Foran DR. Polymerase resistance to polymerase chain reaction inhibitors in bone. J Forensic Sci. 2009;54(5):1001-7.

Engel K, Pinnell L, Cheng J, Charles TC, Neufeld JD. Nonlinear electrophoresis for purification of soil DNA for metagenomics. J Microbiol Meth 2012 1;88:35-40.

Farmen RK, Jaghø R, Cortez P, Frøyland ES. Assessment of individual shedder status and implication for secondary transfer. Forensic Sci Int Genet Suppl 2008 8;1:415-417.

Favre N, Rudin W. Salt-dependent performance variation of DNA polymerases in co-amplification PCR. BioTechniques. 1996 7;21(1):28-30.

Fazekas A, Steeves R, Newmaster S. Improving sequencing quality from PCR products containing long mononucleotide repeats. BioTechniques. 2010 4;48(4):277-85.

Findlay I, Taylor A, Quirke P, Frazier R, Urquhart A. DNA fingerprinting from single cells. Nature. 1997 10;389(6651):555-6.

Forster L, Thomson J, Kutranov S. Direct comparison of post-28-cycle PCR purification and modified capillary electrophoresis methods with the 34-cycle "low copy number" (LCN) method for analysis of trace forensic DNA samples. Forensic Sci Int Genet. 2008 9;2(4):318-28.

Funes-Huacca ME, Opel K, Thompson R, McCord BR. A comparison of the effects of PCR inhibition in quantitative PCR and forensic STR analysis. Electrophoresis. 2011;32(9):1084-9.

Ge J, Budowle B. Modeling one complete versus triplicate analyses in low template DNA typing. Int J Legal Med. 2013 10.

Gill P, Buckleton J. A universal strategy to interpret DNA profiles that does not require a definition of low-copy-number. Forensic Science International: Genetics. 2010 7;4(4):221-7.

Gill P, Curran J, Elliot K. A graphical simulation model of the entire DNA process associated with the analysis of short tandem repeat loci. Nucleic Acids Res. 2005 1;33(2):632-43.

Gill P, Curran J, Neumann C, Kirkham A, Clayton T, Whitaker J, Lambert J. Interpretation of complex DNA profiles using empirical models and a method to measure their robustness. Forensic Sci Int Genet. 2008 3;2(2):91-103.

Gill P, Gusmao L, Haned H, Mayr W, Morling N, Parson W, Prieto L, Prinz M, Schneider H, Schneider PM, Weir BS. Recommendations on the evaluation of STR typing results that may include drop-out and/or drop-in using probabilistic methods. Forensic Sci.Int.Genet. 2012;5(6):679-88.

Gill P, Jeffreys AJ, Werrett DJ. Forensic application of DNA “fingerprints”. Nature 1985 12;318:577-579.

Gill P, Kirkham A, Curran J. LoComatioN: A software tool for the analysis of low copy number DNA profiles. Forensic Sci Int. 2007 3;166(2-3):128-38.

Gill P, Kirkham A. Development of a simulation model to assess the impact of contamination in casework using STRs. J Forensic Sci. 2004 5;49(3):485-91.

Gill P, Puch-Solis R, Curran J. The low-template-DNA (stochastic) threshold--its determination relative to risk analysis for national DNA databases. Forensic Sci Int Genet. 2009 3;3(2):104-11.

Gill P, Sparkes B, Buckleton J. Interpretation of simple mixtures of when artefacts such as stutters are present-with special reference to multiplex STRs used by the forensic science service. Forensic Sci Int. 1998;95(3):213-24.

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 7;112(1):17-40.

Gill P. Application of low copy number DNA profiling. Croat Med J. 2001 6;42(3):229-32.

Goray M, Eken E, Mitchell RJ, van Ooorschot RAH. Secondary DNA transfer of biological substances under varying test conditions. Forensic Sci Int Genet 2010 2;4(2):62-67.

Graham EA. DNA reviews: Low level DNA profiling. Forensic Sci Med Pathol. 2008;4(2):129-31.

Green RL, Roinestad IC, Boland C, Hennessy LK. Developmental validation of the quantifiler real-time PCR kits for the quantification of human nuclear DNA samples. J Forensic Sci. 2005 7;50(4):809-25.

Grisedale K, van Daal A. Comment on kokshoorn, B, and blankers, BJ 'response to grisedale, KS and van daal, A: Comparison of STR profiling from low template DNA extracts with and without the consensus profiling method'. Investig Genet. 2013 1;4(1):2.

Grisedale KS, van Daal A. Comparison of STR profiling from low template DNA extracts with and without the consensus profiling method. Investigative Genet. 2012 7;3(1):14.

Gross V, Carlson G, Kwan AT, Smejkal G, Freeman E, Ivanov AR, Lazarev A. Tissue fractionation by hydrostatic pressure cycling technology: The unified sample preparation technique for systems biology studies. J Biomol Tech. 2008 7;19(3):189-99.

Haned H, Egeland T, Pontier D, Pène L, Gill P. Estimating drop-out probabilities in forensic DNA samples: A simulation approach to evaluate different models. Forensic Science International: Genetics. 2011 11;5(5):525-31.

Hansson O, Finnebraaten M, Heitmann IK, Ramse M, Bouzga M. Trace DNA collection—Performance of minitape and three different swabs. Forensic Science International: Genetics Supplement Series. 2009 12;2(1):189-90.

Haqqi T, Zhao X, Panciu A, Yadav SP. Sequencing in the presence of betaine: Improvement in sequencing of the localized repeat sequence regions. J Biomol Tech. 2002 12;13(4):265-71.

Hedman J, Nordgaard A, Rasmusson B, Ansell R, Radstrom P. Improved forensic DNA analysis through the use of alternative DNA polymerases and statistical modeling of DNA profiles. BioTechniques. 2009 11;47(5):951-8.

Henke W, Herdel K, Jung K, Schnorr D, Loening SA. Betaine improves the PCR amplification of GC-rich DNA sequences. Nucleic Acids Res. 1997 10;25(19):3957-8.

Higuchi R, von Beroldingen CH, Sensabaugh GF, Erlich HA. DNA typing from single hairs. Nature 1988 4;332:543-546.

Hinda H. Forensim: An open-source initiative for the evaluation of statistical methods in forensic genetics. Forensic Science International: Genetics. 2011 8;5(4):265-8.

Hochmeister MN, Budowle B, Borer UV, Eggmann U, Comey CT, Dirnhofer R. Typing of deoxyribonucleic acid (DNA) extracted from compact bone from human remains. J Forensic Sci. 1991 11;36(6):1649-61.

Hochmeister MN, Budowle B, Jung J, Borer UV, Comey CT, Dirnhofer R. PCR-based typing of DNA extracted from cigarette butts. Int J Legal Med 1991;104:229-233.

Hoff-Olsen P, Mevag B, Staalstrom E, Hovde B, Egeland T, Olaisen B. Extraction of DNA from decomposed human tissue. an evaluation of five extraction methods for short tandem repeat typing. Forensic Sci Int. 1999 11;105(3):171-83.

Horakova H, Polakovicova I, Shaik GM, Eitler J, Bugajev V, Draberova L, Draber P. 1,2-propanediol-trehalose mixture as a potent quantitative real-time PCR enhancer. BMC Biotechnol. 2011 4;11:41.

Horsman-Hall KM, Orihuela Y, Karczynski SL, Davis AL, Ban JD, Greenspoon SA. Development of STR profiles from firearms and fired cartridge cases. Forensic Sci Int Genet 2009 9;3:242-250.

Hube F, Reverdiau P, Iochmann S, Gruel Y. Improved PCR method for amplification of GC-rich DNA sequences. Mol Biotechnol. 2005 9;31(1):81-4.

Hudlow WR, Chong MD, Swango KL, Timken MD, Buoncristiani MR. A quadruplex real-time qPCR assay for the simultaneous assessment of total human DNA, human male DNA, DNA degradation and the presence of PCR inhibitors in forensic samples: A diagnostic tool for STR typing. Forensic Science International: Genetics. 2008 3;2(2):108-25.

Huggett JF, Novak T, Garson JA, Green C, Morris-Jones SD, Miller RF, Zumla, A. Differential susceptibility of PCR reactions to inhibitors: An important and unrecognised phenomenon. BMC Res Notes. 2008 8;1:70.

Irwin JA, Leney MD, Loreille O, Barritt SM, Christensen AF, Holland TD, Smith BC, Parsons TJ. Application of low copy number STR typing to the identification of aged, degraded skeletal remains. J Forensic Sci. 2007 11;52(6):1322-7.

Jakubowska J, Maciejewska A, Pawlowski R. Comparison of three methods of DNA extraction from human bones with different degrees of degradation. Int J Legal Med. 2012 1;126(1):173-8.

Jensen MA, Fukushima M, Davis RW. DMSO and betaine greatly improve amplification of GC-rich constructs in de novo synthesis. PLoS One. 2010 6;5(6):e11024.

Juen A, Traugott M. Amplification facilitators and multiplex PCR: Tools to overcome PCR-inhibition in DNA-gut-content analysis of soil-living invertebrates. Soil Biol Biochem. 2006 7;38(7):1872-9.

Kang J, Soog Lee M, Gorenstein DG. The enhancement of PCR amplification of a random sequence DNA library by DMSO and betaine: Application to in vitro combinatorial selection of aptamers. J Biochem Biophys Methods. 2005 8;64(2):147-51.

Kaya M, Toyama Y, Kubota K, Nodasaka Y, Ochiai M, Nomizu M, Nishi N. Effect of DNA structure on the formation of collagen-DNA complex. Int J Biol Macromol 2005 5;35:39-46.

Kelly H, Bright J, Buckleton JS, Curran JM. A comparison of statistical models for the analysis of complex forensic DNA profiles. Science & Justice. 2014 1;54(1):66-70.

Kelly H, Bright J, Buckleton JS, Curran JM. Identifying and modelling the drivers of stutter in forensic DNA profiles. Australian Journal of Forensic Sciences. 2013:1-10.

Kemp BM, Monroe C, Smith DG. Repeat silica extraction: A simple technique for the removal of PCR inhibitors from DNA extracts. Journal of Archaeological Science. 2006 12;33(12):1680-9.

Kim S, Labbe RG, Ryu S. Inhibitory effects of collagen on the PCR for detection of Clostridium perfringens. Appl Environ Microbiol 2000 3;66:1213-1215.

King C, Debruyne R, Kuch M, Schwarz C, Poinar H. A quantitative approach to detect and overcome PCR inhibition in ancient DNA extracts. BioTechniques. 2009 11;47(5):941-9.

King J, LaRue B, Novroski N, Parson W, Budowle, B. Application of Massively Parallel Sequencing for Whole Mitochondrial Genome Forensic DNA Analysis. Int J Legal Med, in submission.

Kloosterman AD, Kersbergen P. Efficacy and limits of genotyping low copy number (LCN) DNA samples by multiplex PCR of STR loci. J Soc Biol. 2003;197(4):351-9.

Kokshoorn B, Blankers BJ. Response to grisedale and van daal: Comparison of STR profiling from low template DNA extracts with and without the consensus profiling method. Investig Genet. 2013 1;4(1):1,2223-4-1.

Kontanis EJ, Reed FA. Evaluation of real-time PCR amplification efficiencies to detect PCR inhibitors. J Forensic Sci. 2006;51(4):795-804.

Koumoto K, Ochiai H, Sugimoto N. Structural effect of synthetic zwitterionic cosolutes on the stability of DNA duplexes. Tetrahedron. 2008 1;64(1):168-74.

Kovarova M, Draber P. New specificity and yield enhancer of polymerase chain reactions. Nucleic Acids Res. 2000 7;28(13):E70.

Kreader CA. Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. Appl Environ Microbiol. 1996 3;62(3):1102-6.

Kurz M. Compatible solute influence on nucleic acids: Many questions but few answers. Saline Systems. 2008 6;4:6.

Ladd C, Adamowicz MS, Bourke MT, Scherczinger CA, Lee HC. A systematic analysis of secondary DNA transfer. J Forensic Sci 1999 11;44:1270-1272.

Lawless CJ. The low template DNA profiling controversy: Biolegality and boundary work among forensic scientists. Soc Stud Sci. 2013;43(2):191-214.

Leclair B, Fregeau CJ, Bowen KL, Fourney RM. Systematic analysis of stutter percentages and allele peak height and peak area ratios at heterozygous STR loci for forensic casework and database samples. J Forensic Sci. 2004 9;49(5):968-80.

Leclair B, Sgueglia JB, Wojtowicz PC, Juston AC, Fregeau CJ, Fourney RM. STR DNA typing: Increased sensitivity and efficient sample consumption using reduced PCR reaction volumes. J Forensic Sci. 2003 9;48(5):1001-13.

Lee HY, Park MJ, Kim NY, Sim JE, Yang WI, Shin K. Simple and highly effective DNA extraction methods from old skeletal remains using silica columns. Forensic Science International: Genetics. 2010 10;4(5):275-80.

Lee SB, Clabaugh KC, Silva B, Odigie KO, Coble MD, Loreille O, Scheible M, Fourney RM, Stevens J, Carmody GC, Parsons TJ, Pozder A, Eisenberg AJ, Budowle B, Ahmad T, Miller RW, Crouse CA. Assessing a novel room temperature DNA storage medium for forensic biological samples. Forensic Sci Int Genet. 2012 1;6(1):31-40.

Lohmueller KE, Rudin N. Calculating the weight of evidence in low-template forensic DNA casework. J Forensic Sci. 2013;58:S243-9.

Lopes MLM, Valente Mesquita VL, Chiaradia ACN, Fernandes AAR, Fernandes PMB. High hydrostatic pressure processing of tropical fruits. Ann N Y Acad Sci. 2010;1189(1):6-15.

Loreille OM, Diegoli TM, Irwin JA, Coble MD, Parsons TJ. High efficiency DNA extraction from bone by total demineralization. Forensic Sci Int Genet. 2007 6;1(2):191-5.

Loreille OM, Parr RL, McGregor KA, Fitzpatrick CM, Lyon C, Yang DY, Speller CF, Grimm MR, Grimm MJ, Irwin JA, Robinson EM. Integrated DNA and fingerprint analyses in the identification of 60-year-old mummified human remains discovered in an Alaskan glacier. J Forensic Sci 2010 5;55:813-818.

Lowe A, Murray C, Richardson P, Wivell R, Gill P, Tully G, Whitaker J. Use of low copy number DNA in forensic inference. Int Congr Ser 2003 1;1239:799-801.

Lowe A, Murray C, Whitaker J, Tully G, Gill P. The propensity of individuals to deposit DNA and secondary transfer of low level DNA from individuals to inert surfaces. Forensic Sci Int 2002 9;129:25-34.

Mamedov TG, Pienaar E, Whitney SE, TerMaat JR, Carvill G, Goliath R, Subramanian A, Viljoen HJ. A fundamental study of the PCR amplification of GC-rich DNA templates. Computational Biology and Chemistry. 2008 12;32(6):452-7.

Marshall PL, King JL, Lawrence NP, Lazarev A, Gross VS, Budowle B. Pressure cycling technology (PCT) reduces effects of inhibitors of the PCR. International journal of legal medicine 2013;127(2), 321-333.

Marziali A, Pel J, Bizzotto D, Whitehead LA. Novel electrophoresis mechanism based on synchronous alternating drag perturbation. Electrophoresis. 2005 1;26(1):82-90.
Masson P, Tonello C, Balny C. High-pressure biotechnology in medicine and pharmaceutical science. J Biomed Biotechnol. 2001;1(2):85-8.

McCoy J, Hubbell WL. High-pressure EPR reveals conformational equilibria and volumetric properties of spin-labeled proteins. Proc Natl Acad Sci U S A. 2011 1;108(4):1331-6.

Michel S, De Bast A, Vandenbroere I, Froment O. Interpretation of low-copy-number DNA profile after post-PCR purification. Forensic Science International: Genetics Supplement Series. 2009;2(1):542-3.

Minton AP. The influence of macromolecular crowding and macromolecular confinement on biochemical reactions in physiological media. J Biol Chem. 2001 4;276(14):10577-80.

Mitchell AA, Tamariz J, O'Connell K, Ducasse N, Budimlija Z, Prinz M, Caragine T. Validation of a DNA mixture statistics tool incorporating allelic drop-out and drop-in. Forensic Science International: Genetics. 2012;6(6):749-61.

Musso M, Bocciardi R, Parodi S, Ravazzolo R, Ceccherini I. Betaine, dimethyl sulfoxide, and 7-deaza-dGTP, a powerful mixture for amplification of GC-rich DNA sequences. J Mol Diagn. 2006 11;8(5):544-50.

Nagai M, Yoshida A, Sato N. Additive effects of bovine serum albumin, dithiothreitol, and glycerol on PCR. Biochem Mol Biol Int. 1998 1;44(1):157-63.

Occhipinti E, Bec N, Gambirasio B, Baietta G, Martelli PL, Casadio R, Balny C, Lange R, Tortora P. Pressure and temperature as tools for investigating the role of individual non-covalent interactions in enzymatic reactions: Sulfolobus solfataricus carboxypeptidase as a model enzyme. Biochimica et Biophysica Acta (BBA) - Proteins & Proteomics. 2006 3;1764(3):563-72.

Opel KL, Chung D, McCord BR. A study of PCR inhibition mechanisms using real time PCR. J Forensic Sci. 2010 1;55(1):25-33.

Pagan F, Lim C, Keglovic M, McNevin D. Comparison of DNA extraction methods for identification of human remains. Australian Journal of Forensic Sciences. 2012 06; 2014 1;44(2):117-27.

Pang BCM, Cheung BKK. Double swab technique for collecting touched evidence. Leg Med 2007 7;9(4):181-4.

Paschke A. Aspects of food processing and its effect on allergen structure. Molecular Nutrition & Food Research. 2009;53(8):959-62.

Paul N. H. Optimizing multiplex and LA-PCR with betaine. Trends Biochem Sci. 1997 6;22(6):225-6.

Pel J, Broemeling D, Mai L, Poon HL, Tropini G, Warren RL, Holt RA, Marziali A. Nonlinear electrophoretic response yields a unique parameter for separation of biomolecules. Proc Natl Acad Sci USA 2009 7;106:14796-14801.

Perlin MW, Legler MM, Spencer CE, Smith JL, Allan WP, Belrose JL, Duceman BW. Validating TrueAllele® DNA mixture interpretation*,†. J Forensic Sci. 2011;56(6):1430-47.

Perlin MW, Szabady B. Linear mixture analysis: A mathematical approach to resolving mixed DNA samples. J Forensic Sci. 2001;46(6):1372-8.

Perlin MW. When good DNA goes bad. J Forensic Res S. 2013;11:2.

Petricevic SF, Bright JA, Cockerton SL. DNA profiling of trace DNA recovered from bedding. Forensic Sci Int 2006 5;159:21-26.

Pfeifer CM, Klein-Unseld R, Klintschar M, Wiegand P. Comparison of different interpretation strategies for low template DNA mixtures. Forensic Science International: Genetics. 2012;6(6):716-22.

Phipps M, Petricevic S. The tendency of individuals to transfer DNA to handled items. Forensic Sci Int 2007 5;168:162-168.

Primorac D, Andelinovic S, Definis-Gojanovic M, Drmic I, Rezic B, Baden MM, Kennedy MA, Schanfield MS, Skakel SB, Lee HC. Identification of war victims from mass graves in croatia, bosnia, and herzegovina by use of standard forensic methods and DNA typing. J Forensic Sci. 1996 9;41(5):891-4.

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 Congr Ser. 2006 4;1288:651-3.

Puch-Solis R, Kirkham AJ, Gill P, Read J, Watson S, Drew D. Practical determination of the low template DNA threshold. Forensic Science International: Genetics. 2011 11;5(5):422-7.

Puch-Solis R, Rodgers L, Mazumder A, Pope S, Evett I, Curran J, Balding D. Evaluating forensic DNA profiles using peak heights, allowing for multiple donors, allelic dropout and stutters. Forensic Science International: Genetics. 2013;7(5):555-63.

Puntharod R, Webster GT, Asghari-Khiavi M, Bambery KR, Safinejad F, Rivadehi S, Langford SJ, Haller KJ, Wood BR. Supramolecular interactions playing an integral role in the near-

infrared raman "excitonic" enhancement observed in beta-hematin (malaria pigment) and other related heme derivatives. J Phys Chem B. 2010 9;114(37):12104-15.

Quinque D, Kittler R, Kayser M, Stoneking M, Nasidze I. Evaluation of saliva as a source of human DNA for population and association studies. Anal Biochem 2006 6;353:272-277.

Radstrom P, Lofstrom C, Lovenklev M, Knutsson R, Wolffs P. Strategies for overcoming PCR inhibition. CSH Protoc. 2008 3;2008:pdb.top20.

Ralser M, Querfurth R, Warnatz H, Lehrach H, Yaspo M, Krobitsch S. An efficient and economic enhancer mix for PCR. Biochem Biophys Res Commun. 2006 9;347(3):747-51.

Ramakers C, Ruijter JM, Deprez RH, Moorman AF. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci Lett. 2003 3;339(1):62-6.

Rees WA, Yager TD, Korte J, von Hippel PH. Betaine can eliminate the base pair composition dependence of DNA melting. Biochemistry. 1993 1;32(1):137-44.

Roeder AD, Elsmore P, Greenhalgh M, McDonald A. Maximizing DNA profiling success from sub-optimal quantities of DNA: A staged approach. Forensic Sci Int Genet. 2009 3;3(2):128-37.

Rohland N, Siedel H, Hofreiter, M. A rapid column-based ancient DNA extraction method for increased sample throughput. Molecular Ecology Resources. 2010;10(4):677-83.

Rutty GN. An investigation into the transference and survivability of human DNA following simulated manual strangulation with consideration of the problem of third party contamination. Int J Legal Med 2002 6;116:170-173.

Sahdev S, Saini S, Tiwari P, Saxena S, Singh Saini K. Amplification of GC-rich genes by following a combination strategy of primer design, enhancers and modified PCR cycle conditions. Mol Cell Probes. 2007 8;21(4):303-7.

Sarkar G, Kapelner S, Sommer SS. Formamide can dramatically improve the specificity of PCR. Nucleic Acids Res. 1990 12;18(24):7465.

Sasaki Y, Miyoshi D, Sugimoto N. Effect of molecular crowding on DNA polymerase activity. Biotechnology Journal. 2006;1(4):440-6.

Schlotterer C, Tautz D. Slippage synthesis of simple sequence DNA. Nucleic Acids Res. 1992 1;20(2):211-5.

Schmedes S, Marshall P, King JL, Budowle B. Effective removal of co-purified inhibitors from extracted DNA samples using synchronous coefficient of drag alteration (SCODA) technology. International journal of legal medicine 2013 7;127(4), 749-755.

Seo SB, Ge J, King JL, Budowle B. Reduction of stutter ratios in short tandem repeat loci typing of low copy number DNA samples. Forensic Science International: Genetics. 2014 1;8(1):213-8.

Seo SB, Lee HY, Zhang AH, Kim HY, Shin DH, Lee SD. Effects of humic acid on DNA quantification with Quantifiler® Human DNA Quantification kit and short tandem repeat amplification efficiency. Int J Legal Med 2011 11;126:961-968.

Sewell J, Quinones I, Ames C, Multaney B, Curtis S, Seeboruth H, Moore S, Daniel B. Recovery of DNA and fingerprints from touched documents. Forensic Sci Int Genet 2008 9;2:281-285.

Smejkal GB, Robinson MH, Lawrence NP, Tao F, Saravis CA, Schumacher RT. Increased protein yields from escherichia coli using pressure-cycling technology. J Biomol Tech. 2006 4;17(2):173-5.

Smejkal GB, Witzmann FA, Ringham H, Small D, Chase SF, Behnke J, Ting E. Sample preparation for two-dimensional gel electrophoresis using pressure cycling technology. Anal Biochem. 2007 4;363(2):309-11.

Smith PJ, Ballantyne J. Simplified low-copy-number DNA analysis by post-PCR purification. J Forensic Sci. 2007 7;52(4):820-9.

So A, Pel J, Rajan S, Marziali A. Efficient genomic DNA extraction from low target concentration bacterial cultures using SCODA DNA extraction technology. Cold Spring Harb Protoc. 2010 10.

Spiess A, Ivell R. A highly efficient method for long-chain cDNA synthesis using trehalose and betaine. Anal Biochem. 2002 2;301(2):168-74.

Spiess AN, Mueller N, Ivell R. Trehalose is a potent PCR enhancer: Lowering of DNA melting temperature and thermal stabilization of taq polymerase by the disaccharide trehalose. Clin Chem. 2004 7;50(7):1256-9.

Staiti N, Giuffrè G, Di Martino D, Simone A, Sippelli G, Tuccari G, Saravo L. Molecular analysis of genomic low copy number DNA extracted from laser-microdissected cells. Int Congr Ser. 2006 4;1288:568-70.

Staiti N, Romano C, Trapani C, Ginestra E, Leo B, Schiavone S. Analysis of LCN DNA from synthetic ropes: A practical approach used in real homicide investigation. Forensic Science International: Genetics Supplement Series. 2008 8;1(1):446-7.

Steele CD, Balding DJ. Statistical evaluation of forensic DNA profile evidence. Annu.Rev.Stat.Appl. 2014;1:20.1,20.24.

Svintradze DV, Mrevlishvili GM, Metreveli N, Jariashvili K, Namicheishvili L, Skopinska J, Sionkowska A. Collagen-DNA complex. Biomacromolecules 2008 1;9:21-28.

Sweet D, Lorente JA, Valenzuela A. PCR-based DNA typing of saliva stains recovered from human skin. J Forensic Sci 1997 5;42:447-451.

Taroni F, Biedermann A, Garbolino P, Aitken CGG. A general approach to bayesian networks for the interpretation of evidence. Forensic Sci Int. 2004 1;139(1):5-16.

Thompson WC, Mueller LD, Krane D. Forensic DNA statistics: Still controversial in some cases.

Tokutomi T, Takada Y, Kanetake J, Mukaida M. Identification using DNA from skin contact: case reports. Leg Med (Tokyo) 2009 4;11:S576-S577.

Tvedebrink T, Asplund M, Eriksen PS, Mogensen HS, Morling N. Estimating drop-out probabilities of STR alleles accounting for stutters, detection threshold truncation and degradation. Forensic Science International: Genetics Supplement Series. 2013;4(1):e51-2.

Tvedebrink T, Eriksen PS, Mogensen HS, Morling N. Estimating the probability of allelic drop-out of STR alleles in forensic genetics. Forensic Science International: Genetics. 2009;3(4):222-6.

Tvedebrink T, Eriksen PS, Mogensen HS, Morling N. Statistical model for degraded DNA samples and adjusted probabilities for allelic drop-out. Forensic Science International: Genetics. 2012 1;6(1):97-101.

Van Eldik R, KlÃrner FG. High pressure chemistry: Synthetic, mechanistic, and supercritical applications. John Wiley & Sons; 2008.

van Oorschot RA, Ballantyne KN, Mitchell RJ. Forensic trace DNA: A review. Investig Genet. 2010 12;1(1):14,2223-1-14.

van Oorschot RAH, Goray M, Eken E, Mitchell RJ. Impact of relevant variables on the transfer of biological substances. Forensic Science International: Genetics Supplement Series. 2009 12; 2(1):547–548.

van Oorschot RAH, Jones MK. DNA fingerprints from fingerprints. Nature 1997 10;387:767.

Vasudevamurthy MK, Lever M, George PM, Morison KR. Betaine structure and the presence of hydroxyl groups alters the effects on DNA melting temperatures. Biopolymers. 2009;91(1):85-94.

Walsh DJ, Corey AC, Cotton RW, Forman L, Herrin GL, Word CJ, Garner DD. Isolation of deoxyribonucleic acid (DNA) from saliva and forensic science samples containing saliva. J Forensic Sci 1992 3;37:387-395.

Wang DY, Chang CW, Lagacé RE, Calandro LM, Hennessy LK. Developmental validation of the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit: an established multiplex assay with improved performance. J Forensic Sci 2012 11;57:453-465.

Warshauer DH, Marshall P, Kelley S, King J, Budowle B. An evaluation of the transfer of saliva-derived DNA. International journal of legal medicine, 2012;126(6), 851-861.

Watson RJ, Blackwell B. Purification and characterization of a common soil component which inhibits the polymerase chain reaction. Can J Microbiol. 2000 7;46(7):633-42.

Weiler NE, Matai AS, Sijen T. Extended PCR conditions to reduce drop-out frequencies in low template STR typing including unequal mixtures. Forensic Science International: Genetics. 2012;6(1):102-7.

Weissensteiner T, Lanchbury JS. Strategy for controlling preferential amplification and avoiding false negatives in PCR typing. BioTechniques. 1996 12;21(6):1102-8.

Westen AA, Nagel JH, Benschop CC, Weiler NE, de Jong BJ, Sijen T. Higher capillary electrophoresis injection settings as an efficient approach to increase the sensitivity of STR typing. J Forensic Sci. 2009 5;54(3):591-8.

Weusten J, Herbergs J. A stochastic model of the processes in PCR based amplification of STR DNA in forensic applications. Forensic Science International: Genetics. 2012;6(1):17-25.

Whitaker J, Tully G, Sullivan K. Response to low level DNA profiling. Forensic Sci Med Pathol. 2009;5(2):102,3; author reply 104-5.

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 12;123(2-3):215-23.

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 5;47(3):442-50.

Wiegand P, Heimbold C, Klein R, Immel U, Stiller D, Klintschar M. Transfer of biological stains from different surfaces. Int J Leg Med 2011 9;125:727-731.

Winter R, Dzwolak W. Temperature-pressure configurational landscape of lipid bilayers and proteins. Cell Mol Biol (Noisy-le-grand). 2004 6;50(4):397-417.

Yang DY, Eng B, Waye JS, Dudar JC, Saunders SR. Technical note: Improved DNA extraction from ancient bones using silica-based spin columns. Am J Phys Anthropol. 1998 4;105(4):539-43.

Ye J, Ji A, Parra EJ, Zheng X, Jiang C, Zhao X, Hu L, Tu Z. A simple and efficient method for extracting DNA from old and burned bone. J Forensic Sci. 2004 7;49(4):754-9.

Zahra N, Hadi S, Smith JA, Iyengar A, Goodwin W. Development of internal amplification controls for DNA profiling with the AmpFlSTR((R)) SGM plus((R)) kit. Electrophoresis. 2011 6;32(11):1371-8.

Zhang AH, Seo SB, Yi JA, Kim HY, Lee SD. Modification of a commercially available kit for the improvement of PCR efficiency. Human Biology. (3):343.

Zhang Z, Yang X, Meng L, Liu F, Shen C, Yang W. Enhanced amplification of GC-rich DNA with two organic reagents. BioTechniques. 2009 9;47(3):775-9.

Zimmerman SB, Harrison B. Macromolecular crowding increases binding of DNA polymerase to DNA: An adaptive effect. Proc Natl Acad Sci U S A. 1987 4;84(7):1871-5.