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Improved Tools and Interpretation Guidelines for Examining Limited Low Copy Number DNA
Obtained from Degraded Single Source Samples: Bones, Teeth, and Hairs

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A handwritten signature in black ink that reads "Bruce Budowle". The signature is written in a cursive, flowing style.

Bruce Budowle, Ph.D.

Abstract

Low copy number (LCN) typing is a technique(s) for typing low quantity DNA samples. Samples with low amounts of template DNA are subjected to exaggerated stochastic effects and these effects impact the reliability of DNA typing results. Current LCN methods are not well-developed and the confidence associated with a DNA profile associated with a sample is not well-defined. Thus, LCN typing needs to be better developed so genetic data from missing persons can be exploited to its full potential and those individuals making identifications will be able to use the genetic information effectively. The goals of this research project were to improve upon the analytical typing process, assess effects of primary and secondary transfer DNA, and to provide insight on the statistical issues needed to assess the significance of a LCN typing result. Each of the areas is separated into distinct studies. The studies were not combined into one process as the variation in samples may require different approaches on a case-by-case basis. Not all procedures may be needed or combining some may be useful. The purpose was to demonstrate that improvements could be made to processes and further work should be considered to enhance the abilities to improve sample recovery, extraction efficiency, and the conditions of the PCR. One area of research effort was to increase the starting template molecules by improving the overall extraction of DNA from skeletal remains and other 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 can be carried out. Improving sample extraction and being able to concentrate the sample can increase DNA yield. An alternative consideration is that the purity of the extract can be enhanced. By removing inhibitors to the PCR, an effective increase in templates is possible. Increased yield can be obtained by reducing inhibition, even though the total number of template molecules for the PCR is the same or reduced. Another area of research was to attempt to improve the robustness of the amplification process by attempting to make the PCR of low template samples more reliable. If there are less stochastic effects generated during the PCR, better quality results can be generated and then more reliable interpretation guidelines can be implemented. The last area of research was a discussion on and an evaluation of guidelines for the interpretation of assay results from LCN work and to provide guidance for development of robust statistical approaches for assessing the weight of evidence for single source LCN typing results. This project was successful in that a series of methods are described for extraction and sample concentration; additives to the PCR were identified that appear to reduce stutter; primary and secondary transfer was demonstrated not to be as simplistic as previously reported; and that a more refined statistical model is proposed.

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Executive Summary

Low copy number (LCN) typing is used to type DNA samples with very little DNA which includes bones from missing persons cases. Methods are employed that increase the sensitivity of detection of an assay so that the DNA typing results can be observed. However, samples with low amounts of template DNA are subjected to exaggerated stochastic effects and these effects can impact the reliability of DNA typing results. Most stochastic effects manifest as heterozygote allele peak height imbalance, allele drop out, and increased stutter. LCN typing has been defined as the analysis of any sample that contains less than 200 or 100 pg of template. This working level is not the true amount of DNA in any one LCN analysis. Once the amount of DNA is determined and it meets a LCN threshold criterion of amount of available DNA, the sample is divided in two or more aliquots for testing. Thus, the stochastic effects are exacerbated by further reduction on the amount of template DNA. The division of samples approach accepts loss of information to gain on reducing the chance of allele drop in. The quantitative stochastic threshold value is operationally defined for extant STR typing systems provided in commercial kits. The threshold template value will change with technology and genetic markers typed and should be determined through validation studies within the laboratory. Therefore 200 pg should not be misconstrued as a threshold that applies to all systems or all laboratories. It is a general value to consider as part of the analysis of LCN DNA samples. However, the value does seem to be a good first approximation for STR typing, which is the primary DNA analysis system used throughout the forensic science community.

A second value to consider when determining whether a sample meets the criteria of LCN typing is the stochastic or interpretation threshold value. The stochastic threshold is based on a value or range of values where the heterozygote allele peak height imbalance becomes exaggerated and is directly related with the initial template DNA. Amplification yield is directly related to the heights of allele peaks in a DNA profile and those peaks below a certain defined relative fluorescent unit (rfu) value are considered the result of LCN analyses. This threshold is determined within the laboratory through proper validation studies for each system employed. Unfortunately, under LCN typing conditions, all results fall below the stochastic threshold and thus implementation of the threshold is unwieldy and avoided by LCN typing practitioners. It is avoided because it is impossible to implement such a threshold. Regardless, the two parameters, starting DNA amounts and final amplified DNA product yield, should both be used in determining if LCN typing applies in a particular analysis. A single starting amount of DNA can be misleading as many forensic samples are mixtures and each component of a total amount is obviously less than the overall starting amount.

Lastly, if the laboratory attempts to manipulate the assay to increase sensitivity of detection there also is a concomitant increased risk of contamination. Special handling and multiple aliquots of a reduced template sample are recommended to minimize the effects of contamination. The plausibility of DNA transfer through handling should be implemented into procedures. All these phenomena need to be considered and addressed appropriately when conveying to the fact finders the significance or weight of the DNA evidence.

Because of these vagaries, LCN typing cannot be considered a robust methodology for identity testing, even for single source samples such as bones (which are more manageable than touch samples). Current LCN methods are not well-developed and the methodology can be improved substantially. In addition, often the confidence associated with a DNA profile associated with a sample is not well-defined and some laboratories have advocated reporting cannot exclude interpretations with no accompanying statistics. Such practice is a step backward for the field of forensic DNA typing that has established itself as the gold standard and distinguished itself by applying statistical weight to inculpatory evidence. LCN typing needs to be better developed so genetic data from missing persons cases can be exploited to its full potential and those individuals making identifications will be able to use the genetic information effectively.

The goals of this research project are to investigate separate and distinct processes that may improve upon the DNA typing process as it applies to low level DNA analyses, assess effects of primary and secondary transfer DNA, and to provide insight on the statistical issues needed to assess the significance of a LCN typing result. The analytical method approaches were undertaken as separate and distinct studies and not combined into one procedure. They were not combined because in some situations the circumstances of the samples may require only one method or multiple methods and these will vary with the sample; the purpose was to demonstrate that improvements could be made and that more resources should be dedicated to developing synergistic processes; and the combination of methods was beyond the resources of the study.

One area of research effort was to increase the starting template molecule by improving the overall extraction of DNA from skeletal remains and other 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. Improving sample extraction and being able to concentrate the sample can increase DNA yield. An additional consideration is that the purity of the extract can be enhanced. By removing inhibitors to the PCR, an effective increase in number of template molecules is possible. In other words, more yield can be obtained by reducing inhibition, even though the total number of template molecules for the PCR is the same or reduced. Another area of research was to attempt to improve the amplification by making the PCR of low template samples more robust. If there are less stochastic effects generated during the PCR, better quality results can be generated and then more reliable interpretation guidelines can be implemented. Methods for enhanced PCR yield were investigated. Primary and secondary transfer was considered as a potential for contamination. Transfer studies are contrived and may not shed light on the potential for contaminating evidence. The last area of research was an evaluation of guidelines for the interpretation of assay results from LCN work and to provide guidance for development of robust statistical approaches for assessing the weight of evidence for single source LCN typing results. An ancillary product of this work is a comprehensive literature list on published work on LCN typing.

The research summary in this Report described a number of findings that can improve LCN analyses. Methods have been evaluated and developed that offer potential practical solutions for improving the yield of DNA (from any source and particularly from bone). While hairs and teeth were initially suggested as additional sources of study, the work concentrated on bones as these samples are far more challenging. The results from the bones directly apply to other missing

persons type evidence. Yield is defined as a combination of quantity and purity of the DNA extract. Use of the Hi-Flow column enables DNA extraction from large volume samples that is substantially free of PCR inhibitors and can be readily implemented into any laboratory. Therefore larger samples can be extracted and washed to generate greater yield of DNA or a cleaner sample with fewer manipulations. Synchronous coefficient of drag alteration (SCODA) is a novel electrophoretic approach which was the most effective method for removing inhibitors (at much higher concentrations than has ever been tested) and can accommodate more than 5 ml of sample volume. This method shows promise and further work is warranted. Throughput and DNA free reagents are needed for the development of the SCODA methodology and work is underway. Pressure Cycling Technology was sought because it was hypothesized that extreme pressure could increase DNA yield from bone substrate. The results did show some yield increases (as trends), but they were not statistically significant. However, pressure was shown to have a notable impact on reducing the effect of inhibitors. Therefore, pressure may be a method of purification without removing inhibitors. More work should be considered on determining the range or classes of inhibitors and conditions that can be neutralized by pressure.

For improving the PCR, three additives were tested: betaine, DMSO and DNAGuard. While some of these additives have been used to improve genetic typing, there has not been a demonstrated effect of using these compounds to improve the PCR of LCN samples. Certainly sensitivity of detection can be increased with additives or for that matter by increasing the number of cycles of the PCR. The real benefits are if the additives can improve peak height imbalance or reduce stutter. Betaine appears to have some effect on reducing the amount of stutter. The effects are evaluated as trends as the variation in results is still wide. The analyses are subject to stochastic effects and thus variation is expected. The majority of low level DNA samples showed a decrease in the percent stutter and there was less excessive stutter with betaine treatment. DMSO did not reduce stutter further in the presence of stutter. DNAGuard did not show any improvement to the PCR and was not pursued further. Additional formulations of additives are available from Biomatrix and should be tested. The results show that additives can have some impact on the quality of PCR products. Given the reduction in stutter, it is anticipated that betaine (and other additives) will be quite useful for facilitating the interpretation of single source samples. Further work is needed to develop a cocktail that substantially reduces the stochastic effects generated during a PCR.

Primary and secondary (and tertiary) transfer is an important issue that impacts interpretation of results from any object that may have been handled (including bones). Most transfer testing has been under ideal and contrived but extremely unlikely conditions, i.e., contact with washed hands conditions. These studies suggest that the last person in contact with an item is generally the major contributor of the sample. In contrast a primary donor could be the prominent contributor if he/she was a “good shedder” and the secondary individual was a “poor shedder.” It was proposed that saliva, a DNA rich source, is a likely source of DNA on people’s hands and thus the impact of saliva transfer should be tested. The findings of transfer (primary, secondary, and tertiary) when saliva is involved provide more insight. Unlike the non-saliva studies where the last individual to come in contact with an object tends to be the major component, if saliva is the source from the primary donor, the primary donor can persist as the major contributor. This finding suggests that caution should be taken about inferences on who may have handled an object and to not to discount secondary transfer as a plausible explanation. In addition, the

concept of good and bad shedders has less relevance (if it ever had relevance because shedder status was never tested on suspects and unknown conditions leading to DNA deposition could influence the amount of DNA deposited). Saliva makes everyone a good shedder. These findings suggest that inferences on transfer with LCN data should be limited at best and that handling of bones from collection onward should be done with proper protective gear.

Statistical inferences are the weakest part of the LCN typing process, although strides have been made (see work of Balding as potential approach). Indeed, some practitioners have undertaken the practice of including individuals but not providing any statistical weight to the findings. This is in direct conflict with recommendations of the NAS Report on the Forensic Sciences (2009) and the DNA typing standards in the US. There are two methods for interpretation: a biological model and a statistical model. Both involve interpretation but only the latter provides 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 is often reported through modeling studies; eventually empirical data will be needed that reflect the range of variables encountered in real samples. A model for single source sample interpretation is provided that recognizes template sampling issues from a sample, stochastic effects during the PCR, and that the events may not be independent from locus-to-locus. This model can be based on the work of Balding and add further dimensions to interpretation of LCN typing results. Future work should be to derive empirical data from a robust technology that can be used to test and apply the single source model.

In conclusion, bones and other low quantity DNA samples are challenging to type. LCN typing, which entails increasing the sensitivity of detection, has been sought to be able to detect alleles that would not be observable under standard DNA typing conditions. However, under LCN conditions, the results are impacted by stochastic effects primarily when obtaining an aliquot from an extract and during the PCR. These excessive stochastic effects contribute to a lack of reproducibility and hence reliability of results obtained by LCN typing. To overcome some of the stochastic effects better methodologies are needed that approach the robustness of standard DNA typing strategies. Once developed, statistical methods that incorporate the uncertainty of the various features can be considered.

The findings herein have implications for policy and practice. The main policy and practice implications are that the data support that methodological improvements can be made that would enable some current LCN samples to be typed with standard conditions. In addition, analytical conditions can be implemented that will reduce stochastic effects. Thus reliability can be improved. More research should be dedicated to further developing the findings reported herein. Some of these methods are being evaluated for implementation into our missing persons program (which is the largest in the country and will have direct impact on the service). These include in particular the Hi-flow columns, SCODA, and pressure treatment. Combinations of additives described herein and others are being studied by us to develop a cocktail that will notably reduce stutter. Given the increase in high volume crime, and thus touch sample analyses, it is incumbent upon decision makers to invest in developing a fully functional LCN methodology, setting standards of operational performance, and defining conditions where LCN typing results apply and do not apply.

Introduction

Low copy number (LCN) typing is used to type DNA samples with very little DNA. Methods are employed that increase the sensitivity of detection of an assay so that the DNA typing results can be observed (1-14). However, samples with low amounts of template DNA are subjected to exaggerated stochastic effects and these effects can impact the reliability of DNA typing results (5,7,8,15). Most effects manifest as heterozygote allele peak height imbalance, allele drop out, and increased stutter. LCN typing has been defined as the analysis of any sample that contains less than 100-200 pg of template DNA (15-18). This quantitative threshold value is operationally defined for extant STR typing systems provided in commercial kits. The template value will change with technology and genetic markers typed (15). Therefore, 100-200 pg should not be misconstrued as a threshold that applies to all systems or all laboratories. It is a general value to consider as part of the analysis of DNA samples. However, the value does seem to be a good first approximation for STR typing, which is the primary DNA analysis system used throughout the forensic science community.

A second value to consider when determining whether a sample meets the criteria of LCN typing is the stochastic or interpretation threshold value (15-17). The stochastic threshold is based on a value or range of values where the heterozygote allele peak height imbalance becomes exaggerated, as stated above, but relies on the yield of amplified DNA product. Amplification yield is directly related to the heights of allele peaks in a DNA profile and those peaks below a certain defined relative fluorescent unit (rfu) value are considered the result of LCN samples. This threshold is determined within the laboratory through proper validation studies for each system employed. The two parameters, starting DNA amounts and final amplified DNA product yield, should both be used in determining if LCN typing applies in a particular analysis.

With degraded DNA, portions of the DNA profile could be above a threshold where the allele peaks would not be considered part of a LCN DNA analysis and other portions of the profile could be considered within the realm of LCN analysis. Furthermore, while the total amount of template DNA may be above 200 pg, mixed samples pose an additional consideration in that component contributors may be below the LCN threshold of 200 pg.

Lastly, if the laboratory attempts to manipulate the assay to increase sensitivity of detection there also is a concomitant increased risk of contamination (5,8,15). Special handling and multiple aliquots of a reduced template sample are recommended to minimize the effects of contamination. By dividing a sample in to multiple aliquots each containing less DNA, the stochastic effects are increased per aliquot compared with not dividing the sample. While this aliquotting of a sample results in loss of information it is sought to reduce the impact of allele drop in.

All these phenomena need to be considered and addressed appropriately when conveying the significance or weight of the DNA evidence. Because of these vagaries, LCN typing cannot be considered a robust methodology for identity testing. Current LCN methods are not well-developed and the confidence associated with a DNA profile associated with a sample is not well-defined. Thus, LCN typing needs to be better developed so genetic data from missing persons can be exploited to its full potential and those individuals making identifications will be able to use the genetic information effectively.

Project Goals

The goals of this research project are to improve upon the DNA typing process as it applies to low level DNA analyses, assess effects of primary and secondary transfer DNA, and to provide insight on the statistical issues needed to assess the significance of a LCN typing result. Initially, bones, hair and teeth were to be investigated. However, efforts were focused on bones as these samples tend to be more challenging and the results obtained can be directly applied to hairs and teeth. One area of research effort was to attempt to increase the starting template molecule by improving the overall extraction of DNA from skeletal remains and other 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) can be carried out. Improving sample extraction yield and being able to concentrate the sample can increase DNA yield. An alternative consideration is that the purity of the extract can be enhanced. By removing inhibitors to the PCR, an effective increase in templates is possible. In other words, more yield can be obtained by reducing inhibition, even though the total number of template molecules for the PCR is the same or reduced. Another area of research was to attempt to improve the robustness of the amplification by reducing the stochastic effects during the PCR. If there are less stochastic effects generated during the PCR, better quality results can be generated and then more reliable interpretation guidelines can be implemented. Methods for increased PCR yield were investigated. The last area of research was an evaluation of guidelines for the interpretation of assay results from LCN work and to provide guidance for development of robust statistical approaches for assessing the weight of evidence for single source LCN typing results. An ancillary product of this work is a comprehensive literature list on published work on LCN typing.

Final Report Results

Literature List

An important aspect of understanding the complexities of LCN typing and monitoring possible improvements in the analysis and interpretation of results is to maintain a current literature list. The references in this report represent a subset of a relatively comprehensive that contains the majority of peer-reviewed publications on the topic of LCN typing as it relates to forensic applications. The articles will be maintained with the PI because copying and disseminating the actual articles may violate copyright laws. These articles will be maintained electronically.

Improving DNA Yield and Enhancing Purity of Extracts

Hi-Flow Columns

Bone Extraction

The three most established methods for DNA extraction are phenol:chloroform, silica-based approaches, and ultrafiltration. The phenol:chloroform method is effective at removing proteins and lipids from bone extract, but tends to be ineffective for removal of hydrophilic compounds. This poses 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). Organic extraction also is 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.

Materials and Methods

The QIAquick/1 ml extraction involved single step overnight decalcification and protein digestion in 1 ml of Yang extraction buffer (YEB - 0.5 M EDTA, 0.5% SDS, 100 µg/ml ProK) at 56°C, followed by DNA recovery via QIAquick silica gel column (as modified from Yang et al., (19)). The QIAquick kit makes use of three buffers: PB buffer containing guanidine hydrochloride which is used to bind the DNA to the silica bed; PE buffer containing ethanol which is used to wash the undesired mixture components from the column; and EB buffer (10 mM Tris-HCl pH 8.5) which is used to elute the DNA bound to the column. Approximately 0.5 g of bone powder was transferred to a 2 ml screw-cap microcentrifuge tube, and 1 ml YEB was added to the bone powder. The mixture was vortexed thoroughly to suspend the bone powder. The suspension was incubated at 56°C with constant agitation overnight (up to 24 hours) in a rotating hybridization oven. The remaining bone powder was pelleted via centrifugation at 12,800 x g for 5 minutes. The supernatant was transferred to a 15 ml conical tube and 5 volumes of PB buffer added. The contents were mixed well by repeated inversion to avoid excessive foaming. In order to bind the DNA to the QIAquick column, 0.75 ml of sample/PB mixture were added to a column that had been placed in one of the 2 ml “catch” tubes supplied with the kit. The unit then was subjected to centrifugation at 17,900 x g for 1 minute, after which the flow through buffer was discarded. The remainder of the sample was loaded 0.75 ml at a time, repeating the centrifugation steps until all of the sample/PB mixture had passed through the column. The column was washed with 0.75 ml PE buffer and centrifuged at 17,900 x g for 1 minute. The binding and washing processes can be accomplished more rapidly by attaching the column to a vacuum manifold using disposable connectors. The flow-through was discarded and the column once more was subjected to centrifugation at 17,900 x g for 1 minute. This “dry” centrifugation step was necessary to remove residual alcohol. After the “dry” centrifugation, the column was transferred to a new 1.5 ml microcentrifuge tube and 50 µl EB buffer (10 mM Tris-HCl pH 8.5) were added directly to the unit’s silica gel membrane. The column was then incubated at room temperature for 1 minute and subjected to centrifugation at 17,900 x g for 1 minute to collect the eluate in a microcentrifuge tube. The elution step was repeated with a fresh microcentrifuge tube. The two elutions were stored separately at -20°C until further analyzed. The above method was repeated with more YEB in an attempt to further demineralize the bone powder. The QIAquick/3 ml protocol differed from the QIAquick/1 ml procedure in that 3 ml of YEB were used instead of 1 ml.

The chemistry for the Hi-Flow Protocol is essentially the same as for the QIAquick protocol, but on a larger scale. To maintain a consistent comparison, 0.5 g of bone powder was subjected to an overnight decalcification and protein digestion at 56°C in 3 ml of Improved Extraction Buffer (IEB - 0.5 M EDTA, 1% sodium lauroyl sarcosinate, 100 µg/ml ProK). Sodium lauryl sarcosinate (sarkosyl) was used instead of sodium dodecyl sulfate (SDS) because the former is soluble in 0.5 M EDTA at room temperature, while YEB (0.5 M EDTA, 0.5% SDS, 100 µg/ml ProK) must be heated before the SDS will completely solubilize. Bone powder was pelleted by centrifugation at 2545 x g for 5 minutes. The supernatant was transferred to a clean 50 ml conical tube and 5 volumes of PB buffer were added. Due to the tube’s larger volume capacity, the entire

volume of crude DNA extract/PB buffer mixture (approximately 18 ml) could be processed with a single centrifugation step at 2545 x g for 10 minutes. The Hi-Flow columns (Generon Ltd., Maidenhead, UK) 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 contained one of two types of glass fiber filter (A or B). Type B glass fiber is more densely “woven” than type A. Five types of experimental prototype Hi-Flow columns were used: type A units constructed with type A glass fiber filter with one, three, or six layers, and type B units constructed with type B glass fiber filter with one or three layers. Volumes of PE wash buffer were initially increased to 1 ml for use with the larger columns. Lower performance of columns containing multiple layers of glass fiber led to the use of increased wash volumes and multiple washing steps. A 5 ml wash single step and multiple washes up to three times (for total wash volumes of 5, 10, or 15 ml, respectively) were used. Collection of the washes was carried out by centrifugation at 2545 x g for 5 minutes, with a final dry column centrifugation at 2545 x g in an empty 50 ml conical tube for 5 minutes. DNA elution was accomplished using 100 µl of EB, which was repeated up to four times per Hi-Flow column, with an extended 5 minute incubation time at 25°C prior to centrifugation at 2545 x g for 10 minutes.

The original extraction method described by Loreille, et al (20,21) used a single step overnight decalcification and protein digestion at 56°C, followed by two rounds of organic extraction with PCIA (25:24:1 phenol: chloroform: isoamyl alcohol), a single butanol extraction, and subsequent volume reduction. A 0.5 g (\pm 0.02 g) of bone powder was transferred to a 15 ml conical tube, then 3 ml of extraction buffer (0.5 M EDTA, 1% sodium lauroyl sarcosinate, 1 mg/ml ProK) were added to the bone powder, and the mixture was vortexed to suspend the bone powder. The suspension was incubated at 56°C with constant agitation overnight (up to 24 hours) in a hybridization oven. Organic extraction was performed using an equal volume (3 ml) of PCIA followed by vortexing to create a uniform emulsion. The tube was centrifuged at 650 x g in a swinging bucket rotor for 3 minutes, and the aqueous layer transferred to a clean 15 ml conical tube. The organic extraction was repeated a second time with PCIA, followed by an extraction with butanol. The final aqueous layer was transferred to either a Centricon 30 or one of the Amicon Ultra-4 ultrafiltration devices. Amicon Ultra-4 devices with three different nominal molecular weight limits (NMWL) were used: 10K (10,000 NMWL), 30K (30,000 NMWL), and 50K (50,000 NMWL). The ultrafiltration devices were centrifuged at 650 x g until the level of retentate approximated 50 µl. Retentate volume was monitored at 30 minute intervals to prevent excessive drying of the membrane. At this time, 2 ml of TE (10 mM Tris-HCl pH 8, 1 mM EDTA pH 8) were added, and the centrifugation process repeated until the retentate volume dropped to approximately 50 µl. The TE wash step was repeated. The final concentrated retentate then was transferred by pipette from the ultrafiltration reservoir into a 1.5 ml microcentrifuge tube. A 50 µl aliquot of sterile, nuclease-free water was pipetted up and down in the emptied reservoir to collect any residual sample and this washing was pooled with the original retentate for a final recovered volume of approximately 100 µl. The different ultrafiltration devices varied in the number of 30 minute centrifugation intervals required to reduce the retained volume to 50 µl, as would be expected due to the variation in membrane pore size (NMWL) and membrane orientation. The Centricon 30 has a single horizontal membrane, while the Amicon Ultra-4 devices have dual vertical membranes designed to reduce problems associated with clogging. The Centricon 30 required a total centrifugation time of 6.5 hours, the Ultra-4 10K 6 hours, and both the 30K and 50K required 4.5 hours.

The modified Loreille, et al protocol differed from the authors 'original extraction method in four ways: generating a bone powder pellet after the overnight digestion step, reduction of the amount of ProK, an increase in the number and type of organic extractions, and the number and composition of washes used on the sample in the ultrafiltration device. The protocol began with a single step overnight decalcification and protein digestion in 3 ml of: 0.5 M EDTA, 1% sodium lauroyl sarcosinate, 100 µg/ml ProK (IEB) at 56°C and subsequent centrifugation at 650 x g for 10 minutes to clear the slurry of any remaining bone powder. This step was followed by two rounds of PCIA extraction, one round of organic extraction (24:1 chloroform:isoamyl alcohol), a single butanol extraction, and subsequent volume reduction using the Centricon 30 or one of the Amicon Ultra-4 ultrafiltration devices. The reduced extract was "washed" twice using 2 ml of TE for each device and once with 2 ml of sterile, nuclease-free water.

The HSC/YM100 protocol using the Microcon[®] YM100 ultrafiltration device (Millipore Corporation, Billerica, MA) involved two separate overnight incubation steps: first bone powder was decalcified overnight at 56°C in 1 ml 0.5 M EDTA, the EDTA was discarded, remaining bone powder was digested overnight at 56°C with 1 ml stain extraction buffer (SEB: 10 mM Tris, 100 mM NaCl, 39 mM DTT, 10 mM EDTA, 2% SDS - to which was added 20 µg of ProK), the bone powder was pelleted, the supernatant was subjected to organic extraction (PCIA), the aqueous phase was reduced to dryness with the Microcon YM100 device (horizontal membrane, nominal molecular weight limit 100,000), and the DNA was eluted from the YM100 device by centrifuging 100 µl of heated (56°C) ddH₂O in the reverse direction through the membrane.

Results of Bone Extraction Studies

The following results were obtained by extracting 0.5 g of bone powder from multiple sections of a single human femur. While some procedures use less bone, 0.5 g was used with the concept that challenged samples would have less DNA and more bone would be extracted (if available) with the concomitant effect of more inhibitor being recovered. Data from the Quantifiler[™] Human DNA Quantification Kit assay (real-time PCR quantification assay; Life Technologies) allowed comparison of the quantity of human DNA retrieved from a bone sample and a qualitative comparison of the level of inhibition in the DNA extract. Shifts in the internal PCR control (IPC) can be used qualitatively to assess purity of a DNA extract. For example, IPC C_T value will increase if the reaction is impeded by PCR inhibitors. To directly compare these values between real-time PCR runs, the data were normalized by comparing each sample's IPC C_T value to the average IPC C_T values of the DNA standards.

Of the silica devices, the least amount of DNA was recovered by the QIAquick columns (an average of 0.3 ng for a 3 ml extraction and 1.8 ng for a 1 ml extraction), but demonstrated negative shifts in IPC C_T values. A negative shift indicated that the IPC reaction was less inhibited than the IPC in the quantification standards.

Hi-Flow column descriptions were given as the type of glass fiber (A or B), followed by the number of layers of glass fiber (1, 3, or 6). The glass fiber type B columns with three layers (B3) recovered the most DNA (from 7.8-9.8 ng), but often had increased shifts in the IPC C_T values (i.e., indication of more inhibition). The other Hi-Flow columns varied considerably in DNA

recovery and IPC C_T shifts depending upon the sample pretreatment method, the number of washes with PE buffer, and the number of elutions. Figures 1 and 5 show DNA recoveries from bleach pretreated bone using various silica devices. The QIAquick/1 ml experiment used 1 ml of YEB, and the QIAquick/3 ml experiment used 3 ml of YEB. All four types of Hi-Flow column extractions started with 3 ml of IEB and were washed with a single 1 ml aliquot of buffer PE. DNA recovered by the QIAquick columns was found in the first elution only, and all subsequent elutions did not yield detectable amounts of DNA. While three layers of type B glass fiber captured by far the most DNA, this type of column also had the greatest positive shift in IPC C_T , which indicated the presence of inhibitory compounds.

Figures 2 and 6 show DNA recovery from bone with various silica devices. The QIAquick/1 ml experiment used 1 ml of YEB, and the QIAquick/3 ml experiment used 3 ml of YEB. All four types of Hi-Flow column extractions started with 3 ml of IEB and were washed with either a 1 ml aliquot of buffer PE (1 ml W), a 5 ml aliquot of buffer PE (5 ml W), 2 separate 5 ml aliquots of buffer PE (10 ml W), or 3 separate 5 ml aliquots of buffer PE (15 ml W). The DNA recovered by the QIAquick columns was observed only in the first elution and the DNA recovered from the Hi-Flow columns was the sum of DNA recovered in the first and second elutions. While three layers of type B glass fiber again captured the most DNA, it continued to have the greatest increased IPC C_T deviation, regardless of the number of washes. The type A glass fiber with 6 layers recovered the second largest quantity of DNA, and this sample exhibited a negative shift in the IPC C_T value when washed 3 times.

Figure 3 shows that DNA was recovered in four sequential elutions (E1, E2, E3, E4) from Hi-Flow columns A1, A3, B1, and B3. All columns were washed with a single 5 ml aliquot of PE buffer. Hi-Flow column descriptions were given as the type of glass fiber (A or B), followed by the number of layers of glass fiber (1 or 3), followed by the elution number (i.e. first elution from 1 layer of type A fiber would be A1/E1). Hi-Flow column A1 appeared to retain very little DNA, as all of it was released in the first 100 μ l elution. Hi-Flow columns A3 and B1 released about 2/3 of the total DNA in the first elution, with the other third recovered in elution 2 for column A3 and elutions 2 and 3 for column B1. Column B3 released only half of the total DNA in the first elution, and the majority of the remaining DNA was released in the second and third elutions. Thus, in general, additional layers of glass fiber filter gave the column additional capacity but required additional elution steps to recover the DNA.

To demonstrate the effects of wash volume on the quantity of DNA recovered, DNA yields from four elutions (E1, E2, E3, E4) from Hi-Flow columns A3, A6, and B3 are shown in Figure 4. Column descriptions were given as the type of glass fiber (A or B), followed by the number of layers of glass fiber (3 or 6), followed by the number of times that column was washed with 5 ml of PE buffer (2 or 3), followed by the elution number (i.e. the first elution from 3 layers of type A fiber washed twice would be designated A3(2)E1. Regardless of the number of washes, the B3 columns captured the largest amount of DNA but continued to generate the largest positive shifts in IPC C_T values. However, the IPC C_T values did improve in subsequent elutions. The most promising balance of high DNA recovery coupled with a negative shift in IPC C_T value was seen with column A6 when washed with 3 x 5 ml of PE buffer. It should be noted that multiple elution steps were performed on all silica devices, but only the Hi-Flow columns had quantifiable DNA in elutions two through four.

The Microcon[®] YM100 ultrafiltration device (Millipore Corporation, Billerica, MA) recovered less DNA (~2 ng) than other ultrafiltration devices, and as was observed with the QIAquick[®] columns, had negative shifts in IPC C_T values. All other ultrafiltration devices recovered between 6 and 12 ng of DNA but had positive IPC C_T shifts of 1-3.5 cycles. Figure 5 shows DNA recovery from bleach pretreated bone using Amicon[®] ultrafiltration devices (Millipore Corporation, Billerica, MA). These devices included the Microcon YM100 (nominal molecular weight limit of 100,000), Centricon[®] 30 (nominal molecular weight limit of 30,000) (Millipore Corporation, Billerica, MA) and the Amicon Ultra-4 ultrafiltration devices with three different nominal molecular weight limits (NMWL) 10K (10,000 NMWL), 30K (30,000 NMWL), and 50K (50,000 NMWL). The Amicon Ultra-4 devices with 30K and 50K NMWL recovered the largest amount of DNA, followed by the Centricon 30, Ultra-4 10K NMWL, and the YM100. With the exception of the YM100, the use of all ultrafiltration devices resulted in sizeable positive shifts in the IPC C_T values ranging from 0.75 to 3.5 cycles. Of these, the Ultra-4 50K had the smallest shift in IPC C_T value.

Figure 6 shows DNA recovery from UV pretreated bone using Amicon ultrafiltration devices. The Amicon Ultra-4 device with the 50K NMWL recovered the largest amount of DNA, followed by the Ultra-4 30K, Centricon 30, Ultra-4 10K NMWL, and the YM100. All ultrafiltration devices yielded DNA that exhibited large positive shifts in IPC C_T values. Of the ultrafiltration devices, the YM100 and the Ultra-4 50K had the smallest positive shifts in IPC C_T value when used to extract DNA from UV pretreated bone. These data suggested that UV treatment (as predicted) is not a desirable pre-treatment process and that bleach is more effective in removing surface contaminants and contaminating exogenous DNA. Also the fact that UV pre-treatment typically works well for naked DNA and not protein-complexed DNA, UV treatment adds little to bone extraction protocols.

Refined Hi-Flow Procedure for Extraction of DNA from Bone

The Hi-Flow extraction method involved a single step overnight decalcification and protein digestion in 3 ml of Improved Extraction Buffer (IEB - 0.5 M EDTA, 1% sodium lauryl sarcosinate, 100 µg/ml ProK). Remaining bone powder was pelleted by centrifugation for 5 minutes at 2545 x g. The supernatant was collected and mixed with 5 volumes of PB buffer (Qiagen Inc.), which contained guanidine hydrochloride and was used to bind DNA to the silica bed in the column. Due to the large capacity, the entire volume of crude DNA extract/PB buffer mixture (approximately 18 ml) could be processed with a single centrifugation step at 2545 x g for 10 minutes. Originally, a 5 ml wash with PE buffer (PE buffer contained ethanol and was used to wash the undesirable components from the extract) was investigated as a single step process, as was a 5 ml wash repeated 2 or 3 times (for total wash volumes of 5, 10, or 15 ml, respectively). The best performance of the column was achieved with 3 washes of 5 ml each; however, repetition of washes might be considered time-consuming and unwieldy (although could be performed and considered to ensure the best yield). So the wash protocol was tested with a single 15 ml wash (Figure 7 a, b, and c). The washes were collected by centrifugation for 5 minutes at 2545 x g, with a final dry column centrifugation in an empty 50 ml conical tube for 5 minutes at 2545 x g. DNA elution was accomplished using a 100 µl of low-salt EB buffer, which was repeated as many as four times per Hi-Flow column, and always with an extended 5 minute incubation time at 25°C prior to centrifugation for 10 minutes at 2545 x g. The IPC C_T

values are shown in Figures 7 and 9 as deviations in number of cycles, with a positive shift indicating inhibition.

Modern Bone Studies

Testing of the Hi-Flow protocol continued with testing of samples from bone samples from 10 different individuals. These samples previously yielded partial profiles using UNTHSC Center for Human Identification standard operating protocols. Bone powder from each individual bone section was sufficient to perform multiple extractions and, at a minimum, each bone was extracted using the Hi-Flow protocol and the newer Loreille, et al plus MinElute method (21).

DNA Quantification

More DNA was recovered using the Hi-Flow method for 7 of 10 samples (Figure 8). Extractions were performed on bone powder homogenized in the same grinding vial for each individual. Of the 10 samples shown in Figure 9, the Hi-Flow method showed less inhibition in 8 of the extraction comparisons.

DNA Profiles

The most important test criterion of any extraction methodology is whether sufficient quality and quantity DNA was recovered for successful DNA profiling. Figure 10 shows the total number of alleles identified from each individual using the Identifiler Plus kit (and 29 cycles of amplification). A detection threshold of 50 RFUs was applied. The same number of alleles was identified from DNA extracts generated using the Hi-Flow protocol and the Loreille, et al protocol in 8 of the 14 bones shown in Figure 10, and more alleles were identified from the Hi-Flow method extracts in 4 of the 14 bones. Another measure of DNA profile quality was peak heights (in RFUs). In Figure 11, the mean heights for all peaks were compared. In over half of the profiles, the Hi-Flow extracted samples had higher RFUs. The Hi-Flow method performed comparably with the Loreille, et al method overall, but required the use of only one device instead of two (resulting in reduced processing time).

Bones from Antiquity

Further testing of the Hi-Flow protocol was carried out on the skeletal remains from 11 individuals that lived in the Aragon region of Spain approximately 1000 years ago. The human bone samples were provided from the Genetic and Anthropological Study of the Aragonese Royal Family (Laboratory of Forensic Genetics, University of Zaragoza). The surface of the bone samples were decontaminated by sanding the outer surface of the bone followed by washing the bone in 10% bleach for 5 minutes, distilled water 2 times for 10 minutes each, and 100% ethanol for 1 minute. The bones were allowed to completely dry before proceeding to grinding.

Previous attempts at STR profiling of DNA extracts obtained from these remains using various other extraction methods had failed entirely or yielded very limited results with the Minifiler kit (Life Technologies). Of the samples from 11 individuals, 8 or more alleles for 5 individuals and 5 alleles for 2 individuals were obtained (22). Thus, the procedure described herein offers the potential to improve typing success of challenged samples. The likely impact is reduction in inhibitors.

DNA Purification

Synchronous Coefficient of Drag Alteration (SCODA)

Some forensic samples can present challenges for successful STR profiling and particularly for LCN typing due to low quantities of template DNA and/or the presence of inhibitors that can interfere with DNA amplification by the PCR. Commonly used extraction methods, such as silica adsorption and phenol:chloroform extraction, have been successful in recovering LCN DNA and removing inhibitors. However, 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 High-Flow columns described previously only small volume samples are accommodated in the extraction process. Synchronous coefficient of drag alteration (SCODA) is a novel technology (Boreal Genomics, Vancouver, BC) that effectively removes inhibitors while simultaneously concentrating DNA. SCODA exploits alternating electrophoretic fields for removing inhibitors from DNA (23-25). 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. In this study SCODA was used to extract, purify, and concentrate the DNA from challenged forensic samples. The SCODA process was evaluated for its ability to effectively remove contaminants that inhibit PCR and concomitantly concentrating 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.

Materials and Methods

To determine if SCODA is capable of effectively concentrating nucleic acids and minimize loss of DNA during manipulations, various concentrations of purified DNA (0ng/μl, 0.62ng/μl, 0.21ng/μl, 0.068ng/μl, and 0.023ng/μl; 50 μl total volume in TE⁻⁴ buffer) were processed using SCODA. Post-SCODA DNA yields were then compared with original starting template quantities using the Quantifiler™ Human DNA Quantification Kit. The initial yields were comparable or better than manual processes (Figure 12). Indeed, the lowest amount of DNA tests yielded the greatest percent recovery.

To test the ability of SCODA to successfully remove inhibitors from nucleic acids, various concentrations of hematin (100μL 6mM hematin), humic acid (300μg), melanin (40μg), and tannic acid (700μg) were mixed with a total of 10ng control DNA and run through the Aurora system. Concentrations of inhibitors and control DNA were chosen based on concentrations previously determined to fully inhibit a complete profile when amplified using the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit (Life Technologies). Prior to purification each “inhibited” sample was amplified using 1ng total DNA. Once the concentration of inhibitor at which dropout could occur was determined, a minimum of 10-fold concentrations of inhibitors were run using SCODA (see results for concentrations). All purified DNA extracts were at a volume of 100μL. One-tenth of the purified extract (10μL) was used with the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit to determine the impact of purification provided by SCODA. Since all concentrations were 10-fold, using one-tenth the extract can be considered representative of a true comparison with the initial non-purified amplification results.

An aliquot of these same contaminated samples also were purified using the QIAquick® PCR Purification Kit (Qiagen, Valencia, CA), a silica-membrane column, according to the manufacturer's recommendations. Elution volumes were brought up to 100µL and 10µL of the extract were amplified using the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit. STR profiles were generated using the 3500xl Genetic Analyzer (LifeTechnologies) and analyzed using GeneMapper® ID-X software (Life Technologies).

Results

STR profiles from purified products were generated for each purification method and compared for successful percentage allele recovery and RFU (relative fluorescence unit) peak height averages (Figure 13a and b). SCODA was able to purify hematin-, humic acid-, melanin-, and tannic acid-contaminated samples equal to and for higher concentrations better than the QIAquick® method. For humic acid- and tannic acid-inhibited samples, SCODA purification successfully cleaned these samples for downstream amplification yielding full profiles while QIAquick® purified DNA only generated partial profiles. In the case of hematin- and melanin-contaminated samples, both the SCODA and QIAquick® purification methods produced full profiles for each sample; however, average peak height RFUs for both hematin and melanin samples were higher when purified using SCODA. It is important to note that hematin-, humic acid-, and melanin-contaminated samples purified by the QIAquick® method were still dark colored, whereas when purified using SCODA these sample extracts were clear. This observation suggests that severely contaminated samples may be cleansed of inhibitors more effectively with SCODA than with the QIAquick® method. The SCODA approach provides an automated, minimal-step approach to successfully remove inhibitors and concentrate DNA from contaminated samples and is a promising technology to pursue further.

Pressure Cycling

Pressure Cycling Technology (PCT) (Pressure Biosciences, Inc., South Easton, MA) uses rapid cycles of hydrostatic pressure (up to 35,000 psi and greater) between ambient and ultrahigh levels to control biomolecular interactions (26-28). 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.

The primary 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 using known amounts of extracted DNA and cultured epithelial cells to determine if PCT can be an effective tool for increasing DNA yield. The experiments included: assessment of loss of DNA during tube transfer, effects of buffer types and volume,

extraction method, and tube coating by siliconization or addition of bovine serum albumin (BSA) or SDS.

Materials and Methods

Samples were: normal peripheral blood mononuclear cells (PBMC) (AllCells, Emeryville, CA) or purified DNA (Quantifiler Human DNA Standard; 200ng/ul). These samples were added to each swab by placing the pipette tip directly into the swab at a depth of 2mm before expelling DNA at the appropriate quantity. Swabs were dried overnight before extraction.

Sample Processing with Maxwell 16 Extraction Method

All samples were incubated in 190µL working Incubation Buffer with DTT and 10uL Proteinase K for 1 hour at 56°C. Note: Samples processed with PCT were cycled after the 1-hour incubation period. Both the swab and the incubating solution were placed in a PULSE tube. After cycling, the tubes were centrifuged. The swabs and solution were removed from the PULSE tubes and processing resumed with the forensic protocol for Maxwell 16® extraction according to the manufacturer's recommendations. Following incubation (or after cycling), 400uL of working Lysis Buffer were added to each sample and the samples were briefly vortexed. All samples were spun down using a spin filter basket inside 1.5mL tubes for 2 minutes at 20000xg. Samples were then loaded into their respective sample cartridges for Maxwell 16® extraction. Following Maxwell 16 extraction, samples were quantified using the Quantifiler kit.

Sample Processing with Qiagen QIamp Extraction Method

400µL of PBS in addition to 20µL Proteinase K and 400µL Buffer AL were added to each sample. Samples were mixed by vortexing for 15s. Samples were incubated for 10 minutes at 56°C. Following incubation, PCT was performed. Following PCT or No PCT, 400µL of ethanol (100%) were added to each sample. 700µL of the mixture were added to the spin column and centrifuged at 6000xg for 1 minute. The filtrate was discarded and this step repeated until all of the mixture was added to spin column. 500µL of Buffer AW1 were added to the QIamp spin column without wetting the rim. The tubes were then centrifuged at 6000xg for 1 minute and the filtrate was discarded. 500µL of Buffer AW2 were added and the tubes centrifuged at full speed at 20000xg for 3 minutes. The filtrate was then discarded. The spin column was then placed in a clean 1.5ml centrifuge tube and 150µL of Buffer AE were added to tube. The tubes were incubated for 1 minute at RT. The tubes were then centrifuged at 6000xg for 1 minute. Following QIAamp extraction, samples were quantified using Quantifiler.

DNA Recovery

To determine DNA yields with varying DNA quantity inputs, PCT was performed on three different known quantities of DNA (1 ng/ul, 0.5ng/ul, and 0.1ng/ul) and compared with samples not subjected to PCT. The experiment was performed in triplicate. The results indicated that, at lower quantities of DNA, no difference is observed between samples exposed to PCT and those without PCT (Figure 14).

To improve the process, sample manipulations were minimized; the sample was kept in the PULSE tube throughout PCT and the extraction process. Reducing sample manipulation

throughout the extraction process reduced DNA loss compared with multiple sample transfer steps (Figure 15). Further investigation demonstrated increased DNA recovery when a 1:2 volume of incubation to lysis buffer was used. In addition, buffer was compared with water (Figure 16), resulting in improved DNA yields when buffer and PCT were used. Results also demonstrated a trend towards increased DNA yield from purified and cellular DNA following PCT compared with samples not exposed to pressure technology. The trend was more pronounced when smaller DNA quantities were tested (Figure 17). PCT can be used in conjunction with commercially available extraction reagents. It is important to note that the results of increased yield with PCT were trends and significant differences were not observed.

DNA recovery may be increased with PCT with process improvements. The PULSE tubes, used for subjecting samples to pressure conditions, were not treated (as manufactured) to prohibit DNA from binding to the side, as well as other parts, of the tubes. A coating of the tube might block non-specific binding of DNA and allow full exploitation of pressure technology which could increase yield. All experiments used approximately 1 ng/μl purified DNA (except the BSA experiment that used 50μL of white blood cells (200 cells/μl or approximately 1.2ng/ μl). The DNA was extracted using the Qiamp^{55%} system and brought to a final volume of 150μl per sample. The DNA after testing was recovered in the same volume (150μl).

In a number of experiments, the 1.5 ml microcentrifuge tubes yielded higher DNA recovery compared with PULSE tubes (Figure 18). Thus further studies were undertaken to enhance recovery of DNA from the pulse tubes. These studies were undertaken to attempt to exploit the potential benefits of pressure for increasing DNA yield from bones and other materials. DNA recovery from Teflon coated 0.2 ml microtubes (Pressure Biosciences, Inc.) was compared with yield from 0.2 ml PCR tubes, polypropylene tubes, and polyethylene PULSE tubes. The results demonstrated increased DNA recovery with the Teflon coated microtube with similar recovery observed in the polypropylene PULSE tube. However, the difference in yield was not significant and only trends are demonstrated (Figure 19).

Two different types of PULSE tubes were tested for DNA recovery. Polypropylene PULSE tubes were compared with Polyethylene PULSE tubes. Although DNA tends to bind preferentially to polyethylene, the results of several experiments did not support binding preferentially to polyethylene (Figure 20). No differences in DNA recovery from polypropylene tubes compared with polyethylene tubes were observed.

PULSE tubes were coated with a blocking agent, UltraTrol LN (Target Discovery), which is a reagent that has been used successfully in blocking microarrays. A 1:10 dilution of UltraTrol was used to treat PULSE tubes for two minutes each per manufacturer's recommendations. Following treatment with UltraTrol, the tubes were allowed to dry under ambient conditions overnight prior to use. While no significant differences were observed between treated and untreated polyethylene tubes, a slight increase in DNA recovery was observed with the UltraTrol treated polypropylene tubes compared with the untreated tubes (Figure 21); again only a trend was observed.

No difference in DNA yield was observed using siliconized vs. non-siliconized tubes. A concern was that organic solvents could leach compounds from the plastic into the solution, and these compounds could negatively impact downstream applications. Therefore, BSA and SDS were

tested as potential coating agents. PULSE tubes were rinsed with either a 1% BSA or SDS solution and dried overnight prior to use. BSA appeared to improve DNA yield (Figure 22), which could be due to surface blocking. In contrast, SDS was ineffective (Figure 23). It may be that under pressure SDS forms tubules and pulls away from the tube surface. This action may expose the sides of the tube for DNA binding sites or leach compounds that impact on downstream processes.

A proprietary solution from Corning also was used to treat PULSE tubes. The treatment of the PULSE tubes was performed by Pressure Biosciences, Inc, prior to shipment to our laboratory. The treated PULSE tubes then were tested to determine if DNA yield was improved and if downstream analyses would be compromised. The treated PULSE tubes had notably lower DNA yields compared with untreated PULSE tubes for both pressured and non-pressured samples (Figure 24). Pressure treated samples yielded more DNA compared with non-pressure treated samples. For both untreated and treated PULSE tubes, no affect was observed on quantitation, amplification, or capillary electrophoresis. Treatment had no detectable impact on electrophoretic results; a full, clean profile was observed using the Identifiler kit.

Even though some preliminary results show that yield of DNA is improved for both bone and hair (See Figures 25-27 below), the results were only trends. Yet, there was an example of a bone sample that appeared to generate substantial yield when exposed to PCT (Figure 25). The inconsistency of this observation with all other results showing only trends suggested that some other phenomenon may be occurring.

Reducing Effects of Inhibition with PCT

While pressure treated materials did not provide a substantial increase in DNA yield, there was a notable increase in results for some bone samples. Thus, there may be an alternate explanation for the successful increase in bone sample results than increased yield. Forensic samples can contain contaminants that may co-purify with the DNA and inhibit the downstream PCR which could portray as reduced DNA yield. Low quantity samples may appear to have an effective low amount of DNA but in reality yield sufficient DNA template for standard DNA analysis. The presence of inhibitors of the PCR can present the sample effectively as a low copy sample. Mollifying the effects of inhibitors may increase the effective yield of challenged low copy samples (28-32). With more yield the samples stochastic effects would be reduced. It was possible that the conditions of extreme pressure may alter the conformation of some inhibitors (or the DNA) and render less effective inhibition.

Materials and Methods

To determine if pressure cycling technology can denature inhibitor compounds, several concentrations of hematin (0, 2.5 $\mu\text{M}/\mu\text{l}$, 5 $\mu\text{M}/\mu\text{l}$, and 7 $\mu\text{M}/\mu\text{l}$) and humic acid (0, 2.5ng/ μl , 5ng/ μl , and 7ng/ μl) were exposed to conditions of extreme pressure. The Quantifiler™ Human DNA Quantification Kit IPC contained within each reaction serves as an indicator of the possible presence of PCR inhibitors and was used to assess the effects of inhibitors on the PCR process.

Results

In the presence of 2.5 $\mu\text{M}/\mu\text{l}$ and 5 $\mu\text{M}/\mu\text{l}$ hematin and 2.5ng/ μl humic acid (HA), the Ct value of a qCR assay increased with increasing concentration of inhibitor. However, following pressure cycling, IPC Ct values were lower for 5uM/ul Hematin and 2.5ng/ul HA (Figure 28). At 7

μM/μl for Hematin and 5ng/μl and 7ng/μl of Humic Acid, the IPC was not amplified for either PCT or non-pressure treated samples.

These initial experiments focused on whether pressure affected the inhibitors directly. The next set of experiments focused on determining if pressure could impact the effect of inhibitors in the presence of DNA (Table 1, Figures 29-30). Clearly, there is a notable affect on reducing inhibition for samples exposed to PCT. These results of pressure effects on inhibitors may explain the notable increased yield in some bone experiments. The increased yield may be due to reducing the effects of inhibitors as opposed to increasing the yield of DNA by extraction. The evaluation of performance was a PCR-based assessment and thus one may not distinguish between yield increase and enhanced PCR efficiency.

PCR Enhancement with Additives

The Use of DNAgard™ Reagent to Improve DNA Stability and/or Recovery from Swabs

DNAgard™ (Biomatrix®, San Diego, CA) is designed for room temperature storage and shipment of DNA in biological samples, such as mammalian cells and tissues. DNAgard™ has unique stabilization properties, permeating cell structures and membranes, and aggressively destroying proteins (33). Enzymes are immediately destroyed so no DNA degradation occurs even during a relatively lengthy extraction process. In addition, the process separates proteins from DNA. Five formulations of DNAgard-like materials were obtained from Biomatrix. These formulations were tested as a stabilizing agent to reduce degradation. The effects were assessed by yield of purified DNA.

Materials and Methods

Purified DNA (Quantifiler Human DNA Standard; 200ng/ul) in concentrations of 1 ng/ul, 0.5ng/ul, and 0.1ng/ul was added to each swab by placing the pipette tip into the swab at a depth of 2mm before expelling DNA at the appropriate quantity. Swabs were dried overnight before extraction.

Five formulations of DNAgard™ (Biomatrix, Inc.) were tested and compared to TE⁻⁴ buffer. Swabs were placed in microcentrifuge tubes containing one of the five formulations of DNAgard™ or TE⁻⁴ buffer. Following overnight storage of the samples at room temperature, the samples were extracted using QIAamp® DNA Mini and Blood Mini (Qiagen). 400μL of PBS in addition to 20μL Proteinase K and 400μL Buffer AL were added to each sample. Samples were mixed by vortexing for 15s. Samples were incubated for 10 minutes at 56°C. Following incubation, 400μl of ethanol (100%) were added to each sample. The swab was added to the spin column and centrifuged at 6000xg for 1 minute. 700μL of the mixture were added to the spin column and centrifuged at 6000xg for 1 minute. The filtrate was discarded and this step repeated until all of the mixture was added to spin column. 500μL of Buffer AW1 were added to the QIamp spin column without wetting the rim. The tubes were then centrifuged at 6000xg for 1 minute and the filtrate was discarded. 500μL of Buffer AW2 were added and the tubes centrifuged at full speed at 20000xg for 3 minutes. The filtrate was then discarded. The spin column was then placed in a clean 1.5ml centrifuge tube and 150μL of Buffer AE were added to

tube. The tubes were incubated for 1 minute at RT. The tubes were then centrifuged at 6000xg for 1 minute. Following QIAamp extraction, samples were quantified using Quantifiler.

Results

In initial experiments, three different concentrations of purified DNA (Quantifiler standard), 1ng/ul, 500pg/ul, and 100pg/ul were tested. All samples were run in triplicate and DNA yields were assessed using the Quantifiler kit. There was no apparent significant difference at either 1ng/ul or 0.5ng/ul purified DNA with no treatment generally trending towards better performance (Figure 32). For the lowest concentration of DNA tested, formulation 2 appeared to have increased DNA yield but further testing is needed, but again this was not significant. Given the results had no impact, no further testing of the formulations was undertaken.

Additives to Reduce Stutter

LCN results are visualized by increasing sensitivity of detection. When increasing the sensitivity of detection to type LCN samples, stochastic effects during PCR are so exacerbated that, for STR analysis purposes, peak height imbalance, allele drop out, and increased stutter will occur (5,8,15,34-42). Stutter could complicate interpretation for single source LCN typing because stutter may present similar as an allele (and this complication is more vexing for mixture interpretations). The most plausible mechanism for generating stutter is strand slippage when the polymerase pauses during extension. 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 its original allele state. Reduction in stutter would facilitate typing 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 generated during PCR amplification (43-56). Two known PCR enhancers, betaine and dimethyl sulfoxide (DMSO), were assessed for their abilities to reduce stutter. Both betaine and DMSO facilitate strand separation. Betaine acts as an isostabilizing agent, equalizing the contribution of GC- and AT-base pairing to the stability of the DNA duplex; while DMSO acts by disrupting base pairing (55, 57-64). It is important to note that betaine has been reported as an additive for DNA typing; however, there are no reports on its use to ameliorate the stochastic effects of low level templates. Therefore, a controlled study was undertaken.

Materials and Methods

Buccal swabs of 100 de-identified individuals were extracted using the AutoMate Express™ Forensic DNA Extraction System (Life Technologies, Carlsbad, CA). All DNA extracts were stored at -20°C until analyzed. The quantity of recovered DNA was determined using a reduced volume protocol of the Quantifiler® Human DNA Quantification Kit and the ABI 7500 Real-Time PCR System according to the manufacturer's instructions. Samples were normalized to 25, 50, and 100pg. Standards were assayed in duplicate and all experimental samples were assayed singularly for statistical analyses. Standard curves were generated for each experimental set, and amplification plots were used to compare individual PCR reaction consistency, efficiency, and results. IPC results from each assay were monitored for the presence of PCR inhibitors. Negative (no template DNA) and reagent blank controls were included on each assay plate.

Betaine (Sigma, St. Louis, MO) was added at the following final concentrations in the amplification reaction mix: 0.5 mol/L, 1.25 mol/L, and 2 mol/L. DMSO (Sigma) was added at

the following final concentrations in the amplification reaction mix: 1%, 5%, and 10%. Mixtures of various concentrations of betaine and DMSO were prepared using 1.25mol/L betaine and 5% DMSO. Negative (no template DNA), positive (9947A), and reagent blank controls were included in all amplifications.

Primers for two specific loci, D18S51 and D21S11, kindly provided by Life Technologies, were used as a model to evaluate the effects of PCR enhancers on amplification of LCN DNA samples. These loci have shown higher levels of stutter in our hands (data not shown). Thermal cycling was performed using the Identifiler protocol on a GeneAmp® PCR System 9700 as follows: initial denaturation at 95°C for 11 min; 34 cycles of 94°C for 1 min, 59°C for 1 min, and 72°C for 1 min; hold at 60°C for 60 min; and an indefinite hold at 4°C.

Prior to electrophoresis, 1 µL of the amplified product or allelic ladder and 0.5 µL of GeneScan™-500 LIZ® size standard (Life Technologies) were added to 8.5 µL of deionized Hi-Di™ formamide (Life Technologies), denatured at 95°C for 5 min, and placed on ice for 5 min. PCR products were separated and detected on an ABI PRISM® 3130 xl Genetic Analyzer (Life Technologies) following the manufacturer's recommendations. Samples were injected for 10 s at 3 kV and separated electrophoretically in performance optimized polymer (POP-4™; Life Technologies) using the HIDFragmentAnalysis36_POP4 Module (Life Technologies) and a 1,500 sec run time. The data were then collected using the ABI PRISM® 3130xl Genetic Analyzer Data Collection Software 3.0. Electrophoresis results were analyzed with GeneMapper® ID software v3.2 (Life Technologies). Allele peaks were called when the peak heights were equal to or greater than 25 RFU. (Note: We are not recommending a 25 rfu detection threshold for routine work. The threshold was lowered to enable detection of stutter to facilitate estimating stutter percentages given the stochastic effects during the PCR). The effects on PCR product concentration (RFU), peak height ratio (heterozygous loci), and degree of stutter and variance of these ratios were evaluated and compared with controls.

Results

Amplification reaction mixes were prepared for four different test groups: Control (no addition of betaine or DMSO), 1.25M betaine, 5% DMSO, and a combination of both 1.25M betaine and 5% DMSO. Primers for the loci D18S51 and D21S11, loci known to present higher levels of stutter, were used to generate amplicons to serve as models to assess if stutter can be reduced by the addition of betaine and/or DMSO in the PCR. Buccal swabs of five individuals were extracted using the AutoMate Express™ Forensic DNA Extraction System (Life Technologies, Carlsbad, CA) and PCR was performed on 1ng/µl and 0.5ng/µl template DNA at both 28 and 34 cycles (for five individuals each). Samples were then analyzed on the ABI Prism® 3130xl Genetic Analyzer (Life Technologies) and data were analyzed with GeneMapper® Analysis software (Life Technologies). Samples were amplified in triplicate and subjected to electrophoresis.

Results

Stutter percentages at both loci (under 28 cycles of PCR) were then evaluated (Figures 33 and 34). The standard error is still large and this is to be expected. The studies are evaluating stochastic effects and these effects by their nature will range widely. Therefore, the results are evaluated as trends. The data indicate that stutter can be reduced with primarily either the

addition of betaine. Betaine and DMSO could be considered but the impact was minor at best. The results for the 34 cycle were similar in trend with that of 28 cycles. However, caution is suggested with using the data for in depth interpretation of these 34 cycle results because the alleles are saturated with 0.5 and 1 ng of template DNA.

The next experiments reduced the template amounts down to that routinely considered as low template DNA levels, i.e., 100pg and 25pg, and used a larger sample size (n = 86 individuals). While no significant decreases in stutter were observed, the trends suggest that stutter was reduced with the addition of betaine under the conditions of LCN DNA and increased PCR cycle number (Tables 2 and 3). Stutter for the majority of the LCN samples, 80 of 84 samples at 100pg and 70 of 77 samples at 25pg, was reduced compared with controls. Although not significant, the fact that the majority of paired samples showed reduced stutter compared with controls shows promise that stutter could be reduced with additives to the PCR. These data support that some improvements can be made impacting stochastic effects. Additional resources should be considered to develop a cocktail of additives and use of alternate cycling conditions. No significant differences in peak height ratio were observed. Thus, additional conditions will be needed to improve peak height imbalance under LCN conditions.

Reducing stochastic effects is a difficult endeavor. It may be that sampling issues of the DNA in an extract and during the PCR may not be possible to overcome substantially. However, given the increase desires to analyze low level samples, further efforts are warranted.

DNA Transfer

Touch DNA analysis, 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 of forensic profiles and the relevance of the information even for single source samples, including bones. 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. A number of studies have been conducted with the intent of investigating primary transfer, which occurs when DNA is directly transferred from an individual to an object or another individual. For instance, it is thought that a person who grips a knife handle is likely to slough off epithelial cells onto the handle, thus depositing his/her DNA.

Another form of DNA transfer is known as secondary transfer. This is an indirect form of DNA transfer in which the individual depositing the DNA does not actually make contact with an individual or an object. Instead, the DNA is transferred through a vector, i.e., an intermediate object or person. An example of secondary transfer would be a situation in which an individual shook hands with a second individual, who then transfers the initial person's DNA onto a doorknob. Many of the studies conducted thus far on the subject of secondary transfer events involving human vectors have indicated that the majority of detectable DNA on the final object originates from the vector (65). Thus, some researchers have concluded that only minimal amounts of DNA are transferred from the primary individual (65). However, most of these secondary transfer studies have been somewhat limited in that they have focused on DNA deposited through the 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/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 occurs when the first individual's DNA is passed from the pen to the second individual's hand. This study is being conducted under the hypothesis that saliva, which is rich in DNA (e.g., see 68), 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-based DNA may result in higher levels of detectable DNA than what has been previously observed. In addition, the genetic profile of the primary individual may indeed be much more prevalent in such cases of secondary transfer due to the saliva-based DNA that is initially deposited.

Therefore, a goal of this study is 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 explanation based on the amount of recovered DNA. To address this question, experiments were conducted in a manner that mimicked natural human behavior in a controlled manner. For instance, male and female subjects were required to lick their thumbs and hold pens in their mouths. Separate sets of experiments were carried out in order to recreate both primary and secondary transfer events. In addition, a third set of experiments represented tertiary transfer events, where DNA deposited from an individual to an object or individual was then transferred to another object or individual. The genetic data collected from the experiments were analyzed to assess the amount of DNA transferred and the relative decrease in the levels of DNA that occurred as the number of vectors increased. DNA profiles that were obtained from these experiments were then analyzed to determine the ratio of DNA recovered from the different contributors following the transfer events. Different drying times were utilized in order to ascertain what effect (if any) this had on the transfer of saliva-based DNA. Sweat was imitated during the experiments to mimic natural conditions and to observe any effects that this may have on DNA transfer.

Subjects

A total of four individuals (two male and two female) over the age of 18 were used for this study. Buccal swab samples were collected from these subjects to serve as references for the study. One male subject was paired with one female subject in a manner that allowed for the greatest number of differences in alleles between the genetic profiles of the individuals within each pair, for maximum value of mixture data interpretation.

Experimental Design

This study was divided into three distinct sets of experiments. The first set of experiments was designed to investigate primary transfer events, the second set of experiments focused on secondary transfer events, and the third set of experiments examined tertiary transfer events. For each experiment set, 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 DNA was determined by using the Applied Biosystems® Quantifiler™ Human DNA Quantification Kit (Applied Biosystems, Inc., Foster City, CA) on an Applied

Biosystems® 7500 Real-Time PCR System, according to the manufacturer's protocol. Amplification of the samples was performed using the Applied Biosystems® AmpFℓSTR® Identifiler® Plus PCR Amplification Kit (Life Technologies Corporation, Carlsbad, CA) on an Applied Biosystems® GeneAmp® PCR System 9700 thermal cycler, according to the manufacturer's recommendations. Capillary electrophoresis was then performed on an Applied Biosystems® 3130xl Genetic Analyzer using POP-4™ polymer, and analyzed using Applied Biosystems® GeneMapper® ID v3.2 software, according to the manufacturer's protocol. In cases where the standard 28 cycles of PCR did not yield interpretable results, the extracted sample was re-amplified in duplicate using 34 cycles. Alleles were only called if they appeared in both replicates and had peak heights equal to or greater than 50 RFUs.

Primary Transfer Trial: Bare Thumb Lick

For the purposes of this trial, each subject washed his/her hands with soap and dried them. Each subject then licked his/her bare thumb by extending his/her tongue and running his/her thumb down the tongue once. The thumb was then allowed to dry, untouched, for 5 minutes. The thumb was swabbed using the double swab technique, in which a sample was obtained through the use of a wet swab followed immediately by a dry swab. This swabbing method has been shown to recover and yield more DNA than traditional swabbing methods (66). The hand-washing and licking procedures were repeated, and the thumb was allowed to dry for 30 minutes, followed by the double swab collection. Both the 5- and 30-minute procedures were performed in duplicate, for a total of 16 samples (4 per subject).

Primary Transfer Trial: Gloved Thumb Lick

The procedure for this trial was identical to that of the Bare Thumb Lick trial, except that each subject wore a latex glove after washing, thus requiring them to lick their gloved thumbs. The thumbs were allowed to dry and then swabbed, as in the previous trial. Both the 5- and 30-minute procedures were performed in duplicate, for a total of 16 samples (4 per subject).

Primary Transfer Trial: Bare Palm Swab

For this trial, each subject washed his/her hands with soap and dried them. After 5 minutes post-wash, each subject's bare palm was swabbed using the double swab technique. The hand-washing procedure was repeated, and the palm was allowed to dry for 30 minutes post-wash, followed by the double swab collection. Both the 5- and 30-minute procedures were performed in duplicate, for a total of 16 samples (4 per subject).

Primary Transfer Trial: Pen in Mouth

For this trial, each subject held the back (non-capped) end of a sterilized pen in his/her mouth for 30 seconds. The pen was allowed to dry, untouched, for 5 minutes. The pen was then swabbed using the double swab technique. The pen-holding procedure was repeated, and the pen was allowed to dry for 30 minutes, followed by the double swab collection. Both the 5- and 30-minute procedures were performed in duplicate, for a total of 16 samples (4 per subject).

Primary Transfer Trial Results

Genetic data obtained from this first set of experiments were used to evaluate the primary transfer of DNA. The quantity of saliva-based DNA obtained from the bare thumbs of the subjects was compared with that obtained from the subjects' gloved thumbs in order to attempt

to determine a rough estimate of the DNA transferred in a single lick or pen-holding event. However, the results of the primary transfer trials, as explained below, eliminated the feasibility of these calculations. The DNA yield from the bare palms of the subjects was also quantified in order to provide a general baseline of native DNA levels for transfer events to individuals.

DNA quantity charts for the primary transfer trials are shown in Figures 35-38. The majority of cases showed that licked gloved thumbs yielded more DNA. This is believed to be due to the smoother, less porous surface of the glove allowing for more efficient collection of DNA via the swabbing technique than the rougher, ridged thumb surface. This is consistent with a previous study by Goray et al. (67) which determined that smooth, non-porous surfaces, such as plastic, yield higher quantities of transferred DNA than rougher, porous surfaces, such as cotton and wool. The effects of the different surface areas on the DNA yield made the estimation of saliva-based DNA quantity in a single lick less meaningful for this study. The effects of a smoother surface composition, in addition to the greater surface area, esupports the observation that DNA is sometimes obtained in larger quantities from pens held in the mouth of a subject as opposed to the subject's licked thumb. Significant variation in DNA yield wass observed from one replicate to another in each trial, suggesting, as expected, that deposited DNA varies from lick to lick. This makes normalization difficult, and inferences made from this study must thus be recognized as generalized estimates and conclusions.

Amplification of the primary transfer trial samples at 28 PCR cycles, followed by capillary electrophoresis, yielded full genetic profiles in almost every case. An example of these findings can be seen in Figure 39. The notable exception to these results was the group of bare palm swab samples, which generally yielded no genetic profiles. Due to the fact that subsequent secondary transfer trials using the subjects' bare palms as a transfer surface would most likely be amplified using 34 PCR cycles, the bare palm swab samples were amplified in such a manner and re-subjected to capillary electrophoresis. Even at these increased amplification cycles, the bare palm swab samples only yielded genetic profiles showing up to 22.2% of the expected alleles (with one notable exception that showed 53.8% of the alleles). An example of the profile percentages at 34 PCR cycles can be found in Figure 40. These results indicated that the sloughed off epithelial cells on the subjects' palms were not sources of abundant DNA.

Secondary Transfer Trial: Licked Bare Thumb to Plastic Tube

For this trial, each subject washed his/her hands with soap and dried them. Each subject then licked his/her bare thumb by extending his/her tongue and running his/her thumb down the tongue once. The thumb was then allowed to dry, untouched, for 5 minutes. The subject then grasped a sterilized plastic tube for 15 seconds, applying moderate pressure with his/her thumb in a specific location on the tube. The tube was then swabbed using the double swab technique. The hand-washing and licking procedures were repeated, and the thumb was allowed to dry for 30 minutes, followed by a tube grasp and the double swab collection. Both the 5- and 30-minute procedures were performed in duplicate, for a total of 16 samples (4 per subject).

Secondary Transfer Trial: Pen to Palm

In this trial, each subject held the back (non-capped) end of a sterilized pen in his/her mouth for 30 seconds. The pen was allowed to dry, untouched, for 5 minutes. The pen was then passed to

the subject's partner, and the partner grasped the pen with moderate pressure for 15 seconds. The partner's palm was then swabbed using the double swab technique. The pen-holding procedure was repeated, and the pen was allowed to dry for 30 minutes, followed by the double swab collection. Both the 5- and 30-minute procedures were performed in duplicate and the whole experiment was repeated, with the roles of the subjects in the pair reversed, for a total of 16 samples (8 per pair).

Secondary Transfer Trial: Licked Gloved Thumb to Plastic Tube

The procedure for this trial was identical to that of the Licked Bare Thumb to Plastic Tube trial, except that the subjects wore a latex glove after washing, thus requiring them to lick their gloved thumbs. After the specified drying time, the subjects grasped a sterilized tube and the tube was swabbed, as in the Licked Bare Thumb to Plastic Tube trial. Both the 5- and 30-minute procedures were performed in duplicate, for a total of 16 samples (4 per subject).

Secondary Transfer Trial: Pen to Moist Palm

The procedure for this trial was identical to that of the Pen to Palm trial, except that the recipient subjects' palms were sprayed once with sterile water from an atomizer right before the recipient subjects gripped the plastic tube, in order to imitate sweat. The palms were then swabbed, as in the Pen to Palm trial. Both the 5- and 30-minute procedures were performed in duplicate and the whole experiment was repeated, with the roles of the subjects in the pair reversed, for a total of 16 samples (8 per pair).

Secondary Transfer Trial: Licked Moist Bare Thumb to Plastic Tube

The procedure for this trial was identical to that of the Licked Bare Thumb to Plastic Tube trial, except that the subjects' thumbs were sprayed once with sterile water from an atomizer after the drying time was complete, in order to imitate sweat. The subjects then grasped a sterilized tube and the tube was swabbed, as in the Licked Bare Thumb to Plastic Tube trial. Both the 5- and 30-minute procedures were performed in duplicate, for a total of 16 samples (4 per subject).

Secondary Transfer Trial Results

Genetic data obtained from this second set of experiments were used to evaluate the secondary transfer of DNA. The quantities of recovered DNA were compared with those assessed in the primary transfer experiments in order to provide a rough estimate of the amount of DNA lost during the steps of the transfer process. The percentages of obtainable profiles for the Licked Bare Thumb to Tube and Licked Moist Thumb to Tube trials, as well as the Pen to Palm and Pen to Moist Palm trials, were compared in order to assess the effects of simulated sweat (moisture) on secondary DNA transfer. In trials that involved two subjects (Pen to Palm and Pen to Moist Palm), the DNA profiles were compared to the subjects' reference profiles in order to assess the relative ratios of primary and secondary contributor DNA, based on the peak height ratios observed in the electropherograms (data not shown).

DNA quantity results for the secondary transfer trials are shown in Figures 41-44. The calculation of DNA quantity loss due to transfer events was determined to be infeasible due to the fact that most of the quantity estimates for the various secondary transfer trial samples were undetermined. However, the quantification results did indicate that even a single transfer event can lead to dramatically reduced DNA yield. The Pen to Palm trial samples did yield sufficient

interpretable DNA quantity values to provide a rough estimate of DNA yield decrease. 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 this single transfer step was 81.2%. This is consistent with the rest of the data that indicated that secondary transfer dramatically reduced the recoverable amount of originally deposited DNA.

The Licked Bare Thumb to Tube, Pen to Palm, and Licked Gloved Thumb to Tube samples were all amplified in the same batch. Amplification at 28 PCR cycles was insufficient to yield genetic profiles from these samples that showed all of the expected alleles. 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 expected allele. The samples were re-amplified at 34 PCR cycles and re-subjected to capillary electrophoresis, and genetic profiles were thus obtained. An example of the percent of obtainable profiles for a set of these secondary transfer trial samples at both 28 and 34 PCR cycles is shown in Figures 45 and 46. The Pen to Moist Palm and Licked Moist Thumb to Tube samples were amplified in a separate batch at 34 cycles, forgoing the 28-cycle amplification, as the previous trials had demonstrated that the recoverable DNA from such samples can be considered "low copy DNA," and required more amplification cycles to provide detectable results (68,69). Based on these observations, subsequent tertiary trial samples were directly amplified at 34 cycles, as well.

In the cases of the Pen to Palm and Pen to Moist Palm trials, which both 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 locus that displayed at least one allele unique to one of the subjects in the pair, and another allele unique to the other subject in the pair was utilized for these calculations. The percentage of major and minor allele contribution was averaged for each drying time replicate in the trial, and the results were displayed in Figures 47 and 48. In all but one of the replicates in the Pen to Palm trial that yielded unique alleles, the original depositor (the subject that held the pen in his/her mouth) was shown to be the primary contributor of DNA. Similarly, the original depositor was shown to be the primary DNA contributor in all but two replicates of the Pen to Moist Palm trial. These were expected, as the DNA quantity estimates from the bare palm swab samples indicated that very little DNA was present on a subject's clean palm. 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 was predominantly from DNA from the saliva.

The effects of simulated sweat on the transfer process were investigated in both the Pen to Moist Palm and Licked Moist Thumb to Tube trials. To do so, the percentages of obtainable profiles for the Licked Bare Thumb to Tube and Licked Moist Thumb to Tube trials were compared. In all but two of the replicates in these trials, the Licked Moist Thumb to Tube samples yielded genetic profiles that displayed more of the expected alleles than those yielded by the Licked Bare Thumb to Tube samples. Of these 14 samples, 10 yielded profiles that displayed an additional 25% or more of the expected alleles from the depositing subject. These results suggested that the moistened surfaces facilitated DNA transfer more efficiently than dry ones. This finding was consistent with the results of a previous study that showed that the DNA transfer rate was significantly higher for wet samples than dry samples. A comparison of the percentages of obtainable profiles for the Pen to Palm and Pen to Moist Palm trials was not as informative, however, due to the fact that samples from both trials often yielded 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 were consistent with the findings obtained in previous trials that indicated that there initially was not a significant quantity of DNA on a clean palm.

Tertiary Transfer Trial: Licked Thumb to Tube to Palm

For this trial, each subject washed his/her hands with soap and dried them. Each subject then licked his/her bare thumb by extending his/her tongue and running his/her thumb down the tongue once. The thumb was then allowed to dry, untouched, for 5 minutes. The subject then grasped a sterilized plastic tube for 15 seconds, applying moderate pressure with his/her thumb in a specific location on the tube. This tube was then passed to the subject's designated partner (i.e., the vector), who held the tube in the palm of their hand with moderate pressure for 15 seconds. The partner's palm was then swabbed using the double swab technique. Due to the fact that the longer, 30-minute drying time replicates seemed to have no effect on the results of previous trials, these longer replicates were not performed for the tertiary trials. The 5-minute procedure was performed in duplicate, and the whole experiment was repeated, with the roles of the subjects in the pair reversed, for a total of 8 samples (4 per pair).

Tertiary Transfer Trial: Licked Thumb to Tube to Moist Palm

The procedure for this trial was identical to that of the Licked Thumb to Tube to Palm trial, except that the recipient subjects' palms were sprayed once with sterile water from an atomizer (to simulate sweat effects) just before the recipient subjects gripped the plastic tube. The palms were then swabbed, as in the Licked Thumb to Tube to Palm trial. The 5-minute procedure was performed in duplicate and the experiment was repeated, with the roles of the subjects in the pair reversed, for a total of 8 samples (4 per pair).

Tertiary Transfer Trial: Pen to Palm to Tube

In this trial, each subject held the back (non-capped) end of a sterilized pen in his/her mouth for 30 seconds. The pen was allowed to dry, untouched, for 5 minutes. The pen was then passed to the subject's partner, and the partner grasped the pen with moderate pressure for 15 seconds. The partner then gripped a sterile plastic tube with moderate pressure for 15 seconds, and the tube was swabbed using the double swab technique. The 5-minute procedure was performed in duplicate and the experiment was repeated, with the roles of the subjects in the pair reversed, for a total of 8 samples (4 per pair).

Tertiary Transfer Trial: Licked Moist Thumb to Tube to Palm

The procedure for this trial was identical to that of the Licked Thumb to Tube to Palm trial, except that the initial subjects' thumbs were sprayed once with sterile water from an atomizer right before the subjects gripped the plastic tube, in order to imitate sweat. The tube was then passed to the subject's partner, who gripped the tube with moderate pressure for 15 seconds, as in the Licked Thumb to Tube to Palm trial. The partner's palms were then swabbed using the double swab technique. The 5-minute procedure was performed in duplicate and the whole experiment was repeated, with the roles of the subjects in the pair reversed, for a total of 8 samples (4 per pair).

Tertiary Transfer Trial Results

Genetic data obtained from this third set of experiments were used to evaluate the tertiary transfer of DNA. The majority of the quantities of recovered DNA were not great enough to be detected by the quantification system used for these trials, making it unfeasible to accurately estimate the amount of DNA lost during the third step of the transfer process. The fact that the quantities were mostly undetected did, however, indicate that a very large portion of the DNA is lost during the tertiary transfer process, which was consistent with the earlier findings which showed that even a single transfer step eliminated over 80% of the DNA. The percentages of obtainable profiles for the Licked Thumb to Tube to Palm, Licked Thumb to Tube to Moist Palm, and the Licked Moist Thumb to Tube to Palm trials were compared to assess the effects of simulated sweat (moisture) on tertiary DNA transfer. As before, the DNA profiles of these tertiary trials 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.

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. These findings were consistent with the concept that tertiary transfer significantly diminished the amount of DNA that was deposited or recoverable, even when 34 cycles of PCR are used. As an example, the tertiary transfer trial profile completion percentages for subject 003 were displayed in Figure 39.

The profile completion percentages shown in Figure 49 serve as an example of the effects of moisture on the tertiary transfer process, as observed in these trials. When the percentages of a primary subject's profile yielded after the Licked Thumb to Tube to Palm trial were compared to those yielded after the Licked Thumb to Tube to Moist Palm trial, it was clear that a greater portion of the primary subject's DNA profile was transferred when the recipient partner's palm was moist. This indicated that more of the primary contributor's DNA could be transferred from the tube's surface to the recipient's palm in the presence of moisture, which was consistent with the earlier findings of this study, as well as a previous publication. It should also be noted that an even higher proportion of the primary subject's profile was transferred to the recipient's palms when the primary subject's thumbs were moistened before he or she gripped the tube. This was also consistent with previous observations of the effects of moisture on transfer events, and indicated that the presence of moisture during the initial deposition of DNA from the primary source played a more facilitating role in the transfer of the DNA than moisture present during subsequent transfer steps.

As with the two-person 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. The results of these comparisons were displayed in Figures 50-53. In all of the replicates in the Licked Thumb to Tube to Palm trial that yielded unique alleles, the secondary depositor (the subject whose palm was swabbed) was definitively shown to be the primary contributor of DNA. Similarly, the secondary depositor was shown to be the major DNA contributor in all but two applicable replicates of the Licked Thumb to Tube to Moist Palm trial. These results agreed with previously published observations of DNA

transfer, and differed 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. These findings were expected, however, as the additional transfer step involved in the tertiary transfer process most likely diminished the amount of DNA deposited by the initial contributor to the point where even the low levels of DNA present on the recipient's clean palm were more abundant in the mixture. It is worth noting that two of the replicates in the Licked Thumb to Tube to Moist Palm indicated that the primary contributor's DNA made up the majority of the mixture. These results were consistent with earlier findings that the presence of moisture at a subsequent transfer step may slightly raise the likelihood of transferring DNA deposited by the primary contributor during tertiary transfer. More importantly, all but two of the replicates in the Licked Moist Thumb to Tube to Palm trial indicated that the primary contributor's DNA made up the majority of the mixture. These results were consistent with the earlier findings of this study, indicating that the presence of moisture during the initial deposition of the primary contributor's DNA may greatly increase the proportion of primary DNA that was transferred, to the point where it was more abundant than the native DNA on a recipient's clean palm. Similar results were obtained from the Pen to Palm to Tube trial, where all but one applicable replicate indicated that the primary contributor was the major source of the DNA in the mixture. It is possible that these results were due to the greater surface area and smoother texture of the pen allowing more efficient transfer of the primary subject's DNA, a concept which was consistent with the earlier findings of this study.

Inferences About Transfer

The transfer study has implications to consider. First, single source samples can be contaminated and collection of bones at crime and anthropological scenes should be performed with proper collection strategies (such as wearing gloves and face masks). Second, most transfer testing has been under ideal and contrived but extremely unlikely conditions, i.e., contact with washed hands conditions. These studies suggest that the last person in contact with an item is generally the major contributor of the sample. In contrast, a primary donor could be the prominent contributor if he/she was a "good shedder" and the secondary individual was a "poor shedder." It was proposed that saliva, a DNA rich source, is a likely source of DNA on people's hands and thus the impact of saliva transfer should be tested. The findings of transfer (primary, secondary, and tertiary) when saliva is involved provide more insight. Unlike the non-saliva studies where the last individual to come in contact with an object tends to be the major component, if saliva is the source from the primary donor, the primary donor can persist as the major contributor. This finding suggests that caution should be taken about inferences on who may have handled an object and to not to discount secondary transfer as a plausible explanation. In addition, the concept of good and bad shedders has less relevance (if it ever had relevance because shedder status was never tested on suspects and unknown conditions leading to DNA deposition could influence the amount of DNA deposited). Saliva makes everyone a good shedder. These findings suggest that inferences on transfer with LCN data should be limited at best and that handling of bones from collection onward should be done with proper protective gear.

LCN Statistical Issues

There are two general approaches to interpret LCN typing results, a biological model (e.g. see 5) or a statistical model (e.g., see 69). The biological model infers the genotype of one or more contributors by typing multiple aliquots and calling alleles if they are present in more than one aliquot. The statistical model incorporates uncertain events (e.g., peak area/height, drop-in, drop-out, etc.) to assess the probability of each aliquot profile(s) from all possible types. Each model has advantages and limitations. The biological model is easier to implement but has not been interpreted correctly by some practitioners (see, 41 who advocated the use of the probability of inclusion for statistical analyses but did not include accommodations for the probability of allele drop out). The statistical method attempts to use more of the data and most of the data generated have been generated by simulation. More empirical data will need to be generated to support simulations. Also assumptions of independence of the loci in a sample regarding allele drop out (or for that matter allele drop in are unsubstantiated). Indeed, if allele drop out is to be invoked to explain of the hypotheses of the likelihood ratio, then it infers that the sample is challenged. Thus, allele drop out is more probable at the other loci as well. Moreover, assuming that all peaks in a LCN profile are true DNA peaks representing DNA from an individual(s) may not be warranted. As sensitivity of detection increases, artifacts that were not previously observed may become visible. To date there has not been sufficient studies to quantify the uncertain events to effectively employ the statistical model. But some work seems promising (see 69).

Biological Model

In the biological model (5), the sample is divided into two or more aliquots (or replicates), each aliquot is “independently” subjected to the LCN typing process (although these aliquots are not independent), and the “reliable” profile (or part of a profile) is determined by consensus, namely, the only alleles reported as reliable are those observed more than a predefined threshold times (e.g., at least observed twice in all aliquots). The basic concept of the consensus approach is that if drop in (and drop out although drop out cannot be random across the loci in a profile) occurs randomly and infrequently, then observing an allele multiple times increases the confidence that the allele is truly derived from the evidentiary sample (but not how it became part of the sample) and reduces the probability of the reported allele being due to allele drop in or contamination. The initial purpose of the consensus approach was to minimize the effects of contamination. However, the consensus approach has been used more so to report what is deemed “true” alleles with 100% certainty.

Gill et al. (5) showed a simple example of the biological model. Fifty “identical” samples, each containing 3pg DNA, were amplified separately using 34 cycles during the PCR (Note: samples diluted to 3 pg cannot be assumed to be equal in concentration – quantitation efficiency, sampling error and pipetting efficiency will make it impossible to obtain the same amount of DNA in each sample and it is unlikely that 3 pg are represented in any of the sample aliquots; indeed any claims of samples at such levels are likely erroneous but can be used to suggest trends). Consensus results were reported for every pair of samples following a “2 out of 2” rule (i.e., only calling alleles that were observed in any pair of samples). In all there were 1225 comparisons and only 4 consensus calls were due to drop-in alleles, but drop-out alleles were not considered further. In the Gill et al study, the phenomenon of drop in was deemed to be a low occurrence and random. If this is so, then alleles seen only once are substantially more likely to be true alleles rather than being drop in or contaminating alleles. By ignoring the single observed alleles, the rarity of a LCN profile may be inflated.

Caragine et al. (41) also used a similar biological model (i.e., in their case observing the allele(s) at least 2 out of 3 replicates). The “2 out of 3” consensus was more successful than a “2 out of 2” based on the criterion that more confirmed alleles were reported and there was additional information for mixture and homozygote assessment in a 24 buccal sample experiment. In another experiment with 9947A control DNA and 5 buccal swab samples, the drop-out rates were 8%, 23%, and 49% for 25pg, 12.5pg, and 6.25pg, respectively, with the “2 out of 3” consensus approach. (Note; Caution should be exercised in making a comparison on efficiency between the two biological model approaches because the analytical methodologies between the two groups were different).

A more comprehensive study of the biological model was performed by Benshop et al. (70). In this study four consensus methods were compared: composite (include all alleles); n-1 (include alleles detected in all but one replicate); n/2 (include alleles detected in at least half of the replicates); and 2× (include alleles detected twice). An experiment was designed with 414 Identifiler profiles to evaluate all possible consensus methods “x out of n”, where n = 1 to 6 and x = 1 to 5. The composite methods had the highest drop in rates, and “2 out of 2” had the highest drop-out rates, both of which were considered unacceptable compared with the LCN consensus methods. The n/2 method was found to be the best of the consensus methods evaluated to improve the accuracy of LCN typing; it had good balance between the drop in and drop out rates. The “2 out of 4” approach was the most functional consensus method for both single sources and 2-person mixtures. Because of the limited amount of DNA available for LCN typing, a “2 out of 3” or “2 out of 2” approach might be a practical strategy.

The biological model is the first approach implemented for LCN typing because of its simplicity. No mathematical model is assumed and the interpretation process is easy. However, there are deficiencies with the biological model. The information presented in each profile is not always considered (e.g., not using or presenting the single appearing alleles observed in each aliquot). For example in Table 1, with “2 out of 3” rule, the consensus type in all the three scenarios is [13, 15]. But, clearly, scenario (A) should have higher confidence in the declared typing result than (B) and (C), because all three aliquots share the exact same alleles and no other alleles were observed (Note: that peak heights and stutter were not included in Table 4 and these data could alter the certainty further). In scenario (C), there are a number of omitted single-observed alleles, which could infer that the profile was composed of more than one individual. For single source assessments, a profile such as scenario (C) should be considered with extreme caution. These extra alleles would reduce the certainty of a call stating that the [13,15] arose from a single individual. Additionally, since a stutter peak can sometimes approach in height or area that of its true partner allele, additional caution is required. A consensus approach needs to consider the impact of stutter (a phenomenon inherent in STR typing) on the certainty of calls. Thus, a more sophisticated model is needed that addresses the degree of confidence of consensus calls, as well as the probability of drop-in alleles.

Another critical (and as of yet insurmountable) issue of the biological model is the detection threshold of the alleles. The biological model generally does not address well the peak area/height information in a consensus allele/profile determination. A high threshold will result in an increase in drop out of true alleles but yield greater confidence in the alleles that exceed the

threshold. A low threshold will increase the potential calling of spurious alleles (i.e., drop in) as well as reduce the number of “single source” samples and thereby increase the number of “mixtures” observed.

There is a fundamental issue about the biological model that has yet to be addressed. If the application of more amplification cycles, increased capillary electrophoresis injection settings, and post-PCR clean up can obtain a profile with a relatively low drop out, then a consensus approach may not be necessary. A single analysis would have the benefit of placing as much DNA as possible in a PCR and thus minimizing the stochastic effects for that particular analysis. This approach would still have to be balanced with the impact of contamination and studies are needed. Given the efforts that purported LCN laboratories undertake to reduce contamination, it would seem reasonable to test a single amplification strategy with a sterile laboratory/reagent/materials environment for the reliability of LCN typing results. To date, no such study has been reported.

Statistical Model

In the statistical model a Bayesian approach is undertaken (see 69). Modeling should be assumed for each of the events that may occur during the LCN analytical process, the probabilities or distributions of drop-out, drop-in and stutter of the alleles should be defined, all possible true genotypes should be considered, and the evidence must be evaluated with a likelihood ratio approach. The most difficult part of the model to satisfy is supporting the data for the parameters with empirical data, such as drop out, drop in and stutter. These parameters are related to the amount of DNA, the quality of the sample, the protocol, the instrumentation, the commercial kits, and other intangibles circumstance factors, such as mixtures.

Gill et al. (5) in their biological model proposed taking account of contamination, allele drop out and stutter, in which the likelihoods of the evidence profiles were considered under two alternative hypotheses: H_1 : the DNA in the crime stain is the suspect's; or H_2 : the DNA in the crime stain is from a random person. The probability of contamination $P(C)$, the probability of drop out $P(D)$ and the probability of stutter $P(St)$ were described (either by some experimentation or assumed) [Note: the ability to model conditions is difficult because of the variability of forensic samples and any model should explicitly state these limitations]. The details of the likelihood ratio calculation were described with some examples. In addition, software called LoComationN (71) was developed to implement the method. The drop out rate $P(D)$ was estimated by simulation with the maximum likelihood method. The drop in rate $P(C)$ was fixed as a constant, and the probability of stutter $P(St)$ was ignored in the software. In Gill et al (5,71), all the drop in alleles were considered as contamination, and spurious alleles that could be due to stutter were ignored. While they proposed a theoretical framework for interpreting LCN typing, no practical values for the parameters (e.g., $P(D)$ and $P(C)$) were suggested or empirically determined. To date it is unclear if the software LoComationN has been used in any crime lab for case work (although there have been suggestions that it has been used for developing investigative leads with the UK national DNA database). It is known that while the model published by Gill et al (5) has been available for ten years, the Forensic Science Service never implemented the approach and instead remained with the biological model. While there is no documentation for the reason of not implementing the statistical approach, it is possible that the simplicity of the biological model is preferred by analysts who have to deal with practical

applications. Balding (69) introduced a similar likelihood ratio framework, but with a different drop out model than described by Gill et al (5,71). Balding (69) separated the drop out events of heterozygotes and homozygotes, because drop out of a homozygote suggests that both alleles had to drop out, which is a lower probability than a single allele dropping out for a heterozygote. The approach described by balding holds promise and may be a sound first step for developing a robust model. Software for the Balding approach is freely available.

The likelihood ratio approach is widely used in statistics and can be valid for interpreting LCN evidence, but to make it useful for LCN typing the stochastic events must be modeled and then supported with empirical data that are relevant. Gross et al. (72) investigated the distributions of allele drop in and drop out. Allele drop in followed a Poisson distribution, suggesting that dropin events are essentially random. Drop out rates were inversely proportional to the levels of input DNA. It should be noted that given these results and easily predictable drop out events for single cell DNA manipulations, results from 3 pg, 6 pg, and other low level amounts that show full or near full profiles from multiple or a majority of samples should be suspect and should be questionable for supporting any approach. Simple sampling effects should predict that a portion of the samples should yield little or no results and yet some studies seem to remarkably yield somewhat incredible results. Tvedebrink et al. (73) proposed a logistic regression model to measure the uncertainty of allele drop-out events. Experimental data showed that the drop out probability was locus dependent, and the parameter of the model should be estimated for each locus independently [Note: clearly allele drop out cannot be independent because if allele drop out is invoked to explain an unobserved allele then loci with a similar or greater amplicon size may have an increased probability of drop out due to at least the phenomenons of degradation and/or contamination with inhibitors]. These observations provided direction to quantify the probability of drop in and drop out events. Gill et al. (74) showed a statistical model for “extreme” allele drop-out only for heterozygous loci, in which both peak height distribution and logistic regression function were calculated based on empirical data. However, the differences among the loci were ignored and no model was developed for homozygous loci. Haned et al. (75) used a simulation approach to evaluate the efficiency of the logistic model. They noticed that in the sampling process, each chromosome was sampled independently instead of as paired chromosomes and the number of chromosomes may not be equal in LCN samples.

The statistical model is more sophisticated compared with the biological model, because in theory all the information (e.g., each observed allele, peak area/height, etc.) in the LCN typing process can be incorporated in a Bayesian framework. Thus, a better assessment of the profiles might be provided. Likely the biological model will be modified or replaced by the statistical model, if the various parameters in LCN PCR can be validly quantified. However, modeling the entire (or even the most relevant aspects of) LCN process is not a trivial endeavor. Although the principle of the likelihood ratio is the same as that used in other statistical and forensic applications, the calculation of the likelihood of the evidence profile requires good fitting models to incorporate the stochastic events, such as different drop out models for heterozygotes and homozygotes, randomness of the drop in alleles, dependence of the drop out and/or drop in events between/among loci, etc. There have been several reports on developing statistical models, but no model has been developed that sufficiently incorporates values (e.g., 69-79).

Proposed Statistical Interpretation Model

Current interpretation of LCN evidence is based mostly on the observation of drop in and drop out events. Little empirical study has been done to determine the process of how such events occur. A framework is needed that is more comprehensive regarding the LCN typing process. In the LCN typing process, the DNA templates are first extracted from the biological material and the extracted DNA templates are quantified. Then, an aliquot of the extracted DNA templates is subjected to PCR amplification. Contamination (defined herein as that which occurs in the laboratory setting) could occur during the extraction and sampling processes, and could lead to drop in alleles. Drop in may be low level DNA inherent in the sample that is stochastically arising and should be distinguished from laboratory contamination. During the PCR amplification process, the specific regions of the DNA templates are amplified repeatedly at a certain number of cycles (e.g. 28-34) to produce large amounts of DNA amplicons for detecting fluorescently-labeled alleles during electrophoresis. Stutter products may be produced during the amplification process. When the number of template molecules is low and slippage occurs at the first few cycles, it will substantially change the proportion of the alleles in the pool of products. A starting allele may drop out and a stutter or drop in allele (with notable peak height) can arise.

Sampling and PCR amplification are the two major processes which require statistic modeling. In the sampling process, the DNA template is extracted and the molecules (template) are sampled randomly. Gill et al. (5) modeled the sampling process as a binomial distribution of each template. However, jointly considering the whole pool of template, the sampling process more likely follows a hypergeometric distribution, because the sampling is a process without replacement. Additionally, there may be more than two alleles per locus for most mixture samples and the copy number of individual alleles may not be equivalent for haploid cells (e.g., sperms). Suppose there are n distinct alleles in the extracted DNA sample, from A_1, \dots, A_n , with m_1, m_2, \dots, m_i number of copies ($i = 1, \dots, n$), respectively, and $m_1 + m_2 + \dots + m_i = M$. Then, the probability of sampling an aliquot, which includes x_1, x_2, \dots, x_n number of copies, $x_i \leq m_i$ and $x_1 + x_2 + \dots + x_n = X$, follows a multivariable hypergeometric distribution (Equation 1):

$$Pr(x_1, x_2, \dots, x_n; m_1, m_2, \dots, m_n) = \frac{\binom{m_1}{x_1} \binom{m_2}{x_2} \dots \binom{m_n}{x_n}}{\binom{M}{X}} \quad (1)$$

For example, suppose the genotype of an individual is (A, B), the probability that an aliquot with 4 As and 3 Bs is sampled from the pool sample with 10 As and 10 Bs is $\frac{\binom{10}{4} \binom{10}{3}}{\binom{20}{7}} = 0.325$.

This model allows for an unequal number of sampled alleles for heterozygotes. When the total number of cells and the number of sampled cells are large (e.g. 1ng DNA as used in standard testing), the number of differences of the sampled two alleles of a heterozygote typically is trivial (or addressed with standard interpretation guidelines), such that the peak height imbalance after PCR amplification is a minor consideration. When the DNA pool is small (i.e., an LCN sample), it is more likely that different numbers of the heterozygous alleles will be in different aliquots. This sampling leads to variation in profiles obtained in different aliquots along with increased peak height imbalance and drop out of less sampled alleles.

In the PCR amplification process, most templates are replicated with the functionally exact same sequences as the starting templates, but a good proportion of new templates may be generated for

STRs during the PCR via the slippage mechanism. Thus, most slippage products are one or two steps greater or less than the starting allele. With LCN typing, a drop in allele (with notable peak height) can be observed if slippage occurs in the first few cycles, which will substantially change the proportion of the alleles in the final pool of products compared with that in the original sample. Figure 54 shows the PCR amplification process for STR loci. e_{pcr} is the PCR efficiency, which varies according to the cycles. PCR efficiencies can be estimated by mathematical models (e.g, Sigmodal model (77,78) and the Logistic model (79)) or can be estimated from experimental data. e_{-1} , e_0 , e_{+1} are the proportions of replications with -1, 0, and 1 step slippage mutations, respectively, which can be initially assumed to be constant across the cycles and estimated from experimental data. $e_{-1} + e_0 + e_{+1} = e_{pcr}$. For one cycle of one allele the model is

$$A_i(j) = A_{i-1}(j-1)e_{+1}e_{pcr} + A_{i-1}(j)e_0e_{pcr} + A_{i-1}(j) + A_{i-1}(j+1)e_{-1}e_{pcr} \quad (2)$$

This model incorporates the major events of the LCN amplification process and can explain the stochastic observations in the LCN typed profiles. However, the model requires allele detection at each cycle, which may not be applicable with current technologies. A simplified model can be developed by ignoring the amplification details in each cycle and directly comparing the starting sample and the products after PCR amplifications. For the whole PCR amplification, a single allele A(0) may be replicated to one step less allele A(-1) with probability p_{-1} , the same allele A(0) with probability p_0 , one step more allele A(+1) with probability p_{+1} , or drop out with probability p_d . Two or more steps slippage events can be ignored in the model because the probabilities of multi-step mutations are low. The p_{-1} , p_0 , and p_{+1} depend on the technologies, markers, kits, etc. GeneMapper ® (Life Technologies, Foster City, CA) uses empirical data to estimate these probabilities and provides these numbers for conventional DNA typing. The same method can be applied to other systems. The drop out rate p_d mainly depends on the amount of DNA before PCR amplification. Logistic regression models can be applied to the drop out events (73-75). In these models, the drop out rate was defined differently for homozygotes and heterozygotes, and was associated with the peak area/height of the detected alleles, which is related to the quantity of DNA. A better logistical regression model can be developed to directly associate the individual drop out rate to DNA quantity (Equation 3). Different drop-out rates for homozygotes and heterozygotes may not be necessary. In equation 3, m is the number of DNA copies, and β_0 and β_1 are the parameters to be estimated with empirical data, which can vary with different systems, markers, kit, etc.

$$p_d = \frac{e^{(\beta_0 + \beta_1 m)}}{1 + e^{(\beta_0 + \beta_1 m)}} \quad (3)$$

The PCR process of multiple copies of a single allele can be modeled as a multinomial distribution (Equation 4) assuming the copies are mutually independent. The y_{-1} , y_0 , y_{+1} , and y_d are the normalized amount of replicated DNA relative to the starting copy numbers for alleles A(-1), A(0), A(+1), and drop out respectively.

$$\Pr(y_{-1}, y_0, y_{+1}, y_d; X; p_{-1}, p_0, p_{+1}, p_d) = \frac{X!}{y_{-1}! y_0! y_{+1}! y_d!} p_{-1}^{y_{-1}} p_0^{y_0} p_{+1}^{y_{+1}} p_d^{y_d} \quad (4)$$

These amounts can be measured by quantifying the PCR products, allele peak area/height, and the PCR efficiency. Based on this model, the evidence can be directly evaluated in the likelihood

ratio framework. The estimates of the y_{-1} , y_0 , y_{+1} , and y_d may not be precise, and therefore the likelihood can be the average of multiple likelihoods with reasonable y -vectors. Expectation-Maximization algorithm can be applied when multiple distinct alleles are present. This model described above incorporates allele drop-in due to slippage events. However, contamination before PCR amplification can also generate allele drop-in. To incorporate contamination, the hypothesized contamination alleles and corresponding amounts can be added in the starting template pool before the PCR process, and the likelihood will be multiplied by a contamination rate (e.g., 5%). Likelihoods calculated from multiple contamination hypotheses can be averaged to reach a final likelihood.

Further studies will be needed to develop quantitative values for the parameters of this model. Large numbers of low quantity samples (e.g., 50 pg, 20 pg) should be aliquoted, amplified, and typed for STR loci. The peak heights (for alleles and stutter products) should be collected. Such research is recommended. One should be cognizant that these data will be helpful for single source estimations but are under pristine conditions. Interpretation of LCN typing currently does not employ any peak height thresholds. A stochastic threshold is the second filter on defining an area where greater uncertainty in data interpretation exists. LCN typing by its nature produces data that reside below the stochastic level of standard DNA typing. It may not be readily simple to transfer pristine data results to some LCN typing procedures.

Conclusions

Bones and other low quantity DNA samples are challenging to type. LCN typing, which entails increasing the sensitivity of detection, has been sought to be able to detect alleles that would not be observable under standard DNA typing conditions. However, under LCN conditions, the results are impacted by stochastic effects primarily when obtaining an aliquot from an extract and during the PCR. These excessive stochastic effects contribute to a lack of reproducibility and hence reliability of results obtained by LCN typing. To overcome some of the stochastic effects better methodologies are needed that approach the robustness of standard NA typing strategies. Once developed, statistical methods that incorporate the uncertainty of the various features can be considered.

The research summary in this Report described a number of findings that can improve LCN analyses. They are presented as independent studies so the reader can evaluate which ones show promise. These methods demonstrate that processes can be improved that can impact LCN typing. The methods offer practical solutions for improving the yield of DNA (from any source and particularly from bone). Yield is defined as a combination of quantity and purity of the DNA extract. The most successful studies were the use of Hi-Flow columns, SCODA, PCT treatment of inhibitors. Use of the Hi-Flow column enables DNA extraction from large volume samples that is substantially free of PCR inhibitors and can be readily implemented into any laboratory. SCODA is a novel electrophoretic approach which was the most effective method for removing inhibitors (at much higher concentrations than has ever been tested). This method shows promise and further work is warranted. Throughput and DNA free reagents are needed for further development of the SCODA methodology. Pressure Cycling Technology was sought because it was hypothesized that extreme pressure could increase DNA yield from bone substrate. The

results did show some yield increases, but they were not significant. The most promising finding to date was the impact of pressure on reducing the effect of inhibitors. Therefore, pressure may be an effective method for reducing the impact of inhibitors. More work should be considered on determining the range or classes of inhibitors that can be neutralized by pressure.

For improving the PCR, three additives were tested: betaine, DMSO and DNAguard. None of these additives have been demonstrated with the PCR of LCN samples. Betaine appears to have some effect on reducing the amount of stutter. The majority of low level DNA samples showed a decrease in the percent stutter and there was less excessive stutter with betaine treatment. DMSO did not reduce stutter further in the presence of stutter. DNAguard did not show any improvement to the PCR and was not pursued further. Additional formulations of additives are available from Biomatrix and should be tested. The results show that additives can have some impact on the quality of PCR products. Given the reduction in stutter, it is anticipated that betaine (and other additives) will be quite useful for facilitating the interpretation of single source samples. More work should be considered that combines multiple additives that may reduce stutter and at the same time increase yield of PCR products. In addition, conditions regarding temperature and time with the cycles should be addressed.

Primary and secondary (and tertiary) transfer is an important issue that impacts interpretation of results from any object that may have been handled (including bones). Most transfer testing has been under ideal but extremely unlikely conditions, i.e., contact with washed hands conditions. It was proposed that saliva is a more likely source of DNA on people's hands and thus the impact of saliva transfer should be tested. The findings of transfer (primary, secondary, and tertiary) when saliva is involved provide more insight. Unlike the non-saliva studies where the last individual to come in contact with an object tends to be the major component, if saliva is the source from the primary donor, the primary donor can persist as the major contributor. This finding suggests that caution should be taken about inferences on who may have handled an object and to not to discount secondary transfer as a plausible explanation. In addition, when the primary donor was the major contributor in transfer studies the experimentation specifically paired a good shedder as the primary donor and a poor shedder as the secondary (or vector) donor. The concept of good and bad shedders in actuality has no relevance as this status was never tested on suspects and the conditions leading up to deposition of a sample are entirely unknown. Saliva makes everyone a good shedder. These findings suggest that inferences on transfer with LCN data should be limited at best and that handling of bones from collection onward should be done with proper protective gear.

Statistical inferences are the weakest part of the LCN typing process. Some practitioners have undertaken the practice of including individuals but not providing any statistical weight to the findings. This is in direct conflict with recommendations of the NAS Report on the Forensic Sciences (2009) and the DNA typing standards in the US. But there are some promising developments. There are two methods for interpretation: a biological model and a statistical model. Both involve interpretation but only the latter provides 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. A model for single source sample interpretation is provided that recognizes template sampling issues from a sample, stochastic effects during the PCR, and that the events may not be independent from locus-to-locus. Future

work should be to derive empirical data from a robust technology that can be used to test and apply the single source model.

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Figures and Tables

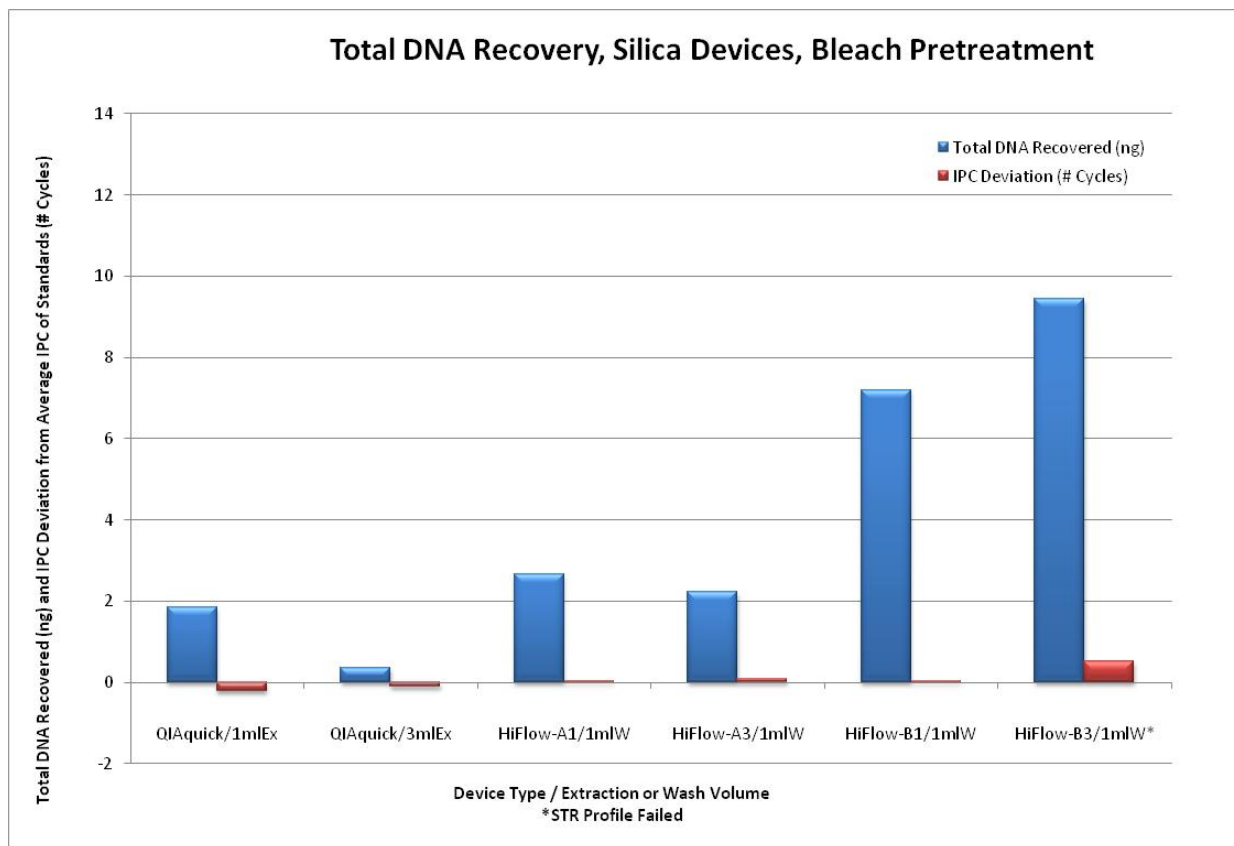


Figure 1. DNA recovery from bleach pretreated samples using silica-based devices, estimated with Quantifiler. The QIAquick/1 ml experiment used 1 ml of YEB, and the QIAquick/3 ml experiment used 3 ml of YEB. All four types of Hi-Flow column extractions started with 3 ml of IEB and were washed with a 1 ml aliquot of buffer PE.

QIAquick/1 ml experiment – bone powder digested with 1 ml Yang extraction buffer, recovered with QIAquick column, washed with 0.75 ml PE buffer

QIAquick/3 ml experiment – bone powder digested with 3 ml Yang extraction buffer, recovered with QIAquick column, washed with 0.75 ml PE buffer

Hi-Flow-A1 – bone powder digested with 3 ml improved extraction buffer, recovered with 20 ml Hi-Flow column containing 1 layer of type A glass fiber

Hi-Flow -A3 – bone powder digested with 3 ml improved extraction buffer, recovered with 20 ml Hi-Flow column containing 3 layers of type A glass fiber

Hi-Flow -B1 – bone powder digested with 3 ml improved extraction buffer, recovered with 20 ml Hi-Flow column containing 1 layer of type B glass fiber

Hi-Flow -B3 – bone powder digested with 3 ml improved extraction buffer, recovered with 20 ml Hi-Flow column containing 3 layers of type B glass fiber

1 ml W – washed with 1 ml PE buffer

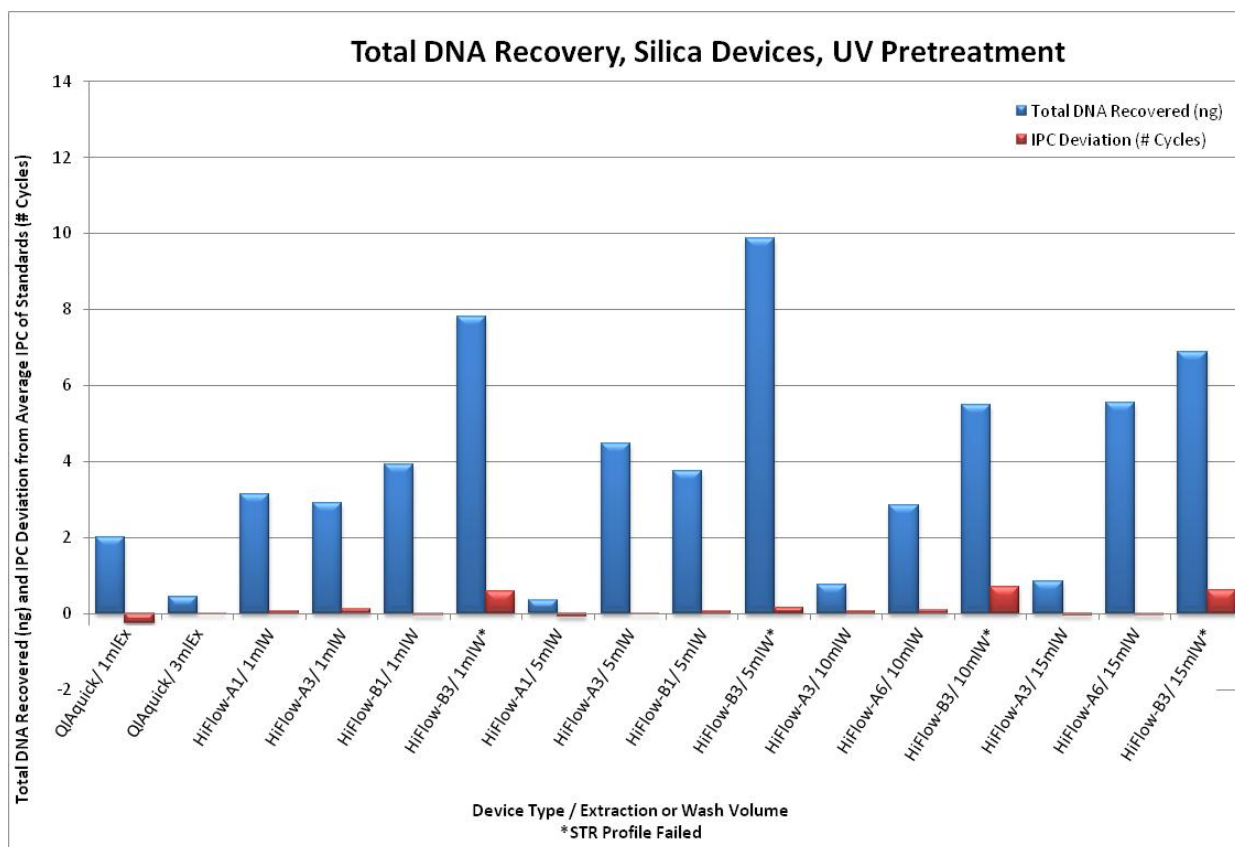


Figure 2. DNA recovery from UV pretreated samples using silica-based devices, estimated with Quantifiler. The QIAquick/1 ml experiment used 1 ml of YEB, and the QIAquick/3 ml experiment used 3 ml of YEB. All four types of Hi-Flow column extractions started with 3 ml of IEB and were washed with either a 1 ml aliquot of buffer PE (1 ml W), a 5 ml aliquot of buffer PE (5 ml W), 2 separate 5 ml aliquots of buffer PE (10 ml W), or 3 separate 5 ml aliquots of buffer PE (15 ml W).

QIAquick/1 ml experiment – bone powder digested with 1 ml Yang extraction buffer, recovered with QIAquick column, washed with 0.75 ml PE buffer

QIAquick/3 ml experiment – bone powder digested with 3 ml Yang extraction buffer, recovered with QIAquick column, washed with 0.75 ml PE buffer

Hi-Flow-A1 – bone powder digested with 3 ml improved extraction buffer, recovered with 20 ml Hi-Flow column containing 1 layer of type A glass fiber

Hi-Flow -A3 – bone powder digested with 3 ml improved extraction buffer, recovered with 20 ml Hi-Flow column containing 3 layers of type A glass fiber

Hi-Flow -B1 – bone powder digested with 3 ml improved extraction buffer, recovered with 20 ml Hi-Flow column containing 1 layer of type B glass fiber

Hi-Flow -B3 – bone powder digested with 3 ml improved extraction buffer, recovered with 20 ml Hi-Flow column containing 3 layers of type B glass fiber

1 ml W – washed with 1 ml PE buffer

5 ml W – washed with 5 ml PE buffer

10 ml W – washed with 2 aliquots PE buffer, 5 ml each

15 ml W – washed with 3 aliquots PE buffer, 5 ml each

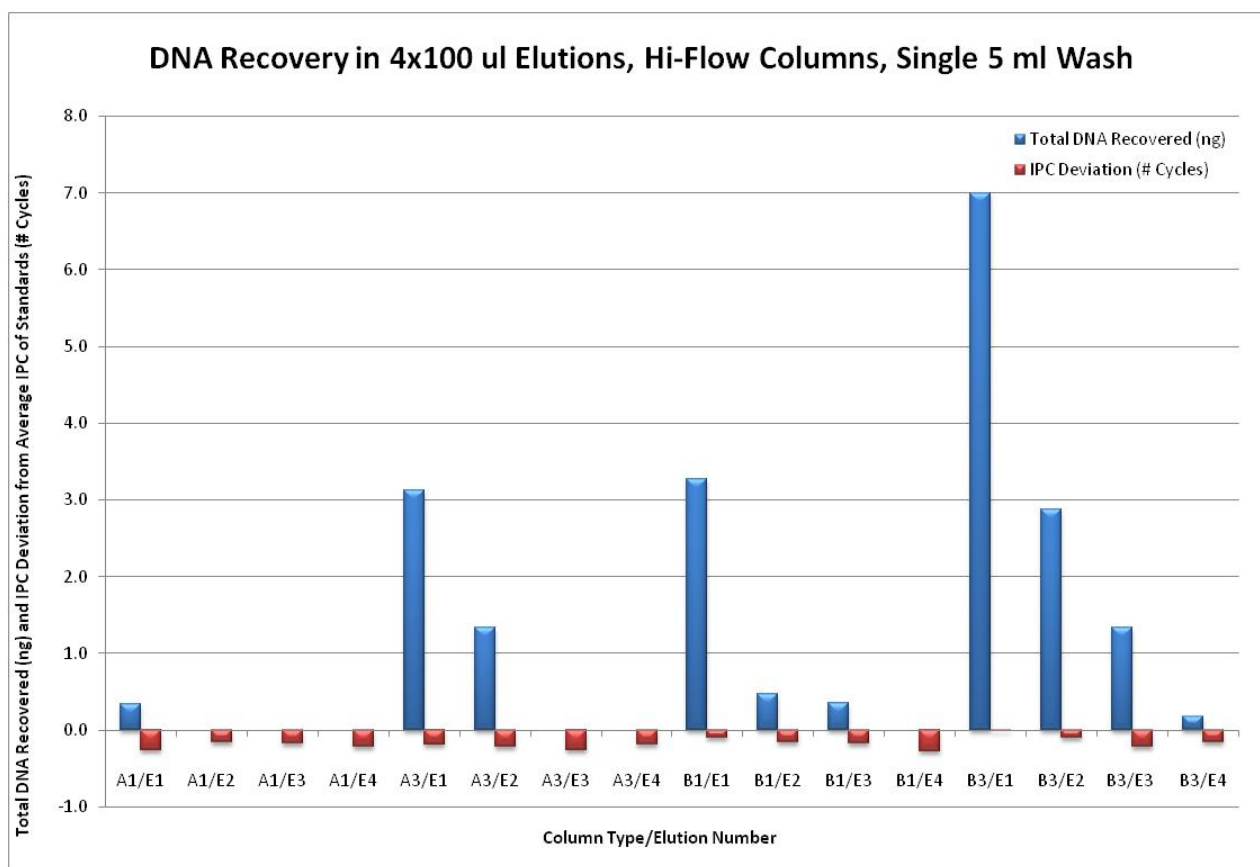


Figure 3. DNA recovery from four elutions (E1, E2, E3, E4) from Hi-Flow columns A1, A3, B1, and B3, estimated with Quantifiler. All columns were washed with a single 5 ml aliquot of PE buffer. Hi-Flow column descriptions are given as the type of glass fiber (A or B), followed by the number of layers of glass fiber (1 or 3), followed by the elution number.

A1 – bone powder digested with 3 ml improved extraction buffer, recovered with 20 ml Hi-Flow column containing 1 layer of type A glass fiber

A3 – bone powder digested with 3 ml improved extraction buffer, recovered with 20 ml Hi-Flow column containing 3 layers of type A glass fiber

B1 – bone powder digested with 3 ml improved extraction buffer, recovered with 20 ml Hi-Flow column containing 1 layer of type B glass fiber

B3 – bone powder digested with 3 ml improved extraction buffer, recovered with 20 ml Hi-Flow column containing 3 layers of type B glass fiber

E1 – First 100 μ l elution

E2 – Second 100 μ l elution

E3 – Third 100 μ l elution

E4 – Fourth 100 μ l elution

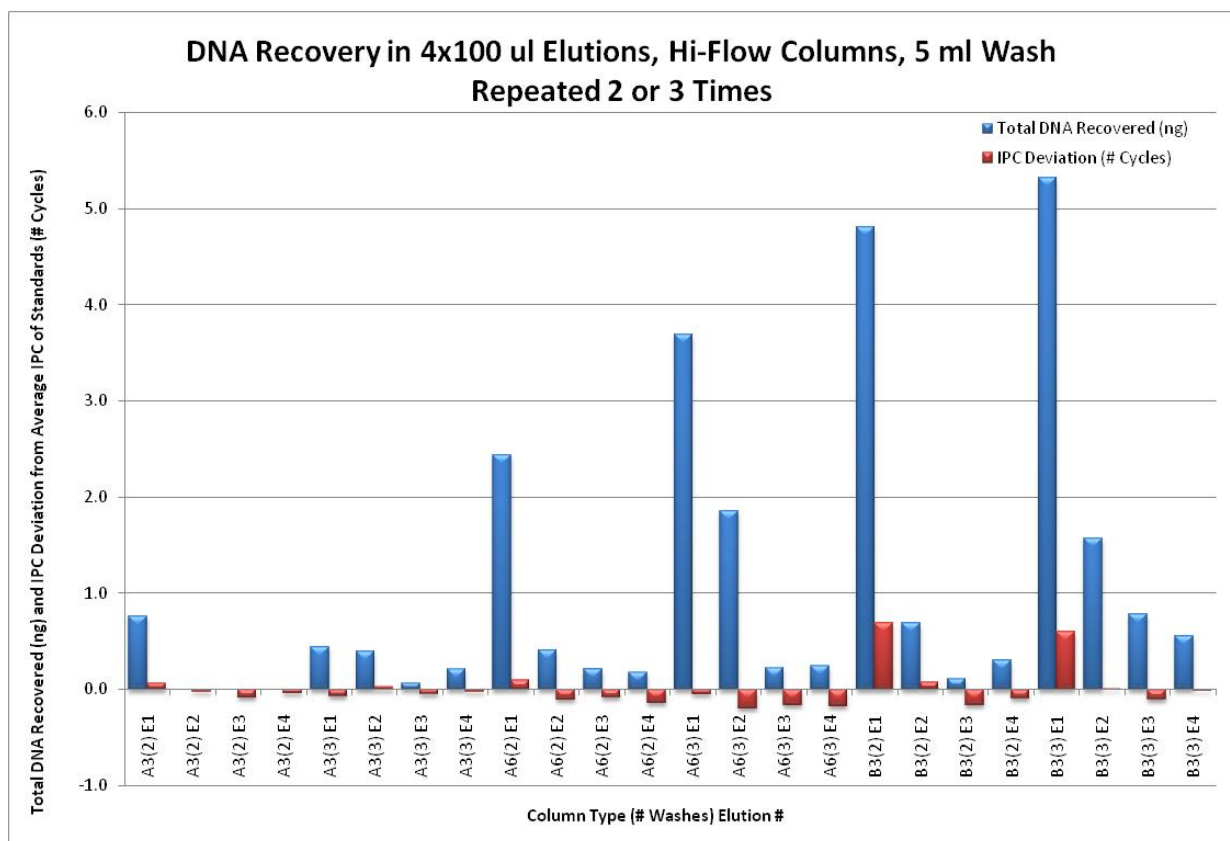


Figure 4. DNA recovery from four elutions (E1, E2, E3, E4) using Hi-Flow columns A3, A6, and B3, estimated with Quantifiler. Column descriptions are given as the type of glass fiber (A or B), followed by the number of layers of glass fiber (3 or 6), followed by the number of times that column was washed with 5 ml of PE buffer (2 or 3), followed by the elution number.

A3 – bone powder digested with 3 ml improved extraction buffer, recovered with 20 ml Hi-Flow column containing 3 layers of type A glass fiber

A6 – bone powder digested with 3 ml improved extraction buffer, recovered with 20 ml Hi-Flow column containing 6 layers of type A glass fiber

B3 – bone powder digested with 3 ml improved extraction buffer, recovered with 20 ml Hi-Flow column containing 3 layers of type B glass fiber

(2) – washed with 2 aliquots PE buffer, 5 ml each

(3) – washed with 3 aliquots PE buffer, 5 ml each

E1 – First 100 µl elution

E2 – Second 100 µl elution

E3 – Third 100 µl elution

E4 – Fourth 100 µl elution

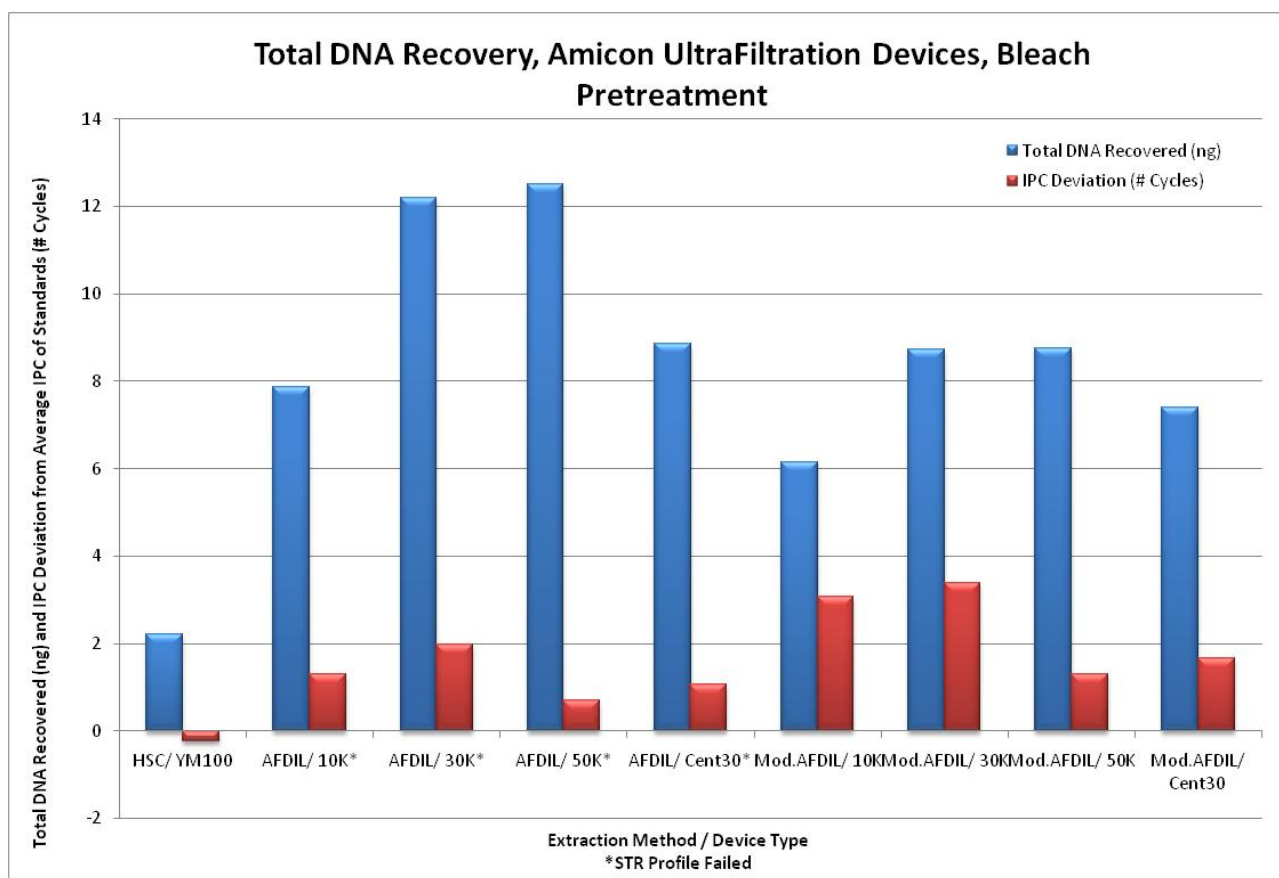


Figure 5. DNA recovery from bleach pretreated bone using Amicon ultrafiltration devices, estimated with Quantifiler[®]. These included the Microcon YM100 (nominal molecular weight limit of 100,000), Centricon 30 (nominal molecular weight limit of 30,000), and the Amicon Ultra-4 ultrafiltration devices with three different nominal molecular weight limits (NMWL) 10K (10,000 NMWL), 30K (30,000 NMWL), and 50K (50,000 NMWL).

HSC/YM100 – bone powder decalcified with 1 ml 0.5 M EDTA, digested with 1 ml stain extraction buffer, subjected to organic extraction, recovered with Microcon YM100 device, horizontal membrane, nominal molecular weight limit 100,000

Loreille et al – bone powder digested with 3 ml improved extraction buffer, subjected to three rounds of organic extraction, ultrafiltrate washed once with TE

Mod. Loreille et al – bone powder digested with 3 ml improved extraction buffer, subjected to four rounds of organic extraction, ultrafiltrate washed twice with TE, once with nuclease-free water

10K – recovered with Amicon Ultra-4 device, vertical membrane, nominal molecular weight limit 10,000

30K – recovered with Amicon Ultra-4 device, vertical membrane, nominal molecular weight limit 30,000

50K – recovered with Amicon Ultra-4 device, vertical membrane, nominal molecular weight limit 50,000

Cent30 – recovered with Centricon 30 device, horizontal membrane, nominal molecular weight limit 30,000

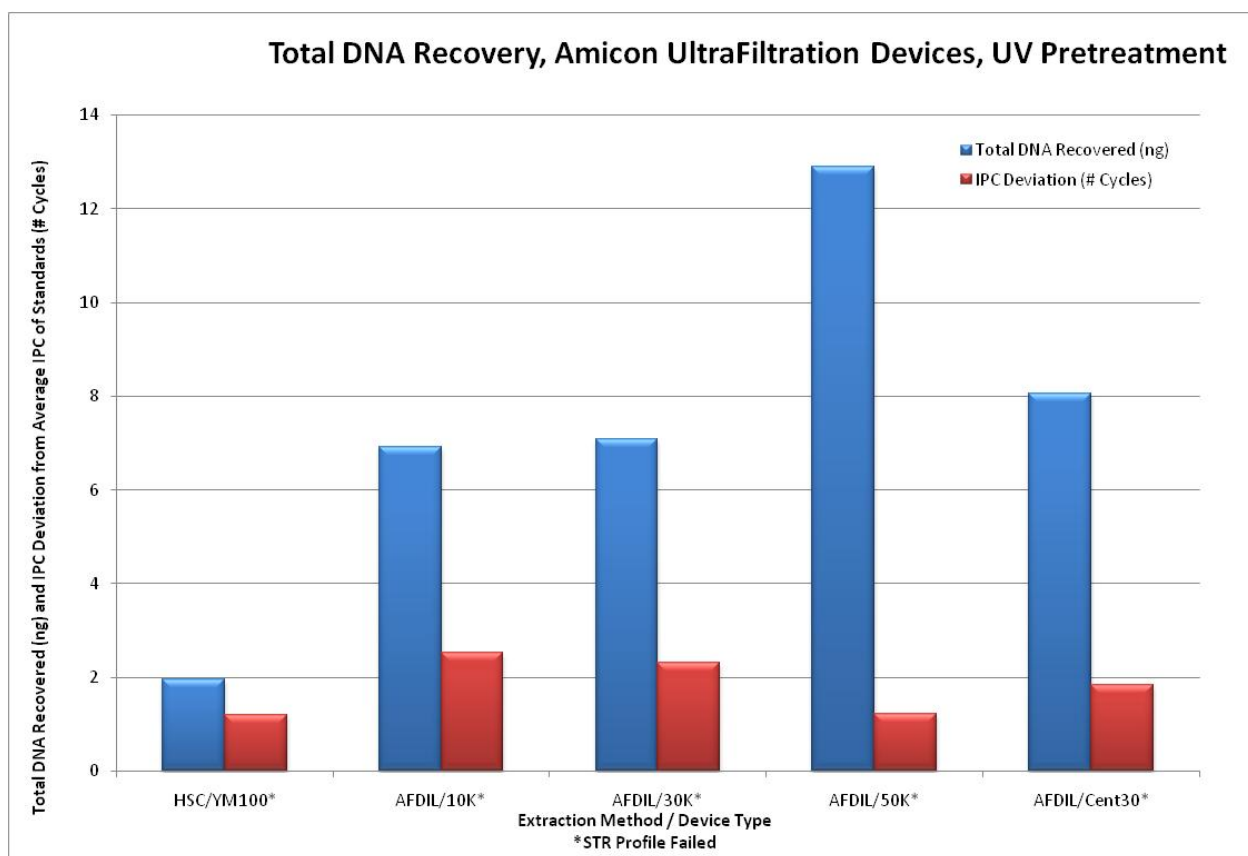


Figure 6. DNA recovery from UV pretreated bone using Amicon ultrafiltration devices, estimated with Quantifiler. The columns represented here include the Microcon YM100 (nominal molecular weight limit of 100,000), Centricon 30, and the Amicon Ultra-4 ultrafiltration devices: 10K (10,000 NMWL), 30K (30,000 NMWL), and 50K (50,000 NMWL).

HSC/YM100 – bone powder decalcified with 1 ml 0.5 M EDTA, digested with 1 ml stain extraction buffer, subjected to organic extraction, recovered with Microcon YM100 device, horizontal membrane, nominal molecular weight limit 100,000

Loreille et al – bone powder digested with 3 ml improved extraction buffer, subjected to three rounds of organic extraction, ultrafiltrate washed once with TE

10K – recovered with Amicon Ultra-4 device, vertical membrane, nominal molecular weight limit 10,000

30K – recovered with Amicon Ultra-4 device, vertical membrane, nominal molecular weight limit 30,000

50K – recovered with Amicon Ultra-4 device, vertical membrane, nominal molecular weight limit 50,000

Cent30 – recovered with Centricon 30 device, horizontal membrane, nominal molecular weight limit 30,000

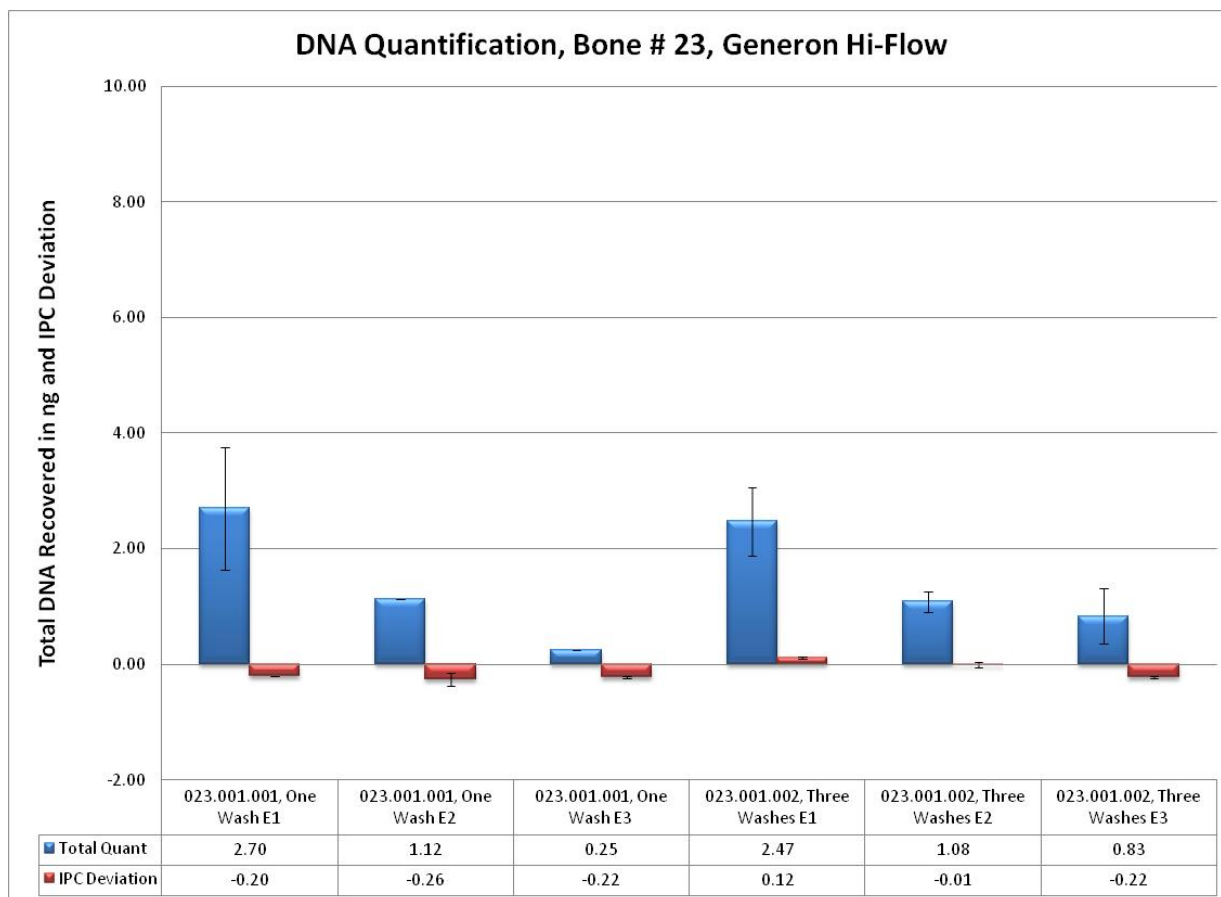


Figure 7a. Comparison of the single 15 ml wash protocol with the triple 5 ml wash protocol. Average DNA recovered (in ng) from the first three elutions are shown for each extraction, with DNA recovery in blue and the deviation of the sample internal positive control (IPC) C_T value from the average IPC C_T value of the standards in red. A positive shift in IPC C_T value indicated that the IPC reaction was inhibited. The single wash method (on the left side of the graph) showed equivalent DNA recovery and less inhibition.

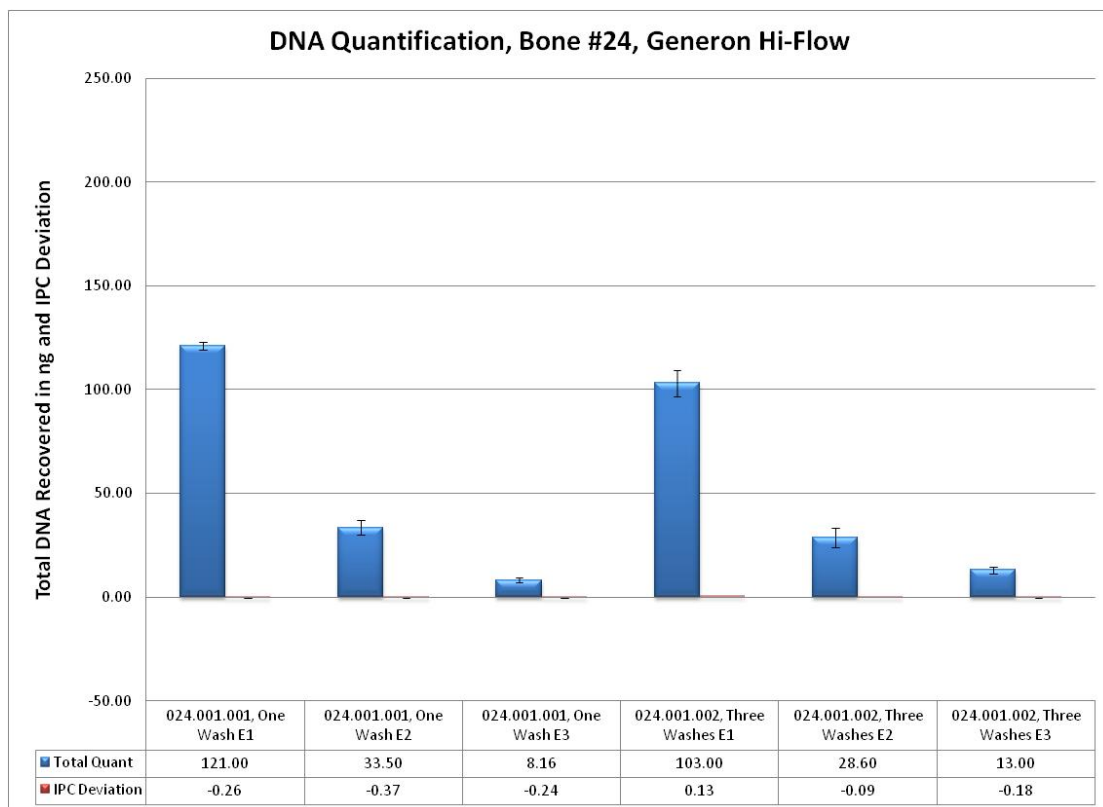


Figure 7b. The single wash method (the first three histograms) showed greater DNA recovery and similar levels of inhibition.

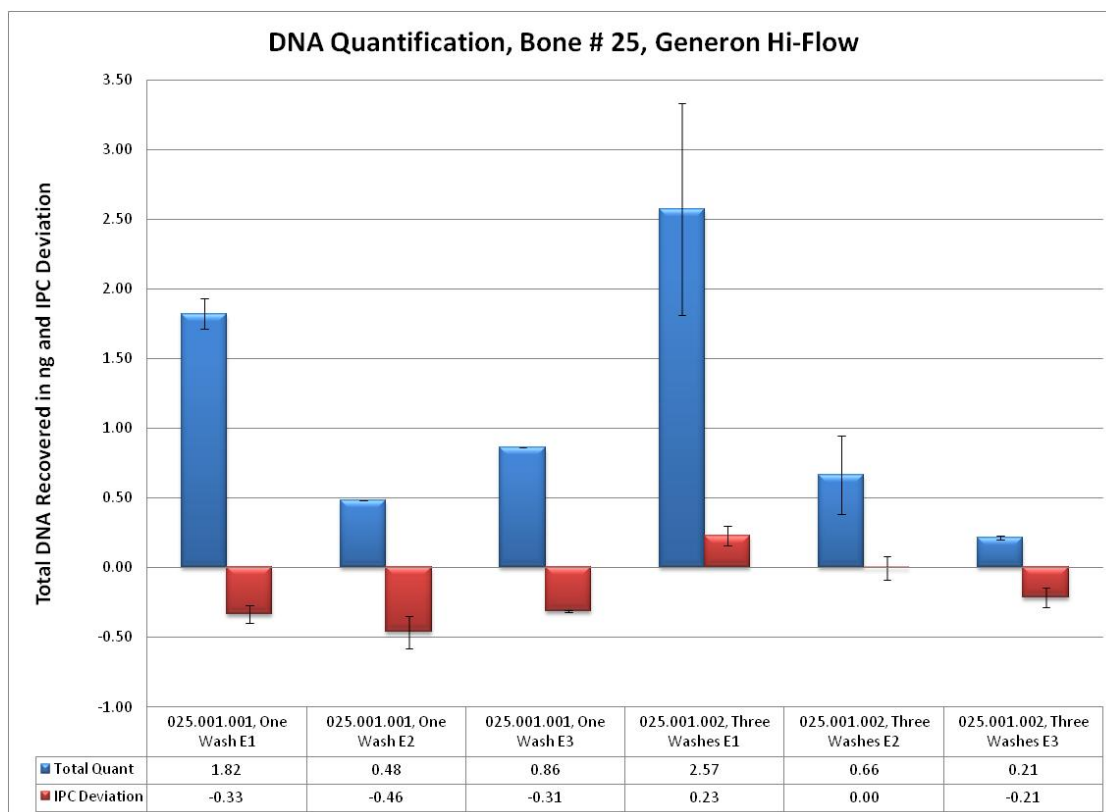


Figure 7c. The single wash method (the first three histogram sets) showed DNA recovery that in total was similar with that of the triple wash method. But the amount of inhibition was less.

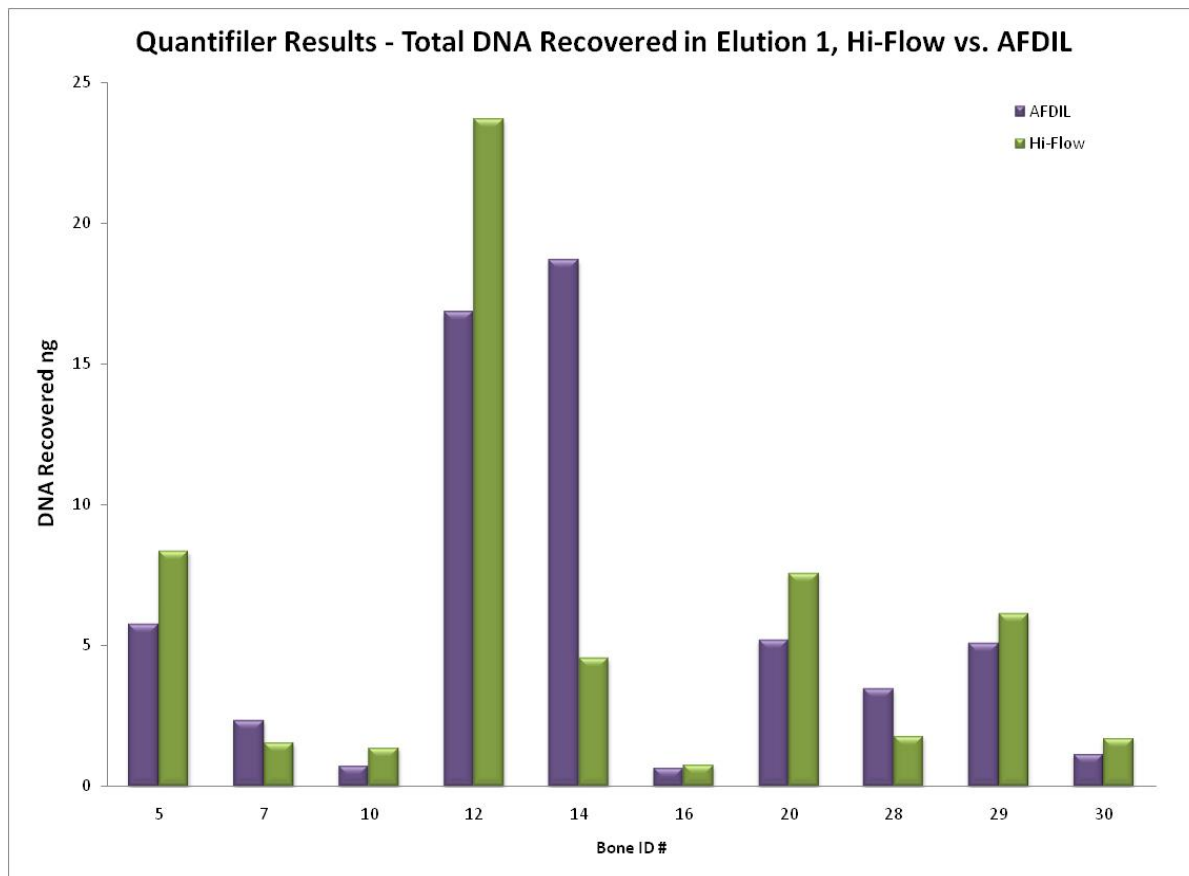


Figure 8. Total DNA recovery from elution one of the Loreille et al + MinElute method (purple) and the Hi-Flow method (green). Extractions were performed on bone powder from a single grind cycle from bones 5, 7, 10, 12, 14, 16, 20, 28, 29, and 30.

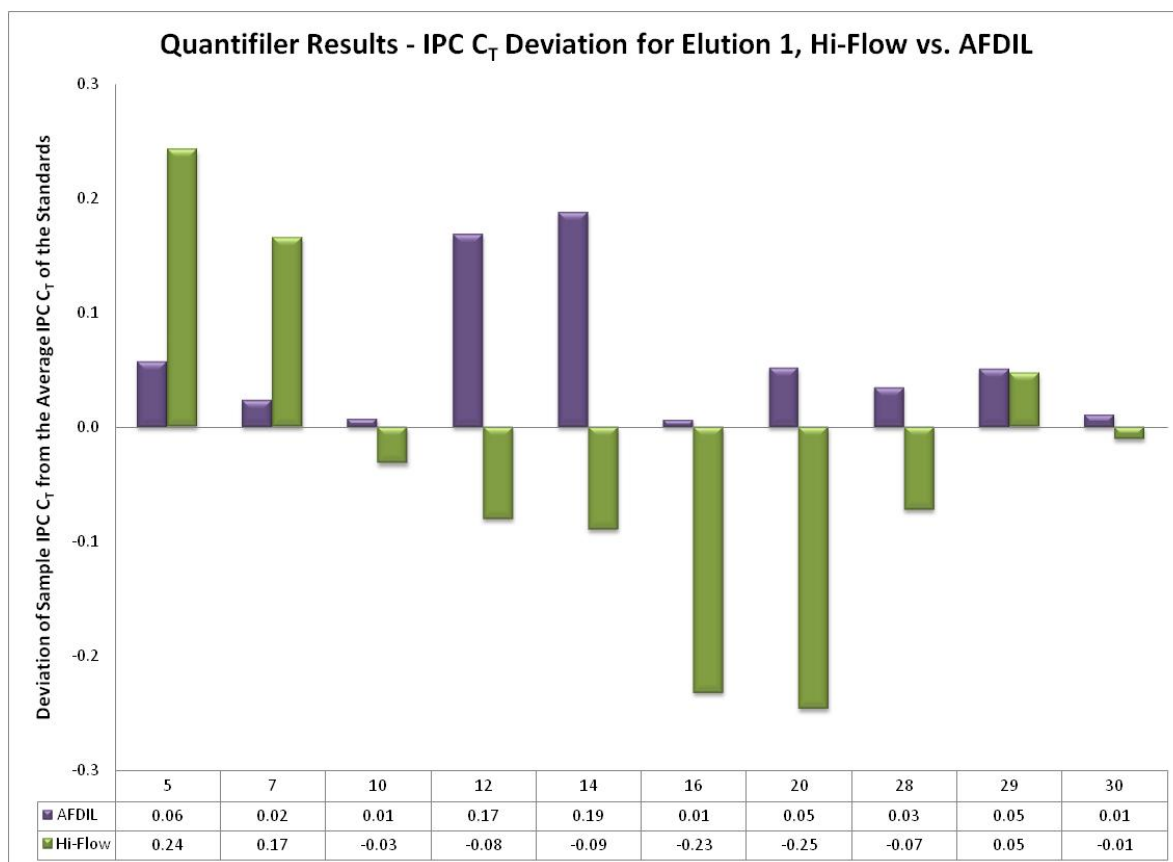


Figure 9. The deviation of the sample IPC C_T value from the average IPC C_T value of the standards is shown for the Oreille et al + MinElute method (purple) and the Hi-Flow method (green). A positive shift in IPC C_T value indicated that the IPC reaction was inhibited.



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

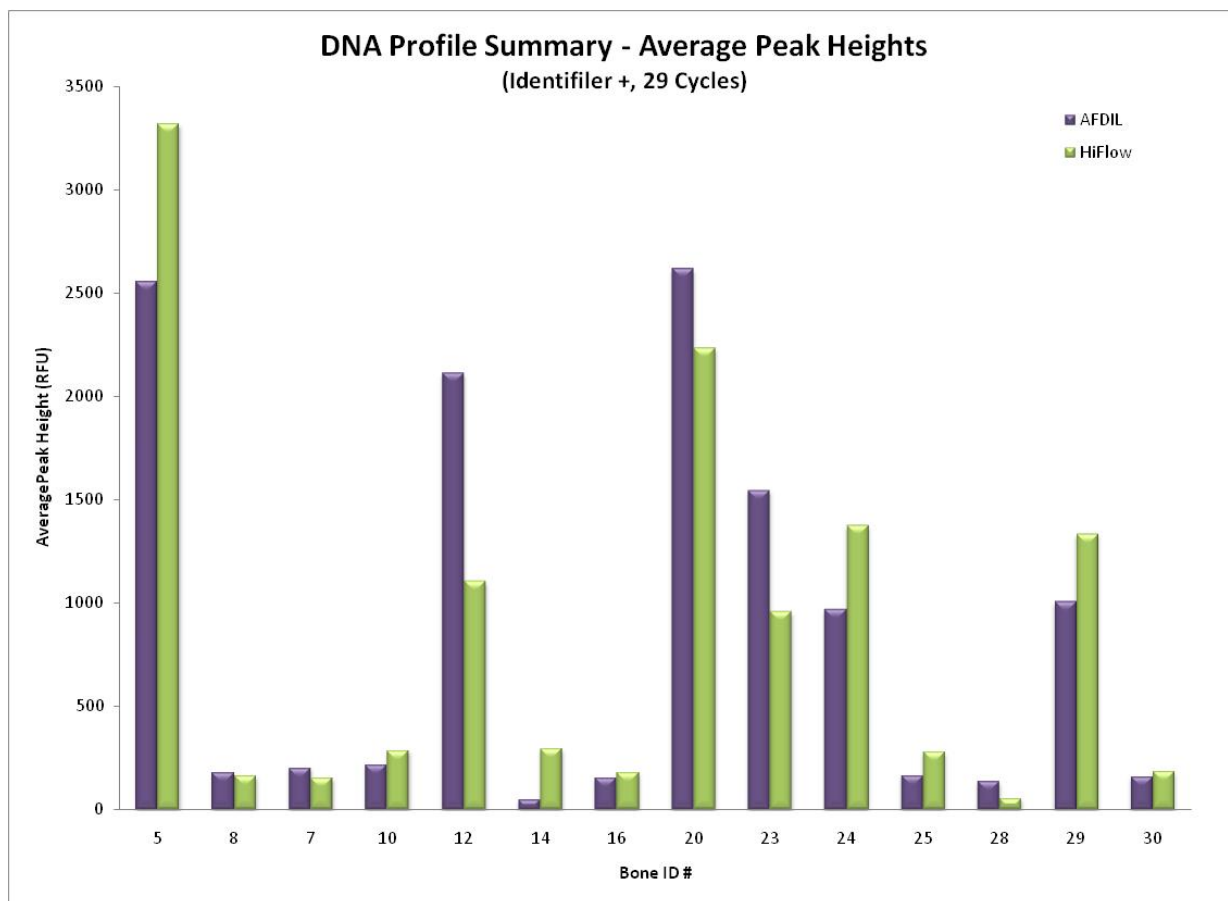


Figure 11. Average peak heights for every peak called in each profile. This permitted a relative comparison of signal strength. The Loreille et al + MinElute samples are represented in purple and the Hi-Flow samples are in green.

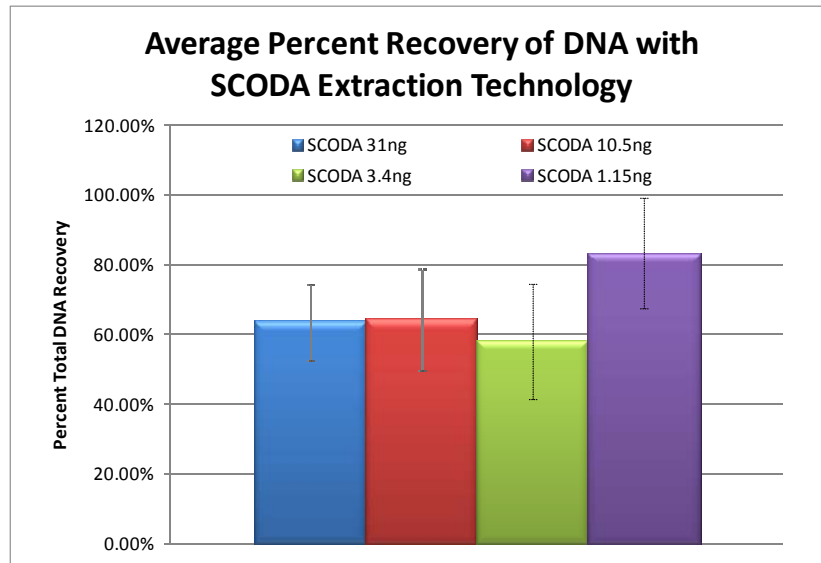
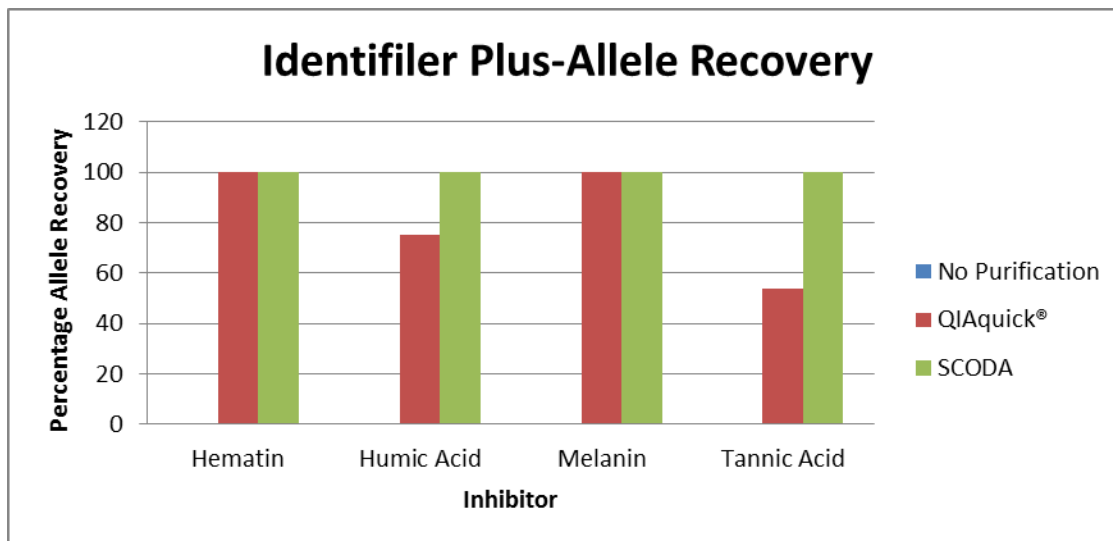
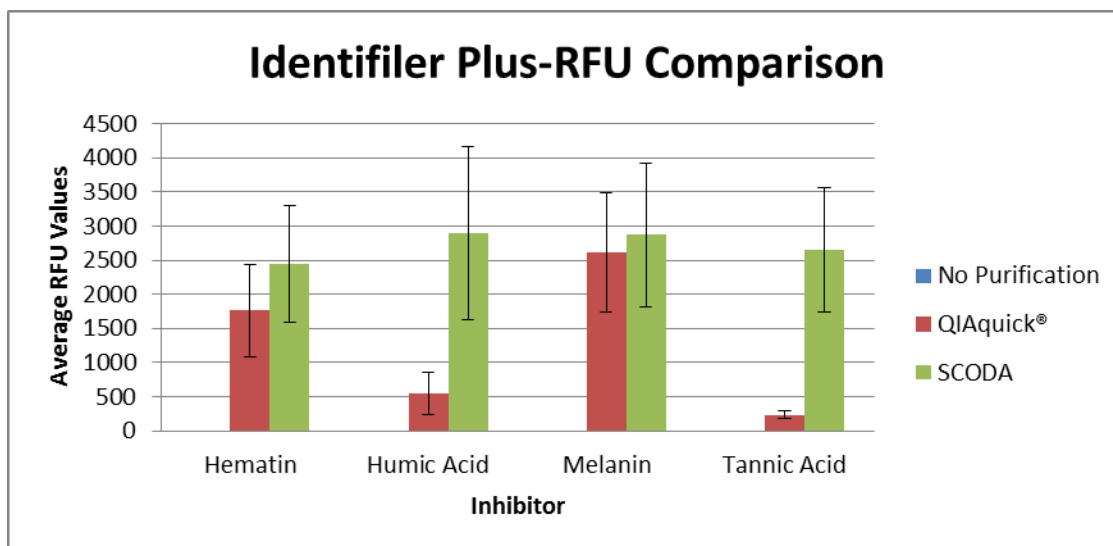


Figure 12. Average Percent Recovery of Total DNA following SCODA. 50 μ l of various concentrations of purified DNA (0.62ng/ μ l, 0.21ng/ μ l, 0.068ng/ μ l, and 0.023ng/ μ l) were placed into SCODA sample cartridges. Experiments were performed in triplicate. Post SCODA DNA yields were compared to Pre-SCODA starting template quantities and results were given as a percentage of the starting quantity.



(a)



(b)

Figure 13. Identifiler® Plus results representing allele recovery for QIAquick® and SCODA purification methods of spiked samples. (a) Percentage allele recovery and (b) average peak height RFU values generated using Identifiler® Plus amplification were compared for no purification, QIAquick® purification, and SCODA purification methods for the following inhibitors: hematin; humic acid; melanin; and tannic acid. The following are final inhibitor and DNA concentration/amounts in 25µL total volume Identifiler® Plus amplification reactions for no purification method: Hematin-2.4mM hematin, 1ng DNA; Humic Acid-30µg humic acid, 1ng DNA; Melanin-4µg melanin, 1ng DNA; Tannic Acid-70µg tannic acid, 1ng DNA. The following are input inhibitor and DNA concentration/amounts added to QIAquick® and SCODA purification methods (10µL of 100µL-elutions for each method were added to each 25µL total volume Identifiler® Plus amplification reaction): Hematin-100µL of 6mM hematin, 10ng DNA;

Humic Acid-300µg humic acid, 10ng DNA; Melanin-40µg melanin, 10ng DNA; Tannic Acid-700µg tannic acid, 10ng DNA.

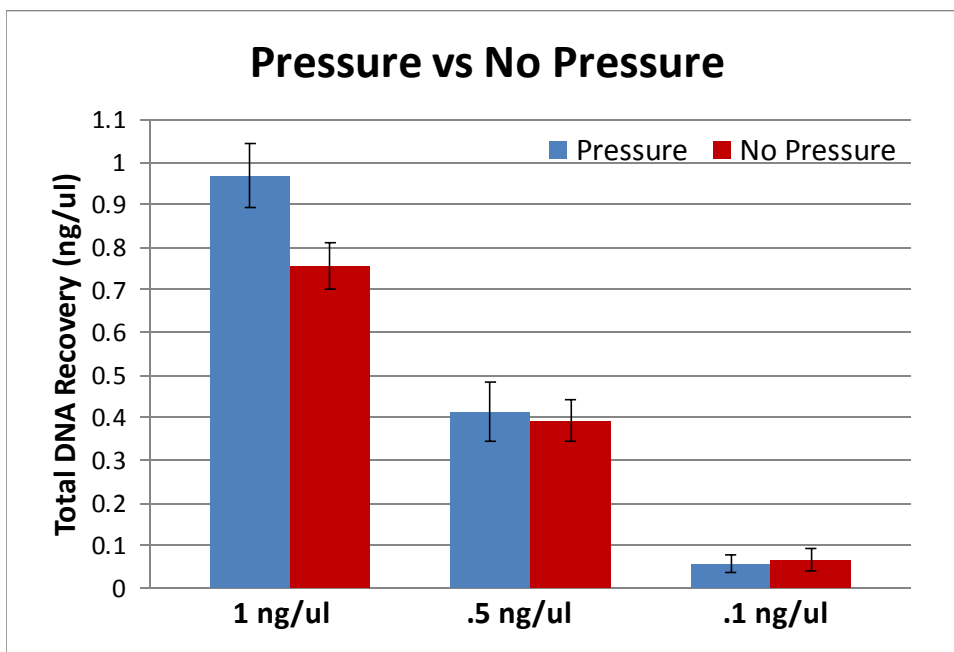


Figure 14. Three concentrations of DNA were tested using PCT and compared with non-pressured samples. 50µl of purified DNA in concentrations of 1ng/ul, 0.5ng/ul, and 0.1ng/ul were added to Pressure Used to Lyse Samples for Extraction (PULSE) tubes and barocycled (30 Cycles [20s at 35k psi and 10s at ambient psi]). Pressured and non-pressured samples were extracted using QIAamp® DNA Mini Kit and quantified using Quantifiler® Human DNA Quantification Kit (reduced volume protocol) on the ABI 7500 Real Time PCR System. Pressure samples were compared with non-pressured samples. Samples were extracted and quantified in triplicate.

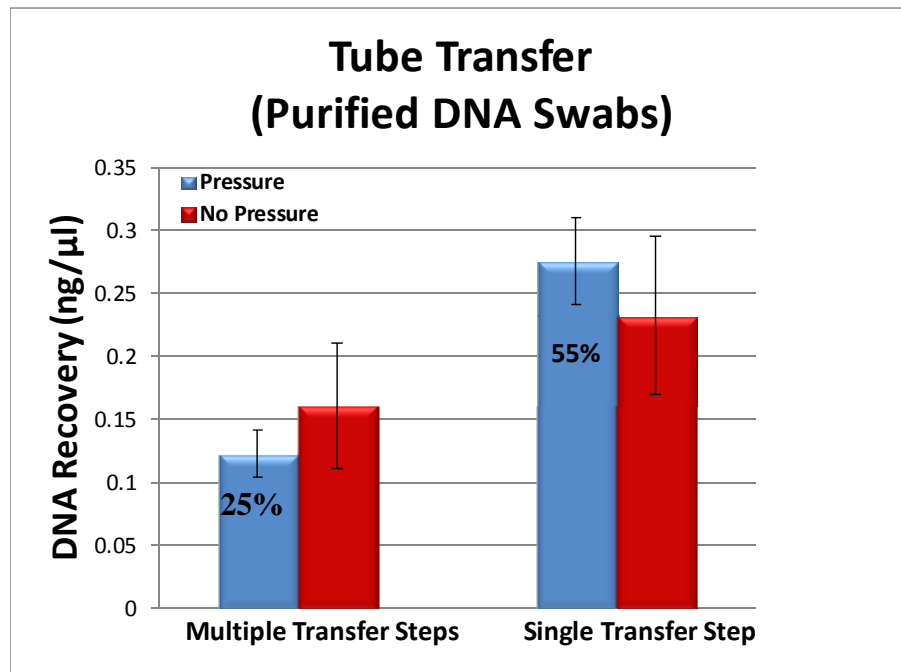


Figure 15. Less Manipulation of Sample. 50μL of purified DNA (500pg/μl) was added to each swab and dried overnight before extraction. Swabs were added to PULSE tubes and either subjected to the original protocol (multiple transfer steps) or the tested protocol of a single transfer step at the end of the extraction process (sample was maintained in the PULSE tube throughout the process). Swabs were then barocycled (30 Cycles [20s at 35k psi and 10s at ambient psi]). Pressured and non-pressured samples were extracted using QIAamp® DNA Mini Kit and quantified using Quantifiler® Human DNA Quantification Kit (reduced volume protocol) on the ABI 7500 Real Time PCR System. Pressure samples were compared with non-pressured samples. Samples were extracted and quantified in triplicate. Yield results tend to be better with less manipulations.

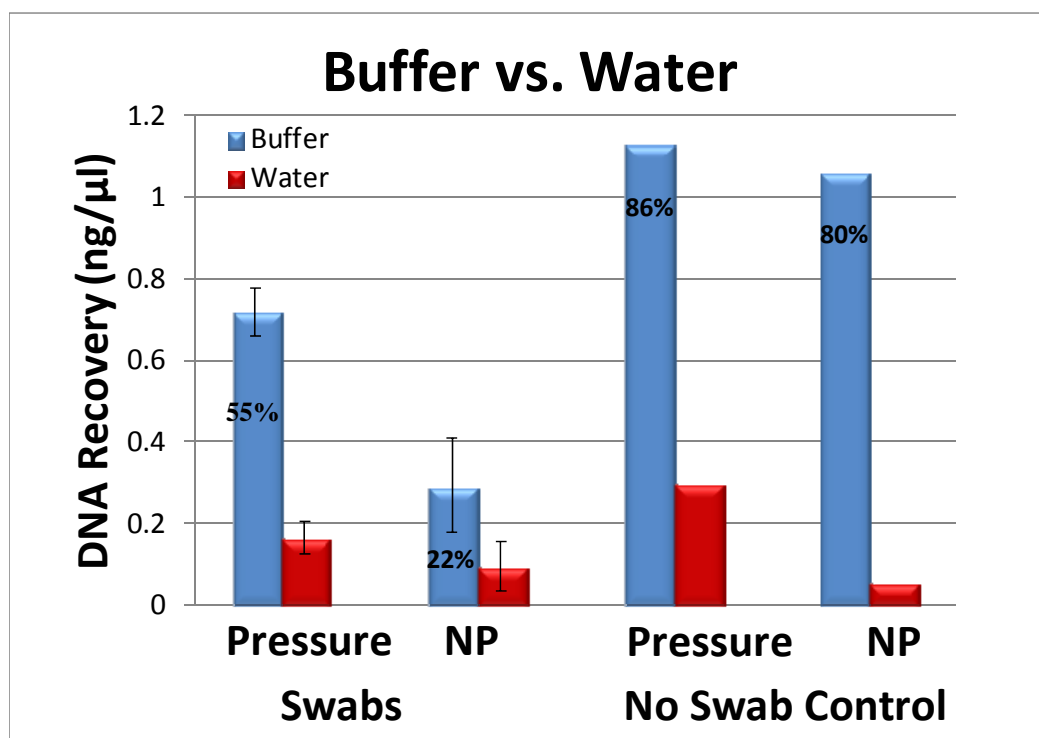


Figure 16. Buffer vs Water. 200 cells/μL of purified DNA (50μL total volume) were added to each swab and dried overnight before extraction. Swabs were added to PULSE tubes and then barocycled (30 Cycles [20s at 35k psi and 10s at ambient psi]). Pressured and non-pressured samples were extracted using QIAamp® DNA Mini Kit and quantified using Quantifiler® Human DNA Quantification Kit (reduced volume protocol) on the ABI 7500 Real Time PCR System. Pressure samples were compared with non-pressured samples in addition to no swab controls. Samples were extracted and quantified in triplicate.

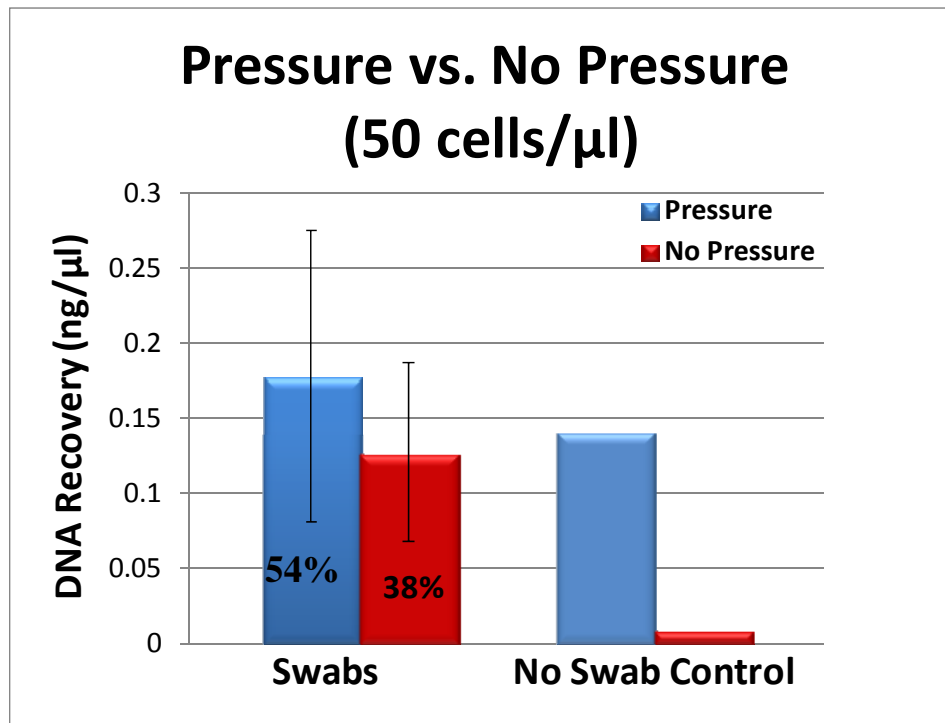


Figure 17. Pressure vs. No PCT. 50 cells/ μ L of purified DNA (50 μ L total volume) were added to each swab and dried overnight before extraction. Swabs were added to PULSE tubes and then barocycled (30 Cycles [20s at 35k psi and 10s at ambient psi]). Pressured and non-pressured samples were extracted using QIAamp® DNA Mini Kit and quantified using Quantifiler® Human DNA Quantification Kit (reduced volume protocol) on the ABI 7500 Real Time PCR System. Pressure samples were compared with non-pressured samples in addition to no swab controls. Samples were extracted and quantified in triplicate.

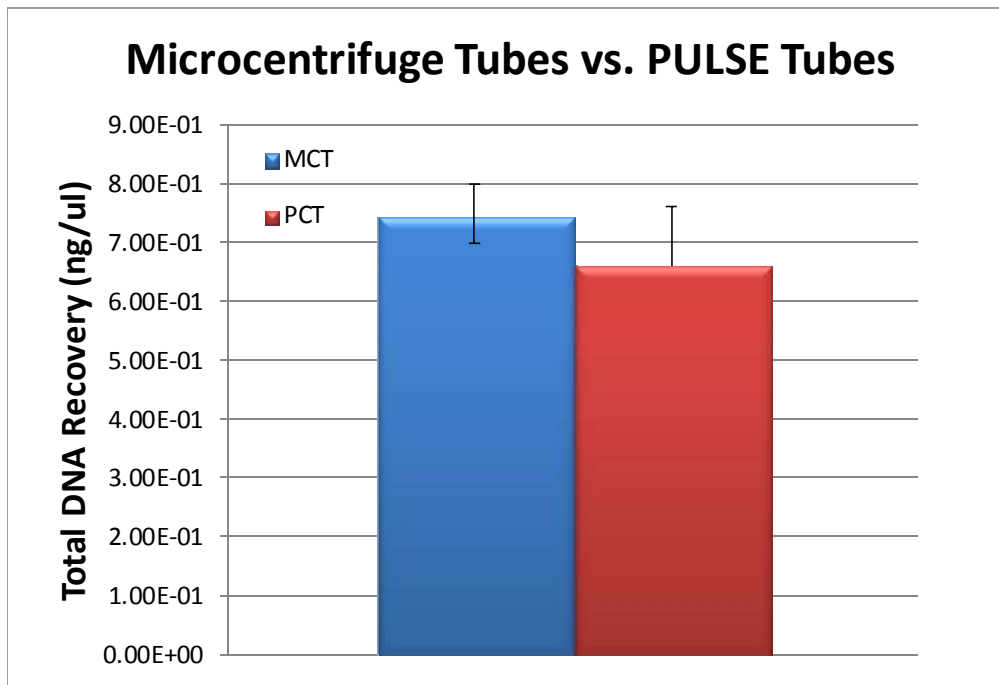


Figure 18. Microcentrifuge Tubes Compared with PULSE Tubes. 50 μ L of purified DNA (1ng/ μ L total volume) were added to 1.5mL Microcentrifuge Tubes or PULSE tubes. Samples were extracted using Maxwell 16 extraction method and quantified using Quantifiler® Human DNA Quantification Kit (reduced volume protocol) on the ABI 7500 Real Time PCR System. Five replicate samples were run for each type of tube. Increased yield of DNA recovered was observed in the Microcentrifuge Tube.

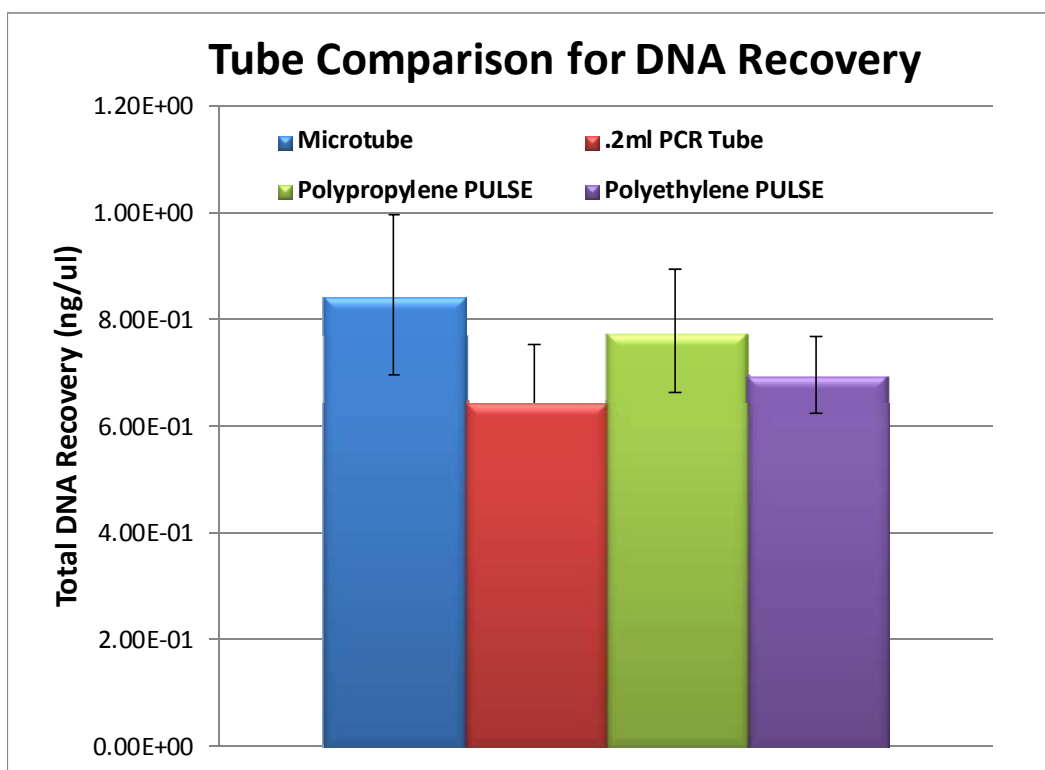


Figure 19. Tube Comparison for DNA Recovery. 150 μ L of purified DNA (1ng/ μ L total volume) were added to Teflon coated 0.2 ml microtubes, 0.2 ml PCR tubes, polypropylene tubes, and polyethylene PULSE tubes. Samples were extracted using the QIamp extraction method and quantified using Quantifiler® Human DNA Quantification Kit (reduced volume protocol) on the ABI 7500 Real Time PCR System. Five replicate samples were run for each type of tube. Increased yield of DNA recovered was observed in the Teflon coated 0.2ml microtubes.

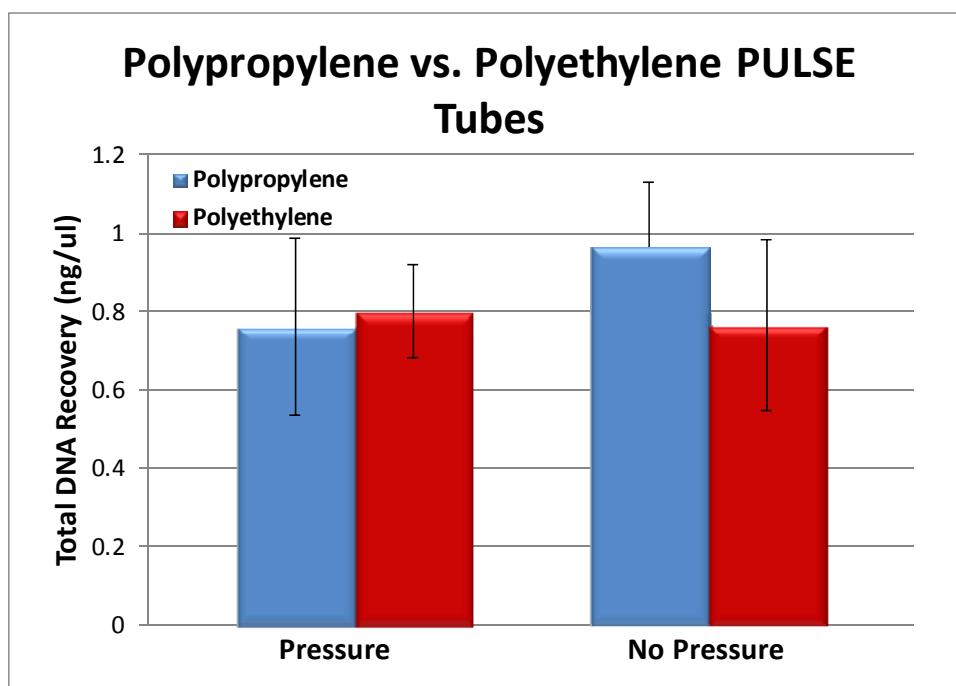


Figure 20. Tube Comparison for DNA Recovery. 150 μ L of purified DNA (1ng/ μ L total volume) were added to polypropylene or polyethylene PULSE tubes. Samples were barocycled and then were extracted using the QIamp extraction method and quantified using Quantifiler[®] Human DNA Quantification Kit (reduced volume protocol) on the ABI 7500 Real Time PCR System. Five replicate samples were run for each type of tube. Increased yield of DNA recovered was observed in the Teflon coated 0.2ml microtubes.

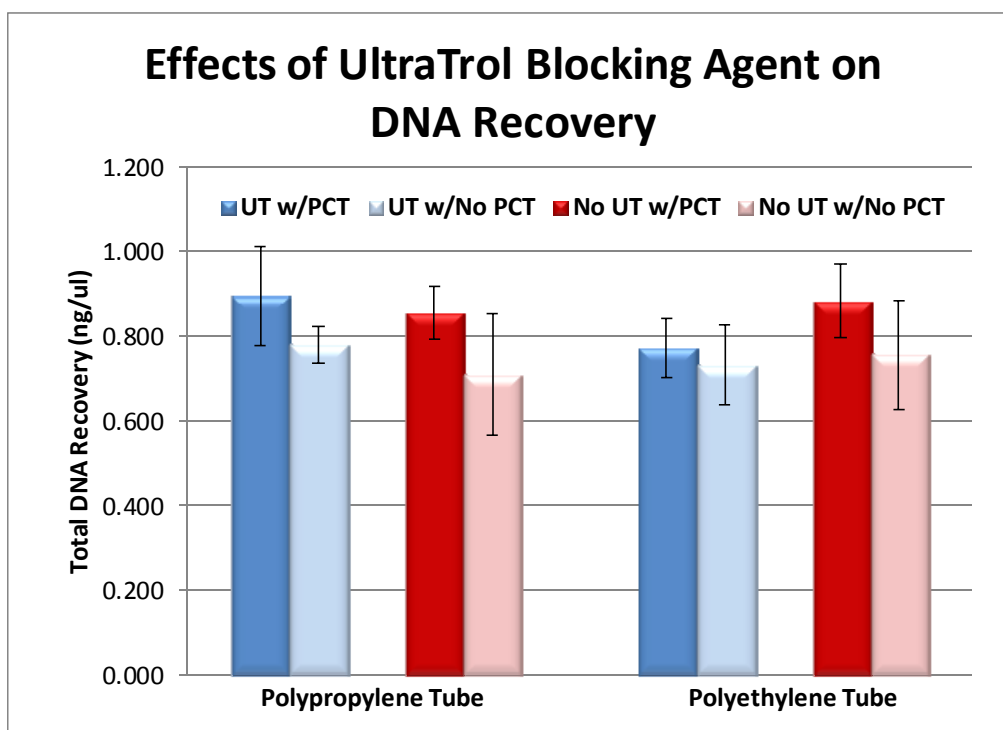


Figure 21. Use of UltraTrol Blocking Agent on PULSE Tubes. 150 μ L of purified DNA (1ng/ μ L total volume) were added to treated (UltraTrol) and untreated (No UltraTrol) PULSE tubes. All samples were barocycled and then were extracted using the QIamp extraction method and quantified using Quantifiler® Human DNA Quantification Kit (reduced volume protocol) on the ABI 7500 Real Time PCR System. Samples were run in triplicate.

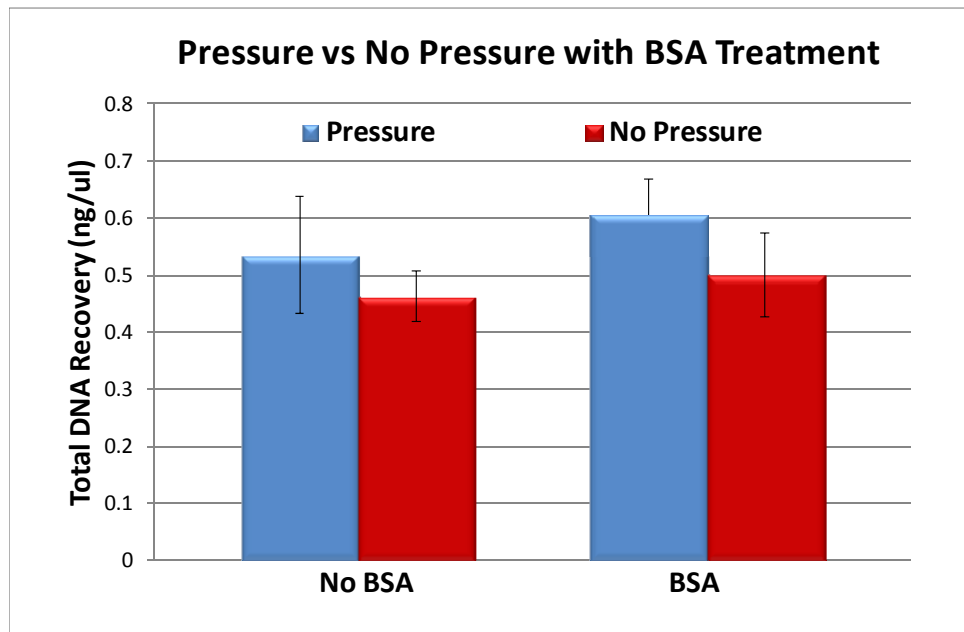


Figure 22. Use of Bovine Serum Albumin on PULSE Tubes. 50 μ L of WBCs (200 cells/ μ L) were added to swabs and swabs dried overnight. PULSE tubes were rinsed in a 1% BSA solution prior to use. Swabs were then placed in treated (BSA) or untreated (no BSA) PULSE tubes. Samples were barocycled and then were extracted using the QIamp extraction method and quantified using Quantifiler® Human DNA Quantification Kit (reduced volume protocol) on the ABI 7500 Real Time PCR System. Samples were run in triplicate. Pressured samples were compared with no pressure samples. Slight increases in DNA yield were observed in both pressure and non-pressure treated samples when the tubes were coated with a 1% BSA solution.

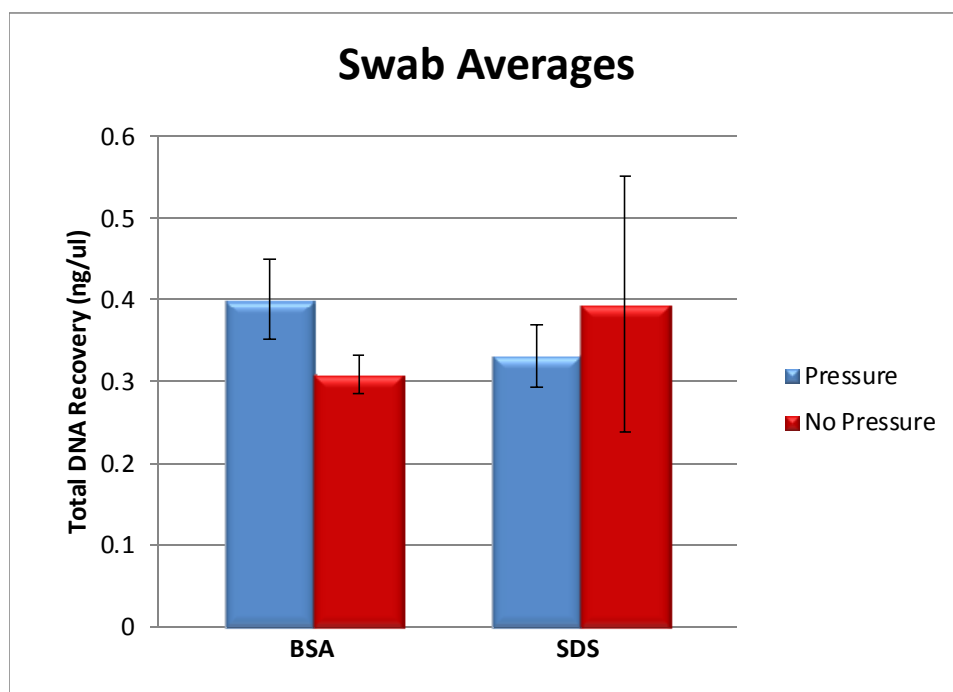


Figure 23. Bovine Serum Albumin vs SDS Treatment on PULSE Tubes. 50 μ L of WBCs (200 cells/ μ L) were added to swabs and swabs dried overnight. PULSE tubes were rinsed in a 1% SDS solution or 1% BSA solution prior to use. Swabs were then placed in treated (BSA) or untreated (no BSA) PULSE tubes. Samples were barocycled and then were extracted using the QIamp extraction method and quantified using Quantifiler® Human DNA Quantification Kit (reduced volume protocol) on the ABI 7500 Real Time PCR System. Samples were run in triplicate. Pressured samples were compared with no pressure samples.

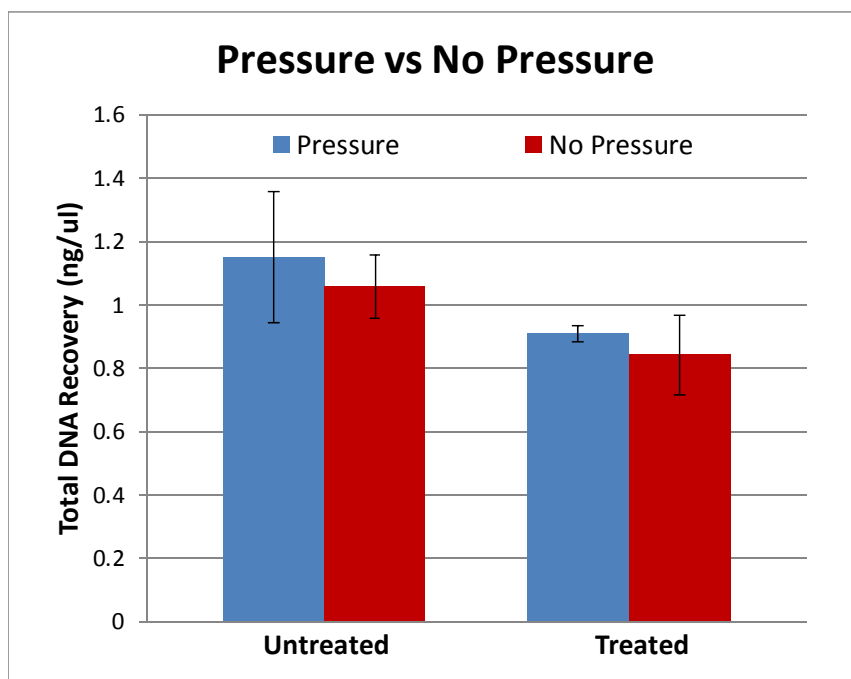


Figure 24. Corning Treatment Effect on DNA Recovery in PULSE Tubes. 50 μ L of purified DNA (1ng/ μ L) were added to untreated and treated (A proprietary solution from Corning) PULSE tubes. Samples were barocycled and then were extracted using the QIamp extraction method and quantified using Quantifiler® Human DNA Quantification Kit (reduced volume protocol) on the ABI 7500 Real Time PCR System. Samples were run in triplicate. Pressured samples were compared with no pressure samples.

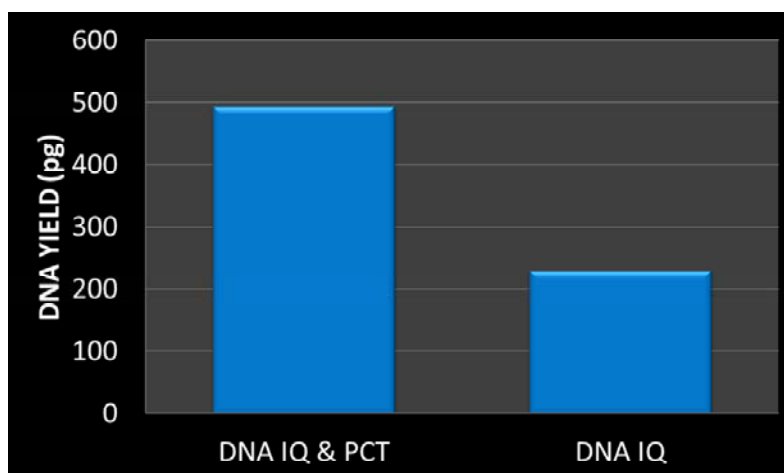


Figure 25. DNA recovery from bones with and without Pressure Cycling. The histogram on the left is with pressure. Both sets of samples were extracted using DNA IQ (Promega Corporation).

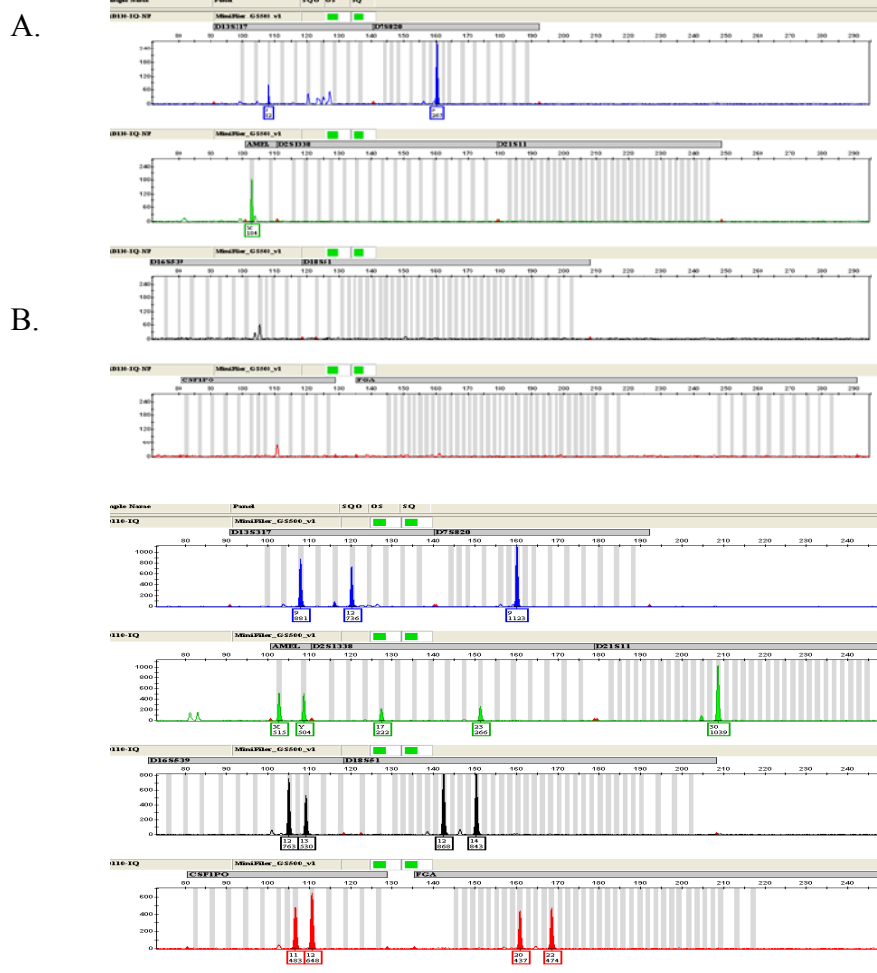


Figure 26. A. Electropherogram of DNA profile from bone under standard extraction and typing conditions. B. Electropherogram of DNA profile from bone under Pressure, standard extraction and typing conditions.

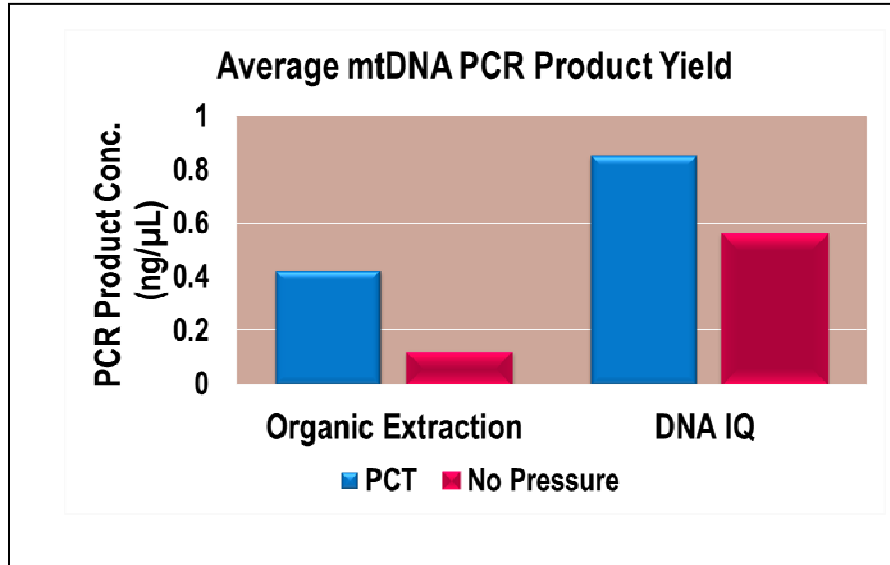


Figure 27. mtDNA yield recovery from hairs with and without Pressure Cycling. The histogram on the left was with pressure. Both sets of samples were extracted using DNA IQ (Promega Corporation).

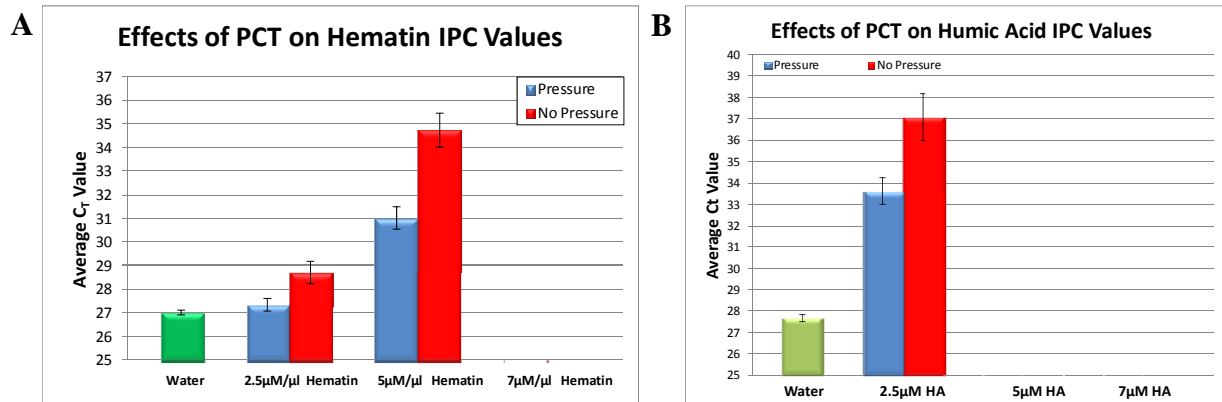


Figure 28. 1mL of Hematin (84.5µM/µl stock) and Humic Acid (500ng/µl stock) was barocycled (30 Cycles [20s at 35k psi and 10s at ambient psi]). Various concentrations of hematin (0, 2.5, 5, and 7 µM/µl) and humic acid (0, 2.5, 5, and 7 ng/µl), A and B respectively, were then added to the Quantifiler master mix (reduced volume protocol) and quantified using ABI 7500 Real Time PCR System [Note: this experiment was done in the absence of DNA]. The data shown in Figure A represents 30 replicates while Figure B represents 5 replicate experiments. The IPC Ct values for each concentration are shown. Pressure samples were compared with non-pressured controls.

Hematin	Average Quant	StDev Quant	Average IPC	StDev IPC
He.0.PC	0.96	0.12	27.50	0.08
HE.0.NP	0.71	0.11	27.84	0.12
HE.2.5.PC	0.76	0.18	27.90	0.09
HE.2.5.NP	0.56	0.03	27.63	0.26
HE.5.PC	0.23	0.13	34.33	1.42
HE.5.NP	0.14	0.03	Undetermined	0.00
HE.7.PC	Undetermined	0.00	Undetermined	0.00
HE.7.NP	Undetermined	0.00	Undetermined	0.00

Humic Acid	Average Quant	StDev Quant	Average IPC	StDev IPC
HA.0.PC	0.75	0.09	27.43	0.13
HA.0.NP	0.90	0.16	27.41	0.05
HA.2.5.PC	Undetermined	0.00	Undetermined	0.00
HA.2.5.NP	Undetermined	0.00	Undetermined	0.00
HA.5.PC	Undetermined	0.00	Undetermined	0.00
HA.5.NP	Undetermined	0.00	Undetermined	0.00

Table 1. 1mL of Hematin (84.5 μ M/ μ l stock) and Humic Acid (500ng/ μ l stock) was barocycled (30 Cycles [20s at 35k psi and 10s at ambient psi]). Various concentrations of hematin (0, 2.5, 5, and 7 μ M/ μ l) and humic acid (0, 2.5, and 5 ng/ μ l), were then added to 50ul purified DNA (1 ng/ μ l). Samples were then quantified using reduced volume Quantifiler protocol using ABI 7500 Real Time PCR System. Samples were run in 5 replicates. The average DNA recovery and average IPC Ct values for each concentration are shown. Pressure samples were compared with non-pressured controls.

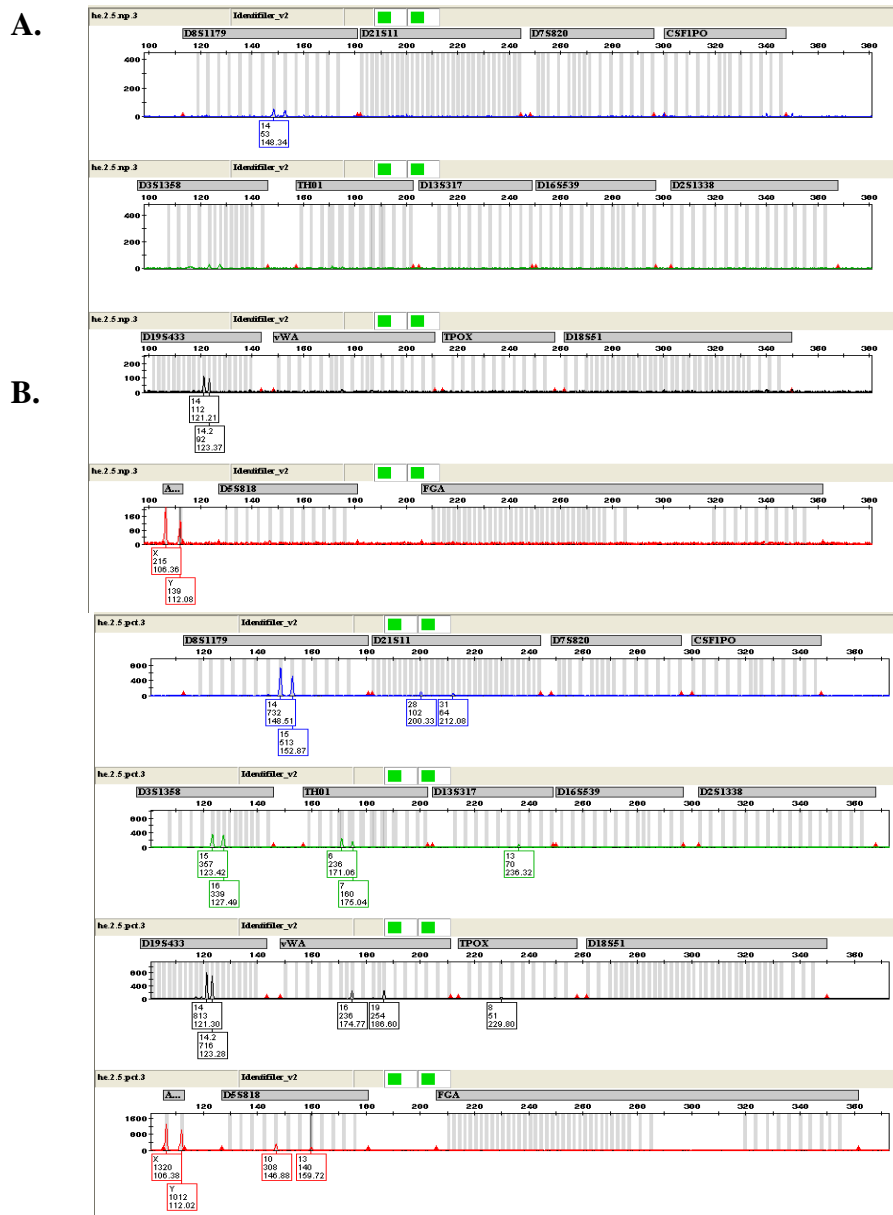


Figure 30. 1mL of Hematin (84.5 μ M/ μ l stock) was barocycled (30 Cycles [20s at 35k psi and 10s at ambient psi]). 2.5 μ M/ μ l of hematin were then added to 50 μ l purified DNA (1 ng/ μ l). A. Electropherogram of DNA profile with non-pressed hematin. B. Electropherogram of DNA profile using hematin that was pressured. More loci are observed in the PCT sample.

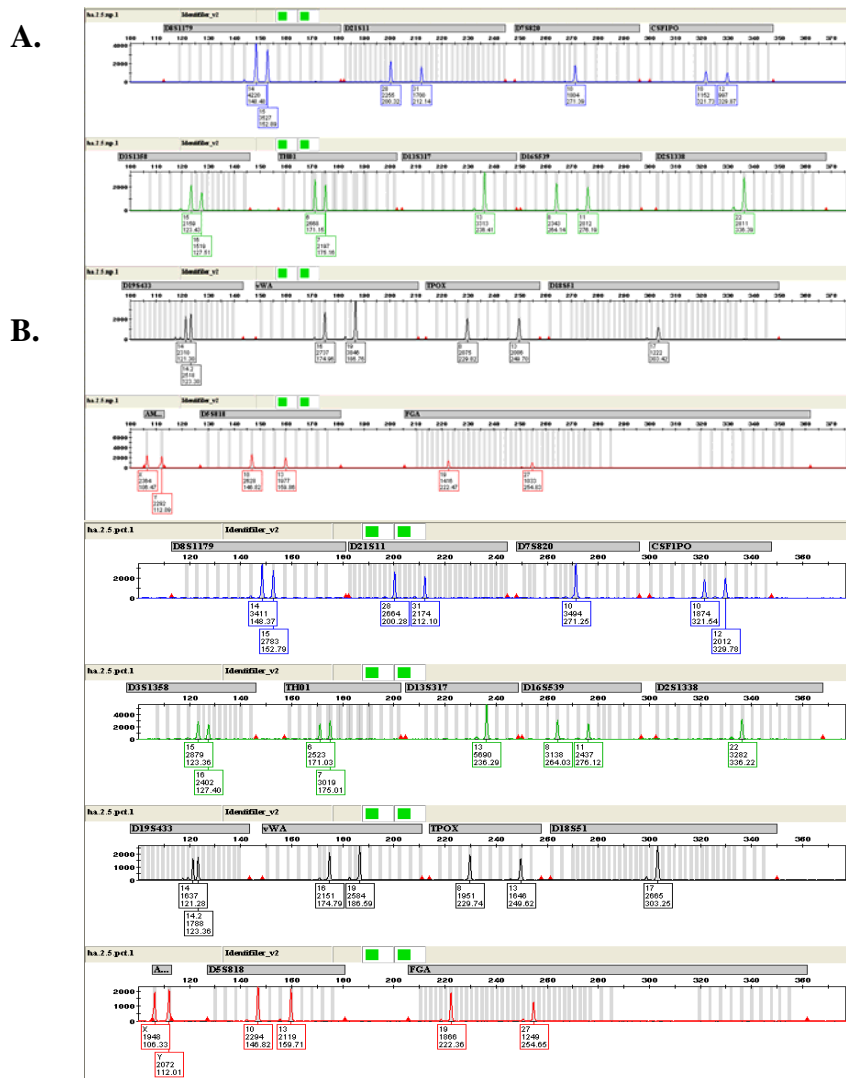


Figure 31. 1mL of Humic Acid (500ng/ μ l stock) was barocycled (30 Cycles [20s at 35k psi and 10s at ambient psi]). 2.5ng/ μ l of humic acid were then added to 50 μ l purified DNA (1 ng/ μ l). A. Electropherogram of DNA profile with non-pressed humic acid. B. Electropherogram of DNA profile using humic acid that was pressured. While both gave full profiles, relative fluorescent units (RFUs) in the pressured sample were higher and there was no ski-slope effect observed in the pressured sample as compared with the non-pressed sample.

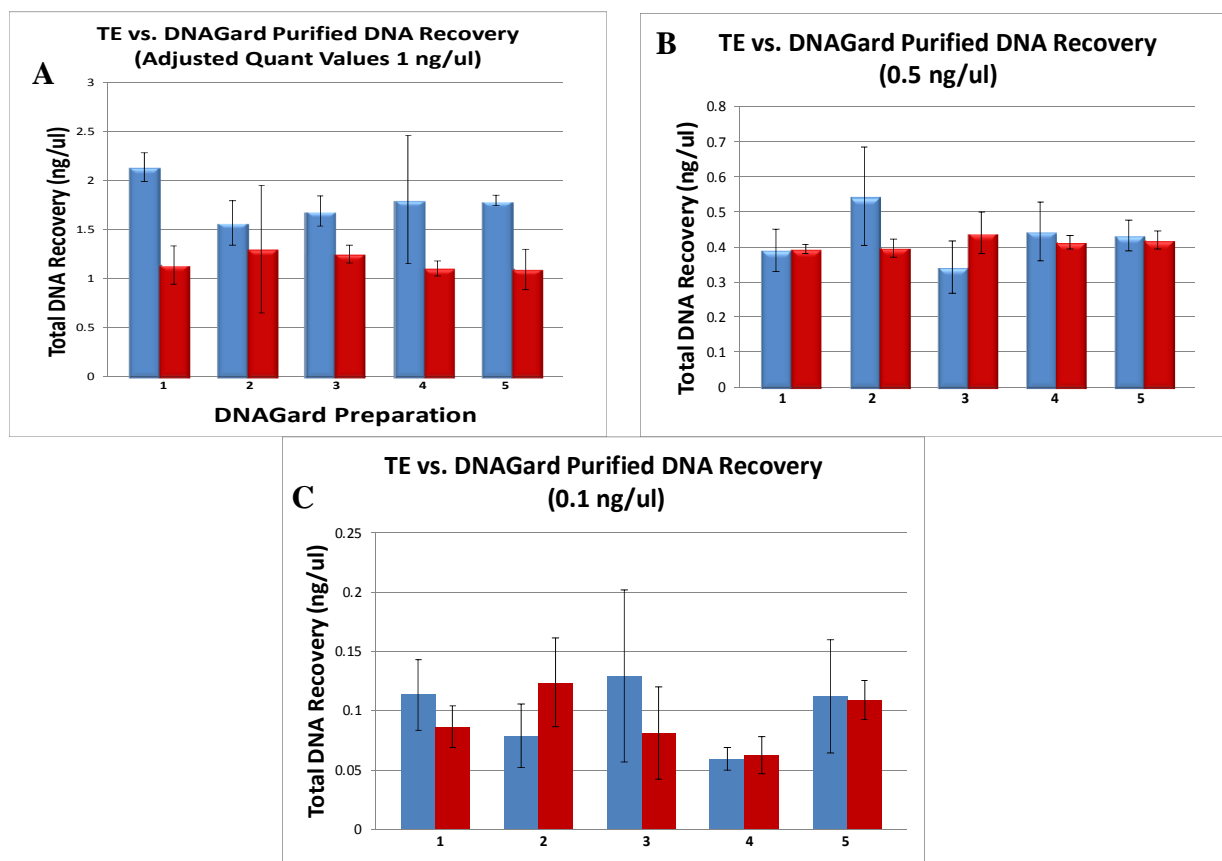


Figure 32. Five formulations of DNAGard™ were compared with TE⁻⁴ buffer. Three concentrations of purified DNA, 1ng/ul, 0.5ng/ul, or 0.1ng/ul, Figures A-C respectively, were added to swabs, dried overnight, and placed into either TE⁻⁴ buffer or one of five formulations of DNAGard™. The swabs were left in the tubes overnight and extracted the following day using QIAamp® DNA Mini and Blood Mini following the manufacturer's protocol. DNA yields were then determined using a reduced-volume Quantifiler™ reaction and quantified using ABI 7500 Real Time PCR System. Samples were run in triplicate. The blue bars represent TE-4 buffer while the red bars represent DNAGard™.

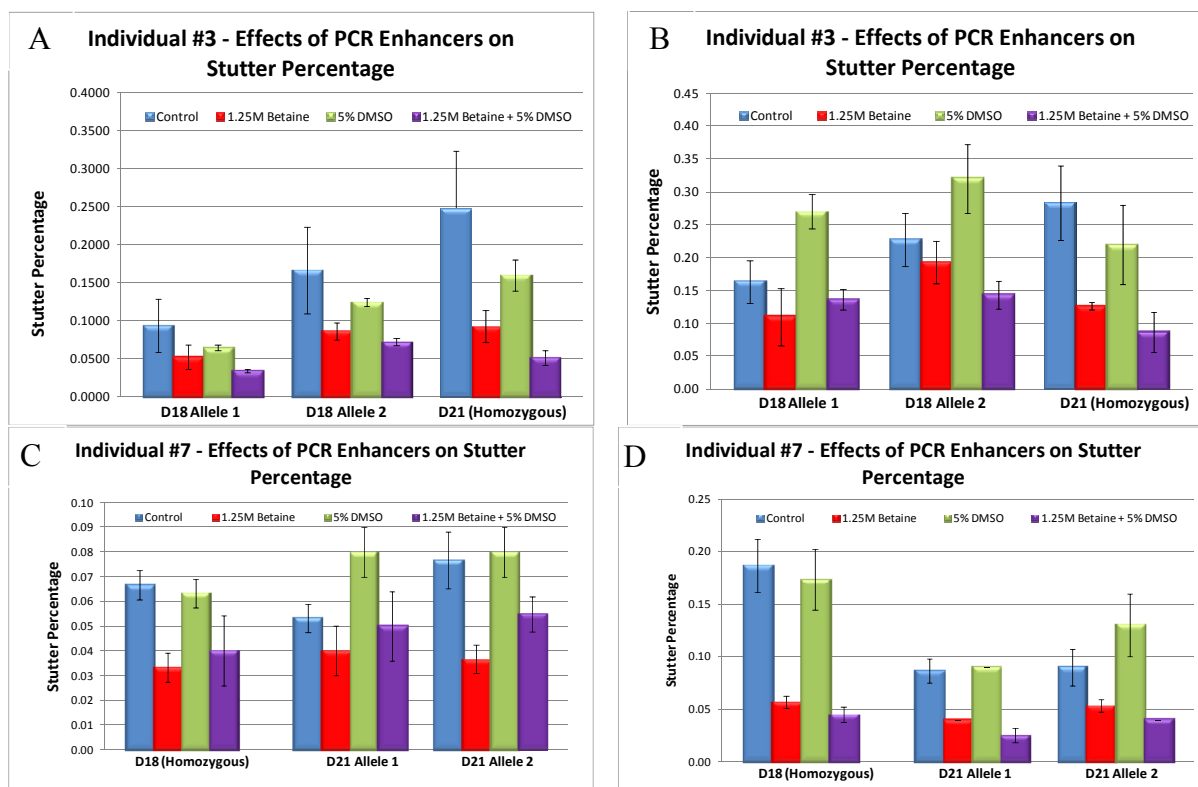


Figure 33. Buccal samples from five individuals were extracted using AutoMate Express™. DNA extracts in concentrations of 0.5 and 1ng/μl were added to custom amplification reaction mixes containing: Control – no PCR enhancer, 1.25M Betaine, 5% DMSO, or a mixture of 1.25M Betaine and 5% DMSO, and amplified using TaqGold polymerase. Samples were then run on the 3130xl and analyzed with GeneMapper®. Stutter percentages were then calculated. A-D represent samples from two individuals: A) 1ng/μl of DNA at 28 cycles. B) 1ng/μl of DNA at 34 cycles. C) 0.5ng/μl of DNA at 28 cycles. D) 0.5ng/μl of DNA at 34 cycles.

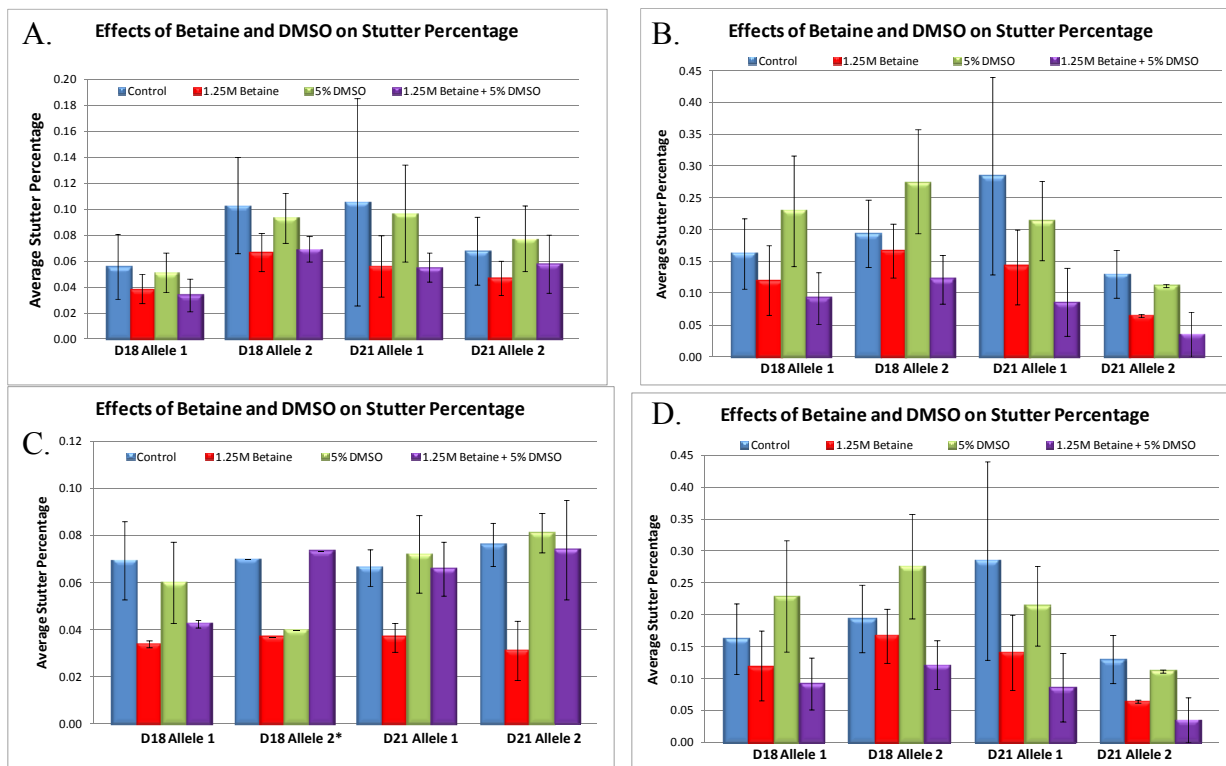


Figure 34. Buccal samples from five individuals were extracted using AutoMate Express™. DNA extracts in concentrations of 0.5 and 1ng/μl were added to custom amplification reaction mixes containing: Control – no PCR enhancer, 1.25M Betaine, 5% DMSO, or a mixture of 1.25M Betaine and 5% DMSO, and amplified using TaqGold polymerase. Samples were then run on the 3130xl and analyzed with GeneMapper®. Stutter percentages were then calculated. A-D represent averages across five individuals: A) 1ng/μl of DNA at 28 cycles. B) 1ng/μl of DNA at 34 cycles. C) 0.5ng/μl of DNA at 28 cycles. D) 0.5ng/μl of DNA at 34 cycles. *For figures XA-B, only two of the five individuals are heterozygous at the D21 locus. *For C-D, only one individual is heterozygous at the D18 locus.

A	Loci (separate alleles)	D18S51 Betaine Stutter	D18S51 Betaine True Allele	D18S51 Control Stutter	D18S51 Control True Allele
	Average RFUs	116	1735	177	1757
	St Dev	87	1395	127	1249
	Stutter %	0.05		0.09	
	St Dev (%)	0.02		0.05	

B	Loci (separate alleles)	D21S11 Betaine Stutter	D21S11 Betaine True Allele	D21S11 Control Stutter	D21S11 Control True Allele
	Average RFU's	152	2347	214	2421
	St dev	133	1721	169	1788
	Stutter %	0.07		0.08	
	St Dev (%)	0.09		0.03	

C	Loci (separate alleles)	D18S51 Betaine Allele 1	D18S51 Betaine Allele 2	D18S51 Control Allele 1	D18S51 Control Allele 2
	Average RFU's	1584	1861	1519	1946
	St dev	1234	1514	1045	1368
	PHR (%)*	0.59		0.61	
	St Dev (%)	0.26		0.24	

D	Loci (separate alleles)	D21S11 Betaine Allele 1	D21S11 Betaine Allele 2	D21S11 Control Allele 1	D21S11 Control Allele 2
	Average RFUs	2165	2504	2266	2547
	St Dev	1641	1783	1745	1823
	PHR (%)*	0.64		0.59	
	St Dev (%)	0.25		0.27	

Table 2 (A-B). Buccal samples from 86 individuals were extracted using AutoMate Express™. One µL of DNA extracts in concentrations of 100pg/µl was added to custom amplification reaction mixes containing: Control – no PCR enhancer or Betaine (1.25M), and amplified using TaqGold polymerase. Samples were then run on the 3130xl and analyzed with GeneMapper© Software. Average peak heights and stutter ratios at two markers, D18S51 and D21S11, were then calculated. Two of the 86 samples failed to amplify. Averages were across 84 individuals. *For stutter percentage tables, an average was taken for all alleles at the locus, including both heterozygous and homozygous peaks, for a single “stutter” and “true allele” average. **A)** Average Stutter Ratio at D18S51 and **B)** Average Stutter Ratio at D21S11 for 100 pg of template DNA at 34 cycles. **C)** Average Peak Height Ratio at D18S51 and **D)** Average Peak Height Ratio at D21S11 with 100 pg of template DNA 34 cycles.

A	Loci (separate alleles)	D18S51 Betaine Stutter	D18S51 Betaine True Allele	D18S51 Control Stutter	D18S51 Control True Allele
	Average RFUs	63	720	90	889
	St Dev	48	534	70	637
	Stutter %	0.06		0.10	
	St Dev (%)	0.03		0.05	

B	Loci (separate alleles)	D21S11 Betaine Stutter	D21S11 Betaine True Allele	D21S11 Control Allele 1	D21S11 Control True Allele
	Average RFUs	70	1024	89	1054
	St Dev	64	864	71	833
	Stutter %	0.06		0.08	
	St Dev (%)	0.02		0.03	

C	Loci (separate alleles)	D18S51 Betaine Allele 1	D18S51 Betaine Allele 2	D18S51 Control Allele 1	D18S51 Control Allele 2
	Average RFUs	625	772	896	884
	St Dev	430	582	552	694
	PHR (%)*	0.58		0.61	
	St Dev (%)	0.23		0.29	

D	Loci (separate alleles)	D21S11 Betaine Allele 1	D21S11 Betaine Allele 2	D21S11 Control Allele 1	D21S11 Control Allele 2
	Average RFU's	901	1119	883	1178
	St dev	884	845	701	903
	PHR (%)*	0.59		0.57	
	St Dev (%)	0.26		0.26	

Table 3. Buccal samples from 86 individuals were extracted using AutoMate Express™. One μ L of DNA extracts in concentrations of 25pg/ μ L was added to custom amplification reaction mixes containing: Control – no PCR enhancer or Betaine (1.25M), and amplified using TaqGold polymerase. Samples were then run on the 3130xl and analyzed with GeneMapper© Software. Average peak heights and stutter ratios at two markers, D18S51 and D21S11, were then calculated. Two of the 86 samples failed to amplify. An additional seven samples failed at either one locus or both loci and were excluded. Averages are across 77 individuals. *For stutter percentage tables, an average was taken for all alleles at the locus, including both heterozygous and homozygous peaks, for a single “stutter” and “true allele” average. **A)** Average Stutter Ratio at D18S51 and **B)** Average Stutter Ratio at D21S11 for 25pg of template DNA at 34 cycles. **C)** Table – Average Peak Height Ratio at D18S51 and **D)** Average Peak Height Ratio at D21S11 with 25pg of template DNA.

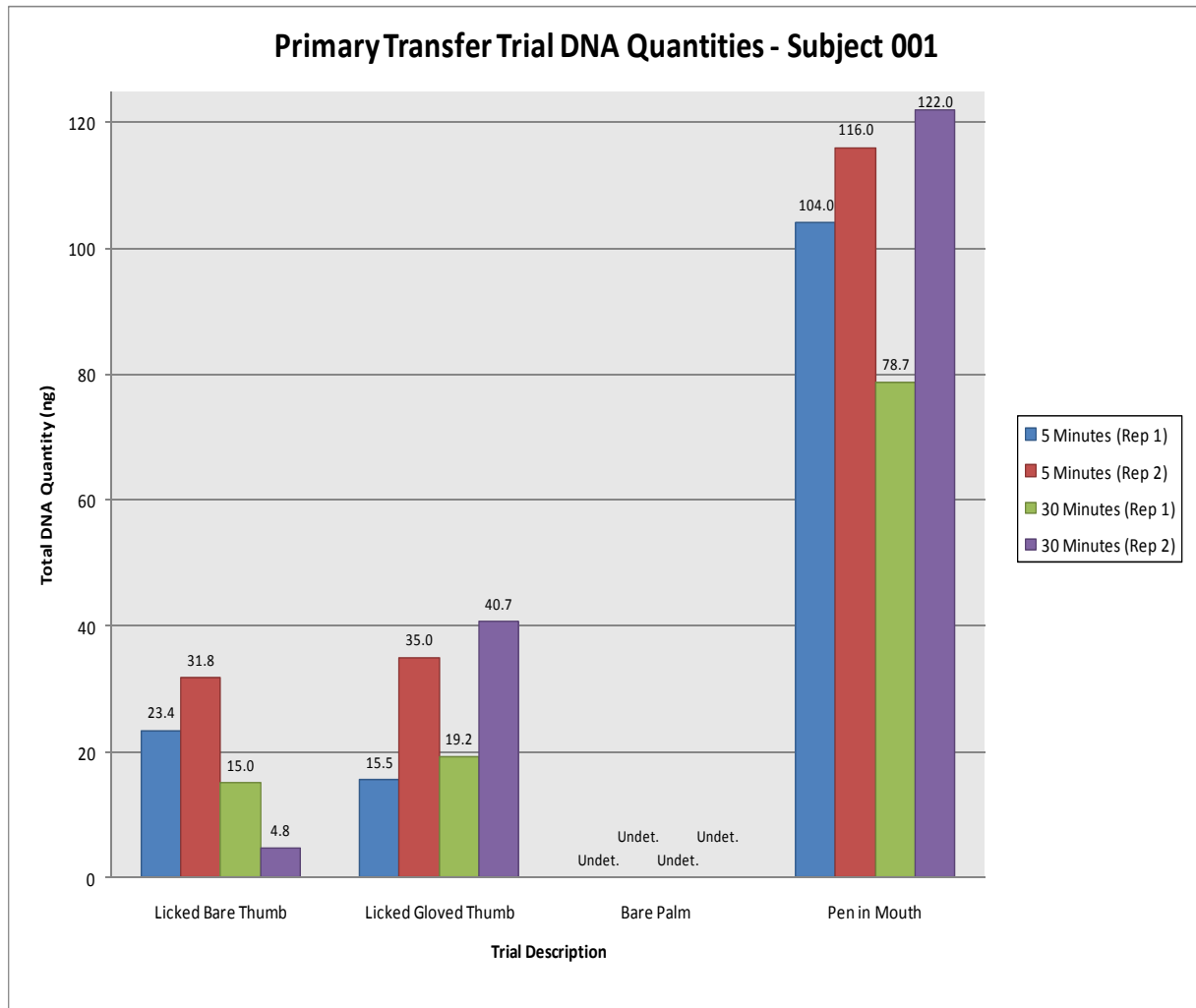


Figure 35. Primary Transfer Trial DNA Quantities – Subject 001. DNA was obtained in generally higher quantities from licked gloved thumbs as opposed to licked bare thumbs. Bare palms do not yield detectable levels of DNA. Pens held in a subject’s mouth yielded high quantities of DNA. Drying time did not appear to affect DNA yield.

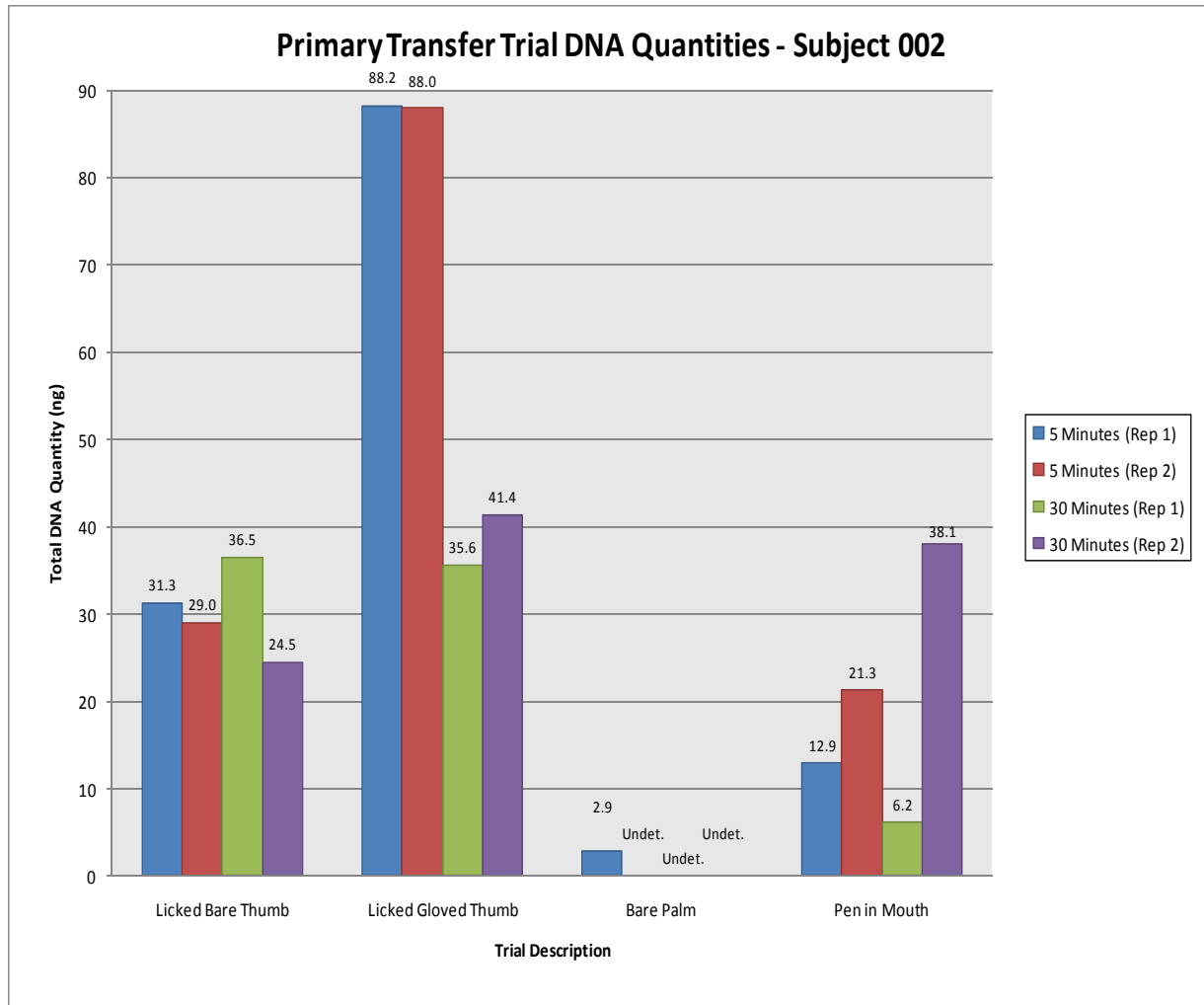


Figure 36. Primary Transfer Trial DNA Quantities – Subject 002. DNA was obtained in generally higher quantities from licked gloved thumbs as opposed to licked bare thumbs. Bare palms did not generally yield detectable levels of DNA. Pens held in a subject’s mouth yielded observable quantities of DNA. Drying time did not substantially affect DNA yield.

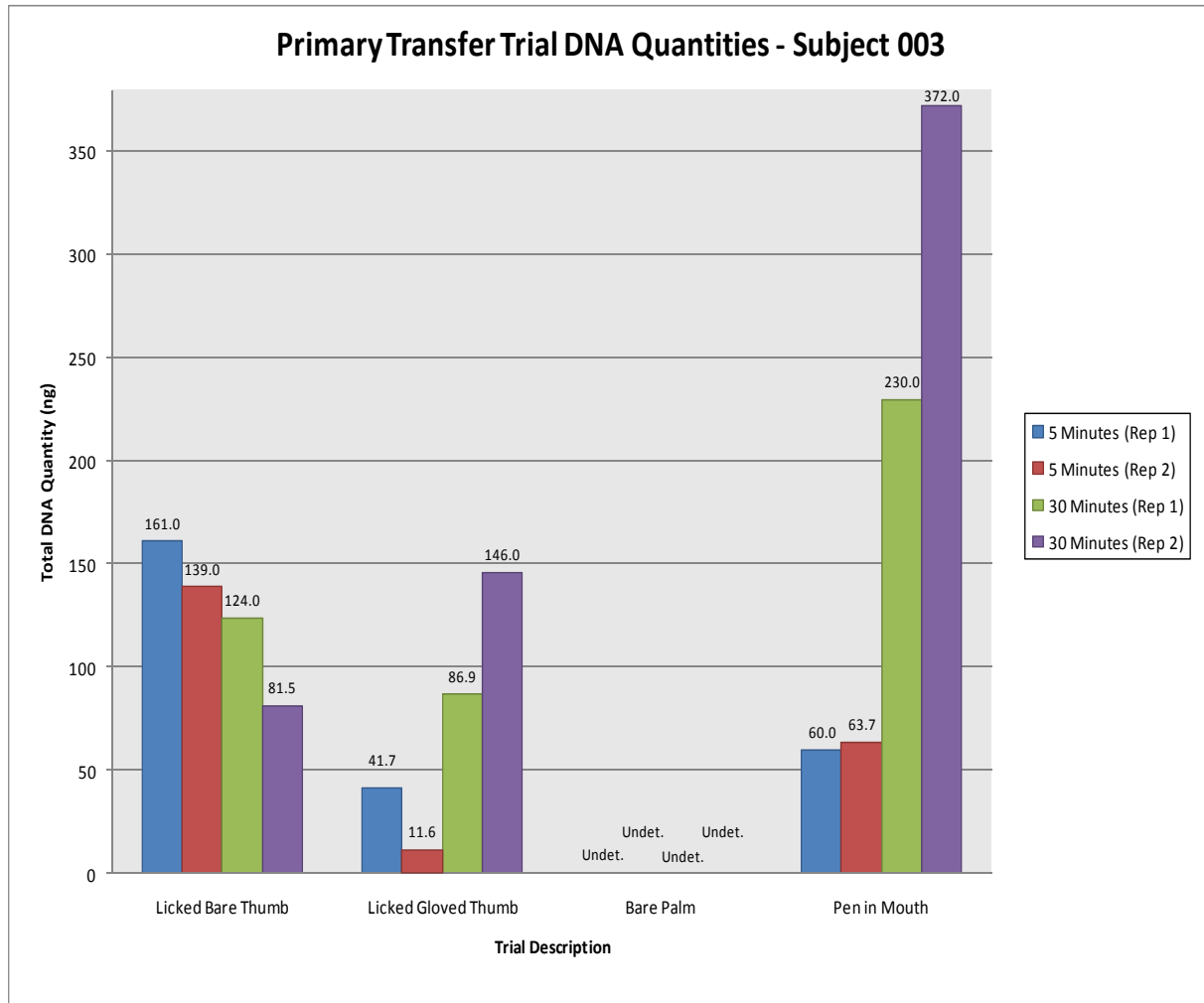


Figure 37. Primary Transfer Trial DNA Quantities – Subject 003. In this case, DNA was obtained in generally higher quantities from licked bare thumbs as opposed to licked gloved thumbs. Bare palms did not generally yield detectable levels of DNA. Pens held in a subject’s mouth yielded fairly high quantities of DNA. Drying time did not appear to affect DNA yield.

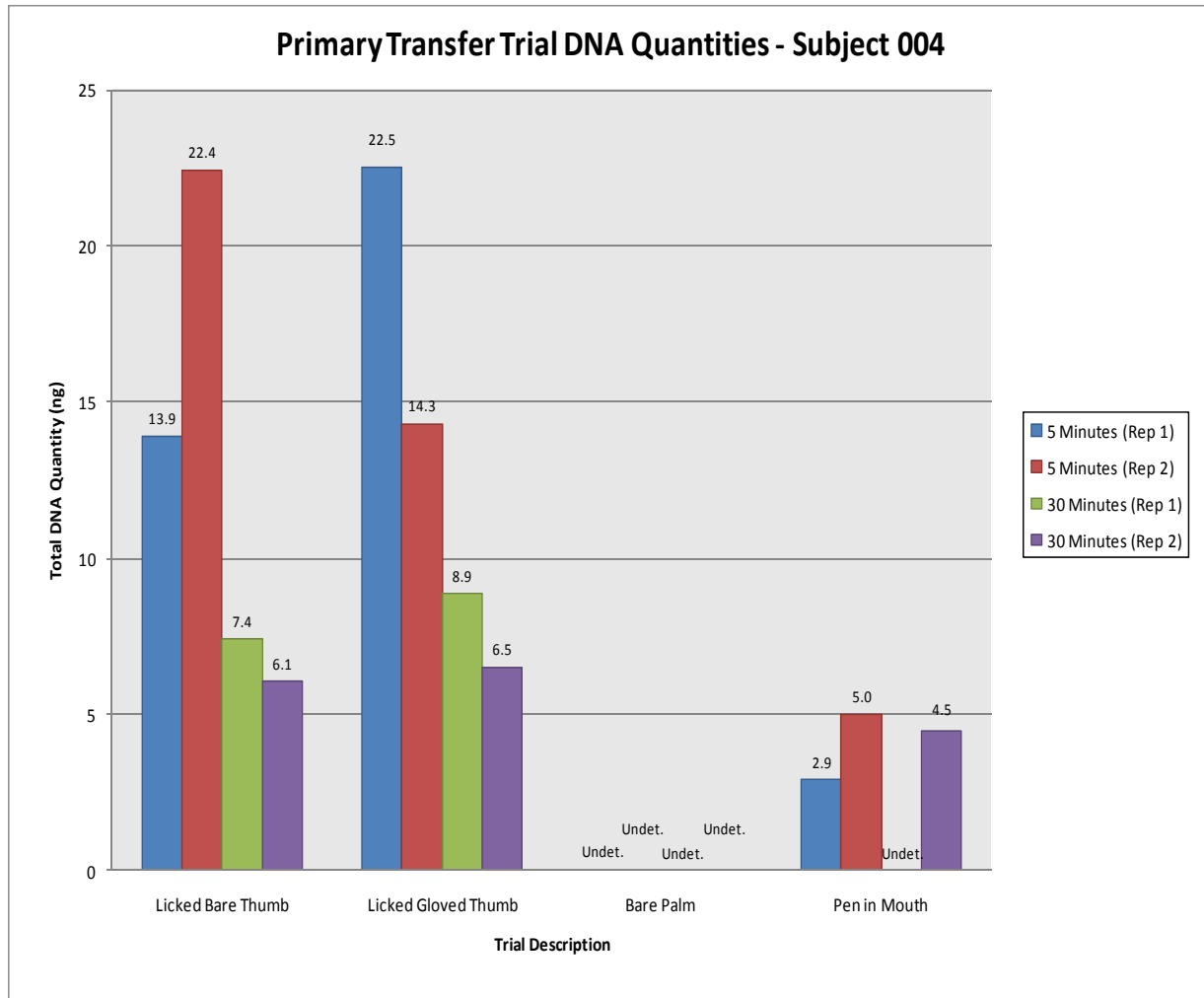


Figure 38. Primary Transfer Trial DNA Quantities – Subject 004. DNA was obtained in generally higher quantities from licked gloved thumbs as opposed to licked bare thumbs. Bare palms did not yield detectable levels of DNA. Pens held in a subject’s mouth generally yielded observable quantities of DNA. Drying time showed some effect on DNA yield, as longer drying times in this case were associated with lower quantities of DNA.

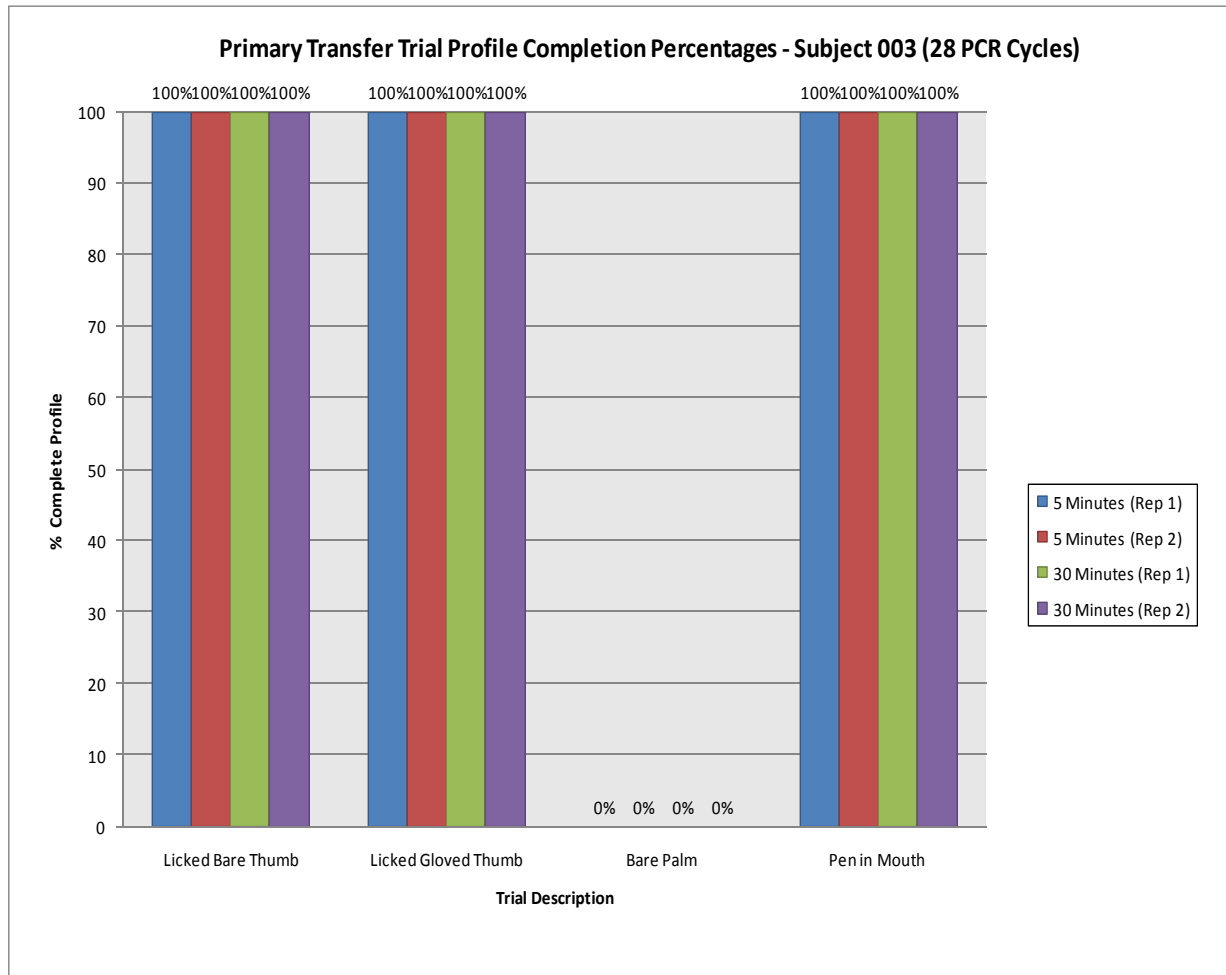


Figure 39. Primary Transfer Trial Profile Completion Percentages - Subject 003 (28 PCR Cycles). The genetic profiles obtained from the licked bare thumb, licked gloved thumb, and pen samples all showed 100% of the expected alleles, while the bare palm swab samples yielded no profiles at 28 PCR cycles.

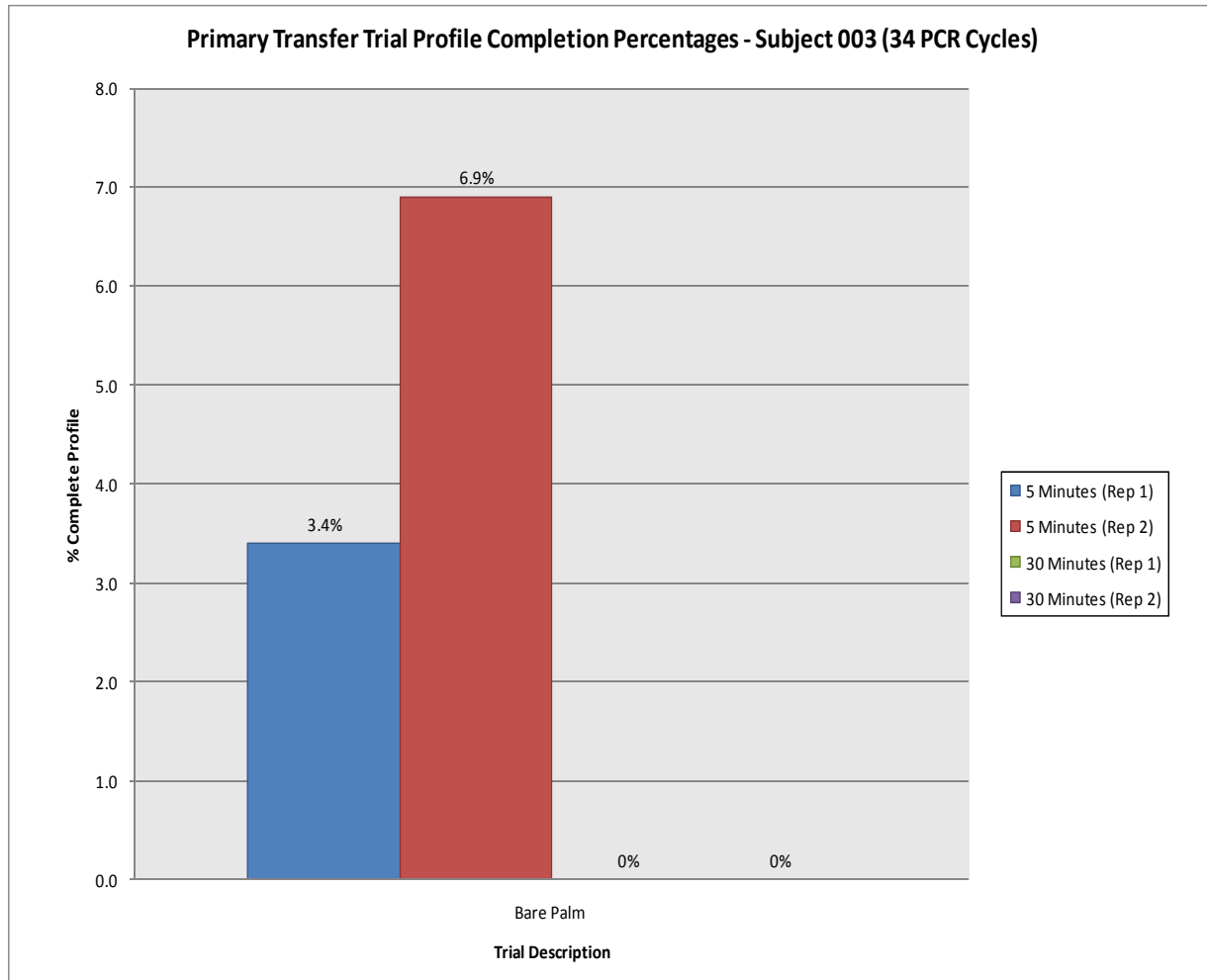


Figure 40. Primary Transfer Trial Profile Completion Percentages – Subject 003 (34 PCR Cycles). When amplified at 34 PCR cycles, the bare palm swab samples still yielded genetic profiles showing an extremely low amount of the expected alleles, or no alleles.

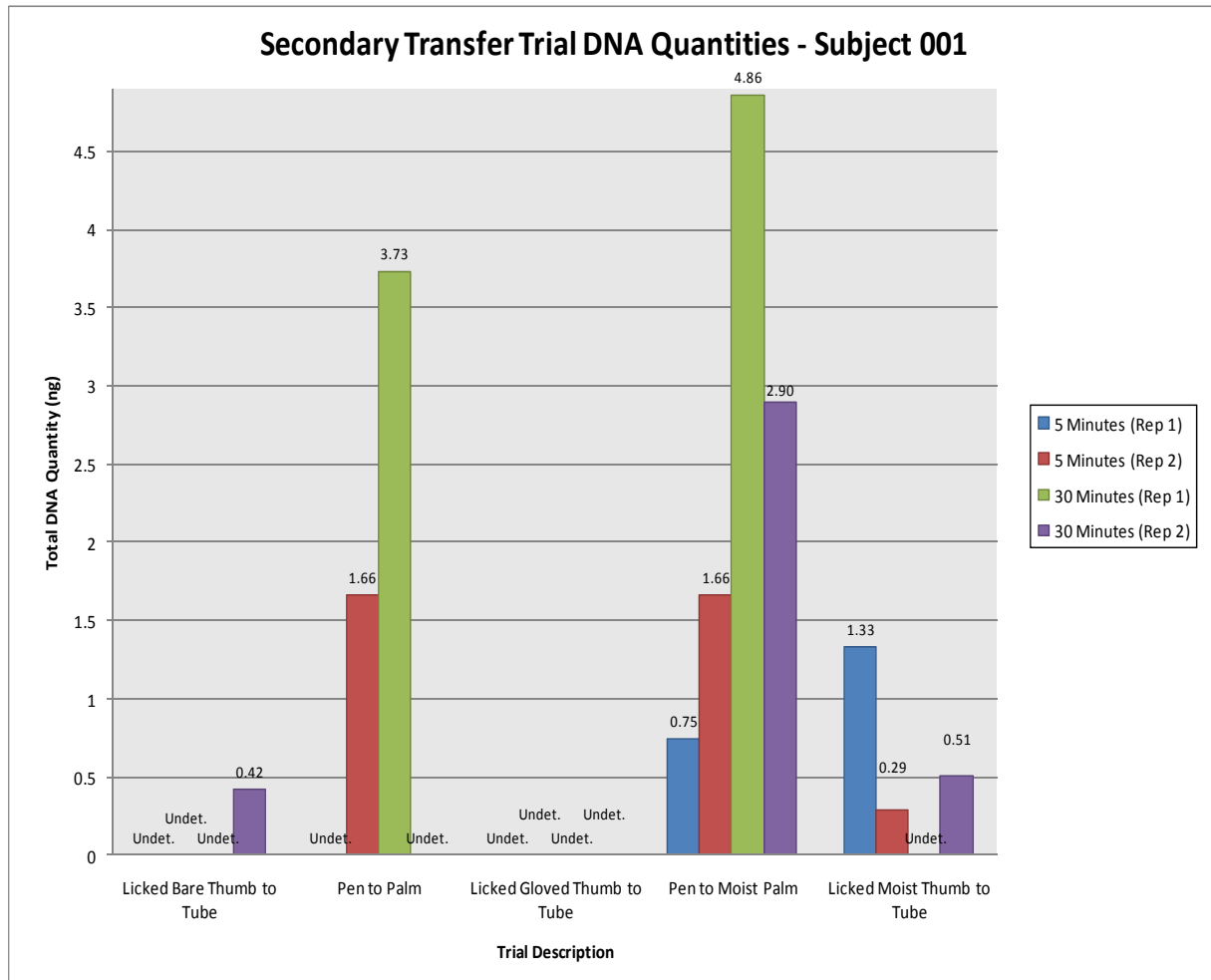


Figure 41. Secondary Transfer Trial DNA Quantities – Subject 001. The DNA present in the majority of the Licked Bare Thumb to Tube and Licked Glove Thumb to Tube samples was found at such low levels that the quantity estimates were generally undetermined. The Pen to Palm, Pen to Moist Palm, and Licked Moist Thumb to Tube trials all yielded low DNA quantity estimates.

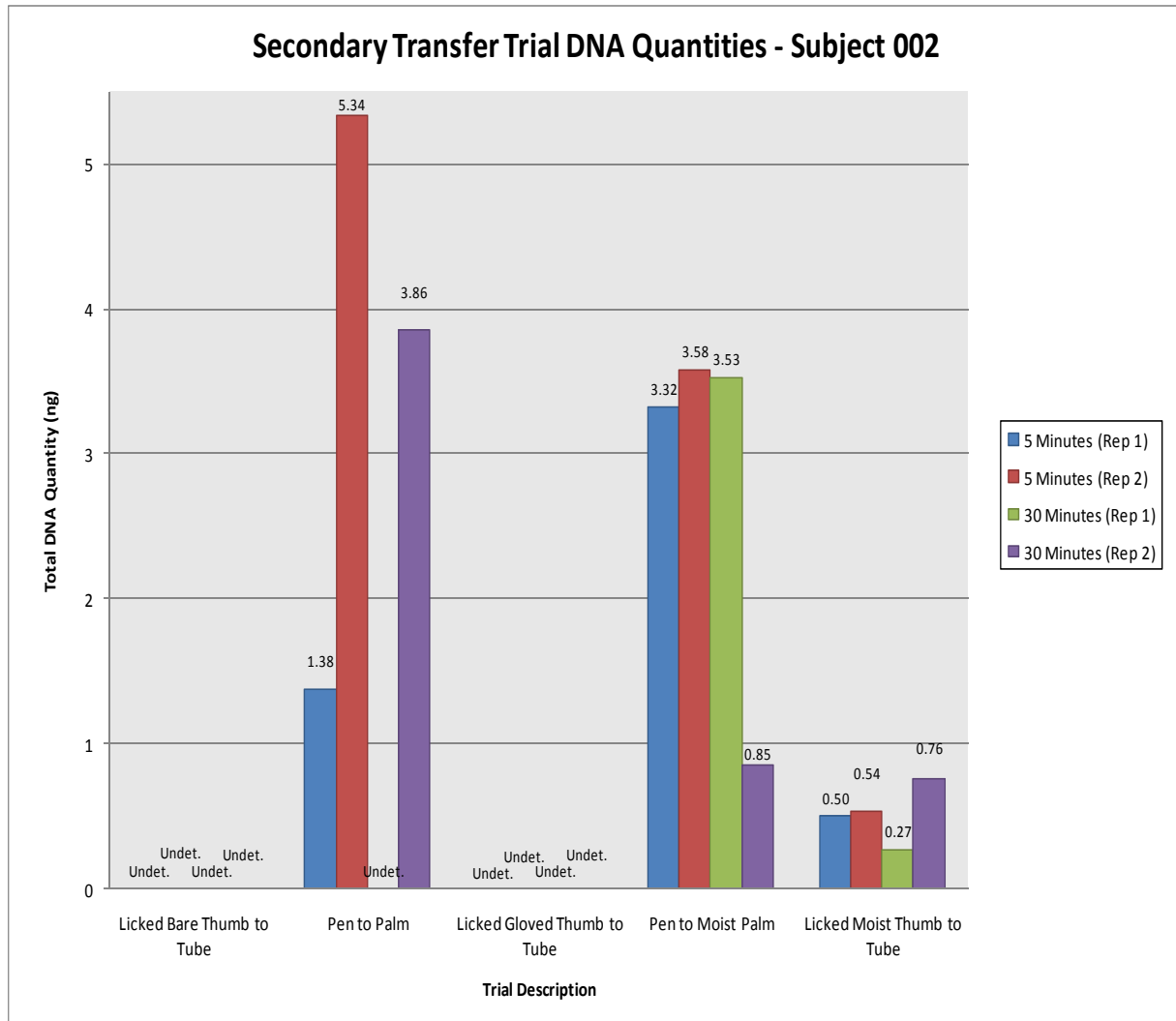


Figure 42. Secondary Transfer Trial DNA Quantities – Subject 002. The DNA present in the Licked Bare Thumb to Tube and Licked Glove Thumb to Tube samples was found at such low levels that the quantity estimates were undetermined. The Pen to Palm, Pen to Moist Palm, and Licked Moist Thumb to Tube trials all yielded low DNA quantity estimates.

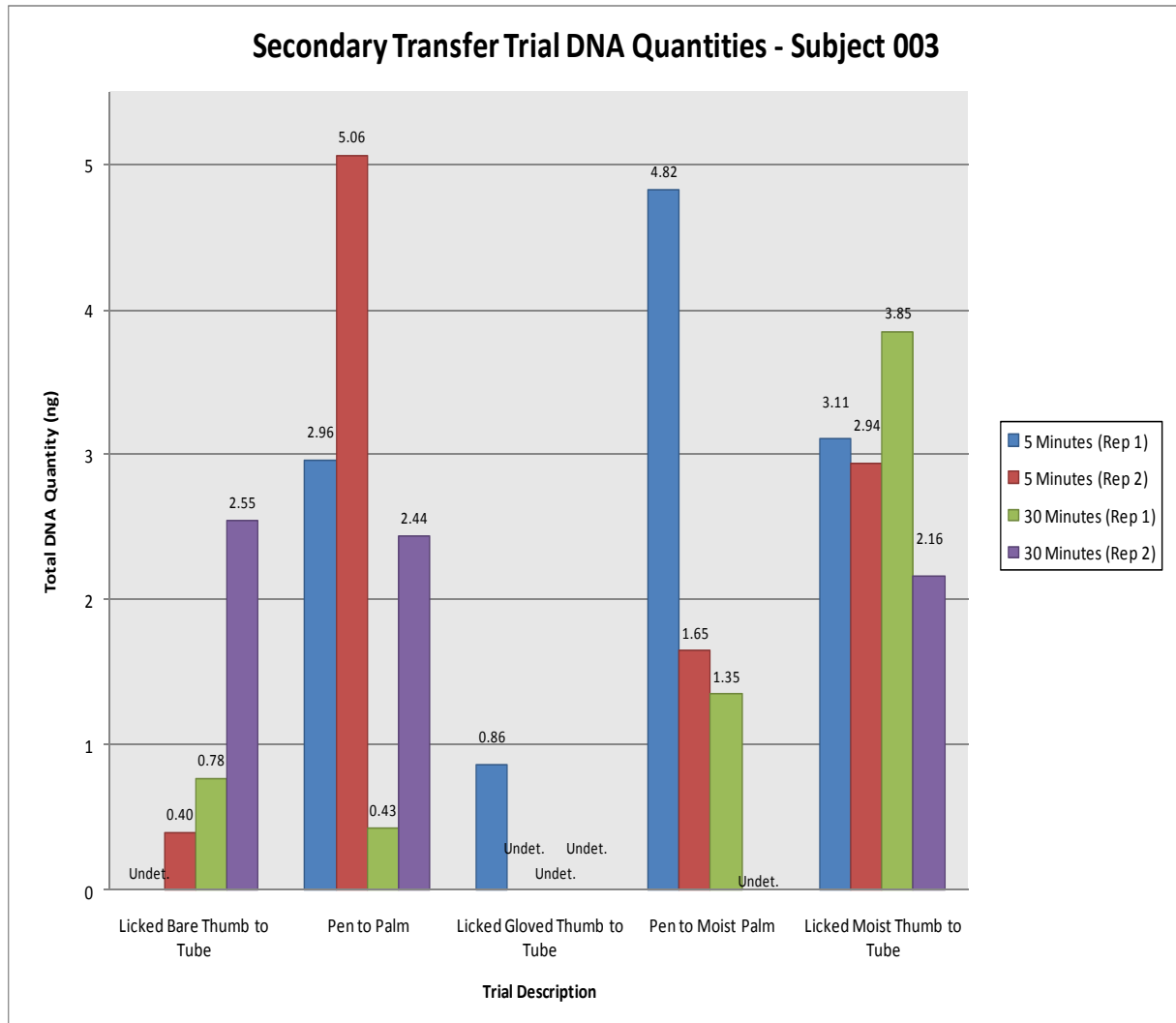


Figure 43. Secondary Transfer Trial DNA Quantities – Subject 003. The DNA present in the majority of the Licked Glove Thumb to Tube samples was found at such low levels that the quantity estimates were generally undetermined. The Licked Bare Thumb to Tube, Pen to Palm, Pen to Moist Palm, and Licked Moist Thumb to Tube trials all yielded low DNA quantity estimates.

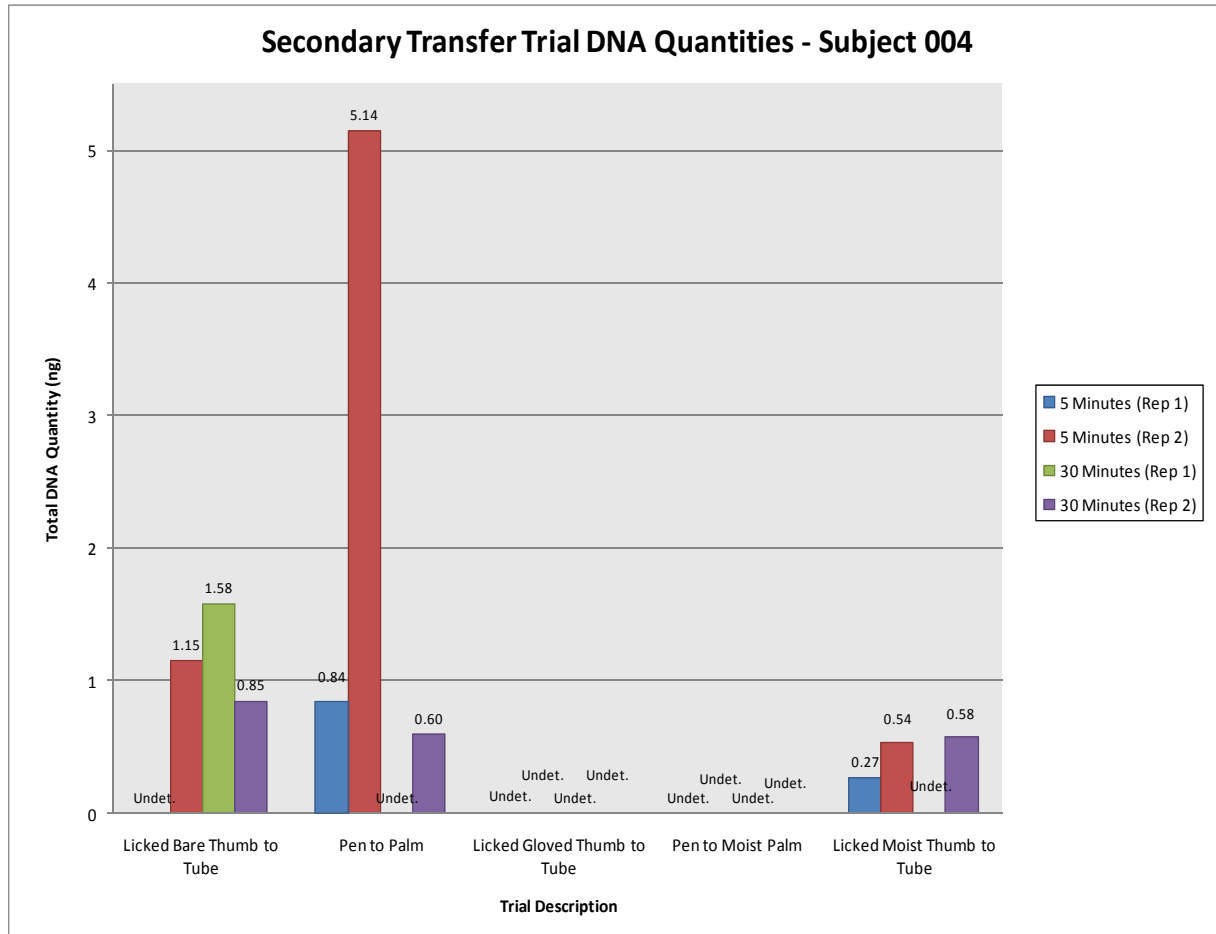


Figure 44. Secondary Transfer Trial DNA Quantities – Subject 004. The DNA present in the Licked Glove Thumb to Tube and Pen to Moist Palm samples was found at such low levels that the quantity estimates were undetermined. The Licked Bare Thumb to Tube, Pen to Palm, and Licked Moist Thumb to Tube trials all yielded low DNA quantity estimates.

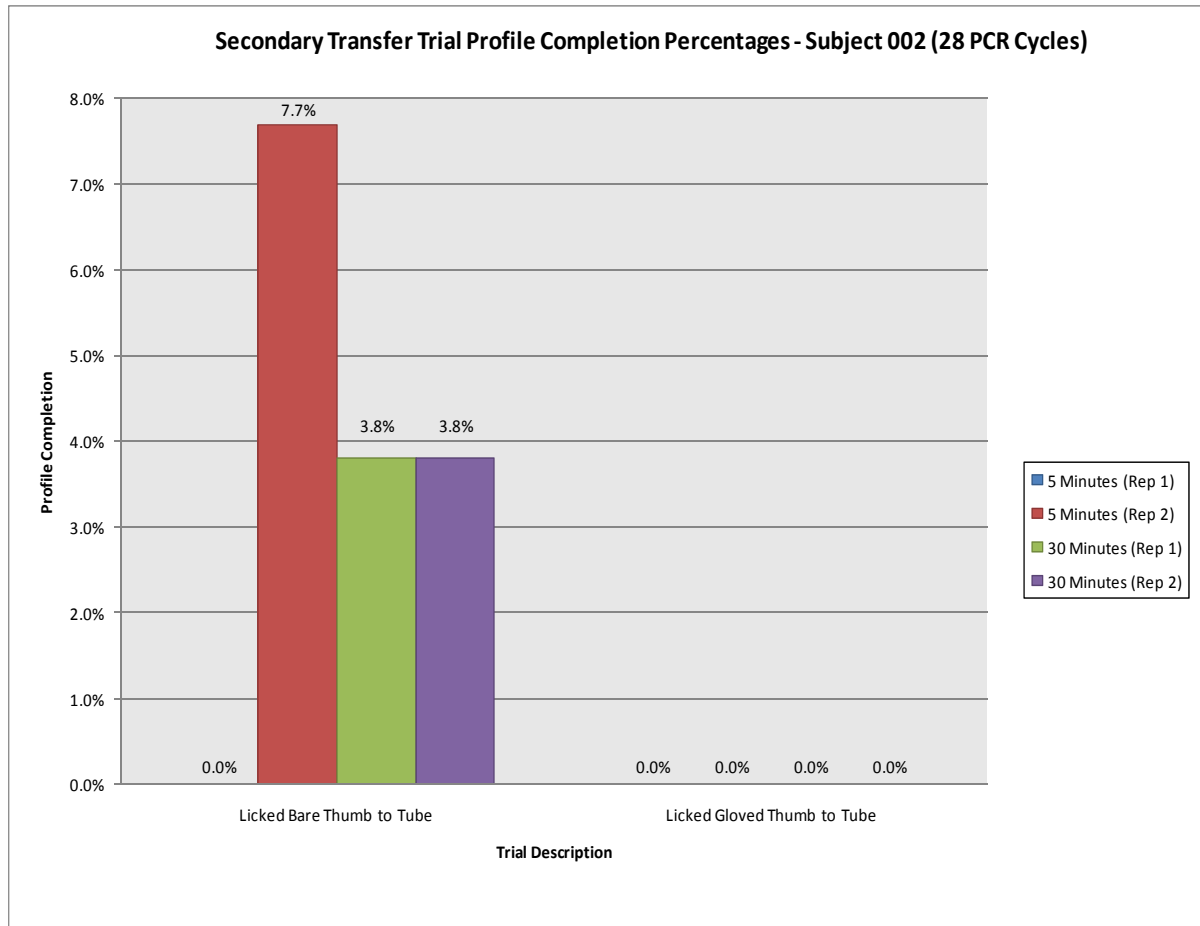


Figure 45. Secondary Transfer Trial Profile Completion Percentages – Subject 002 (28 PCR Cycles). At only 28 cycles of PCR, the Licked Gloved Thumb to Tube samples yielded no genetic profiles, while the Licked Bare Thumb to Tube samples yielded genetic profiles that showed no more than 7.7% of the expected alleles.

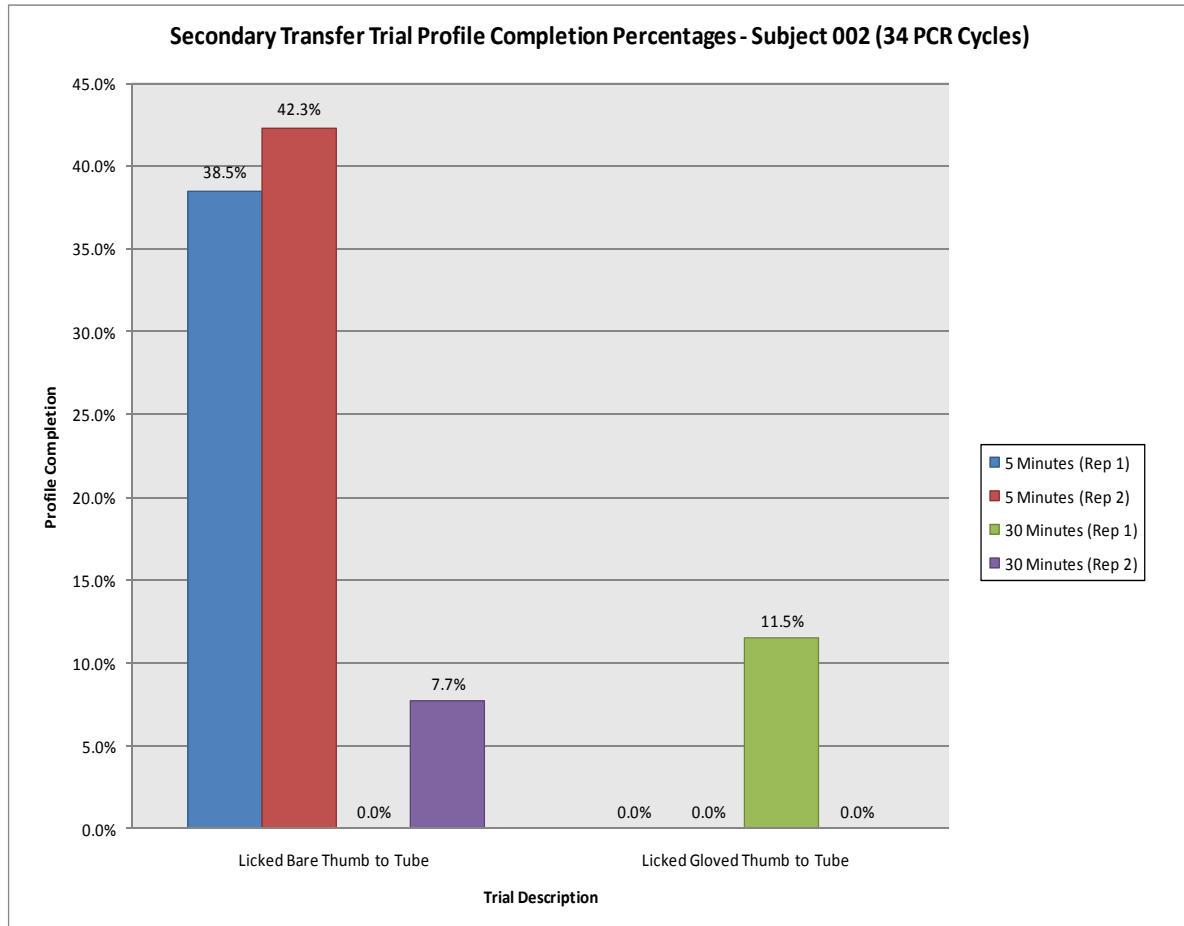


Figure 46. Secondary Transfer Trial Profile Completion Percentages – Subject 002 (34 PCR Cycles). At 34 PCR cycles, the secondary transfer trial samples yielded genetic profiles that showed a much higher proportion of expected alleles than those yielded after 28 PCR cycles.

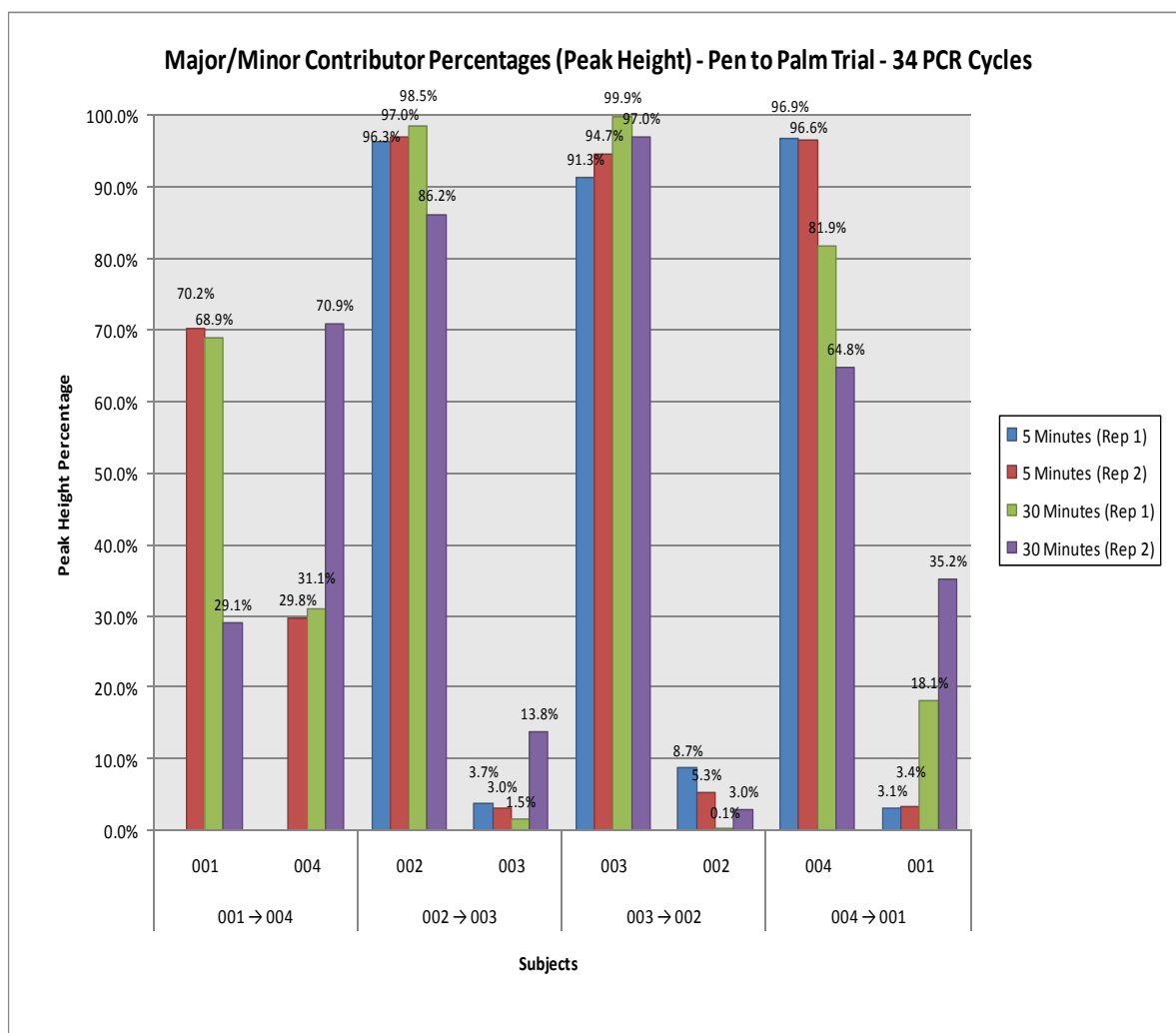


Figure 47. Major/Minor Contributor Percentages (Peak Height) – Pen to Palm Trial – 34 PCR Cycles. In all but the second 30-minute drying time replicate on the 001→004 trial, the original depositor was the primary DNA contributor. It was possible that lick-to-lick variation was responsible for the higher second subject contribution in the second 30-minute 001→004 replicate.

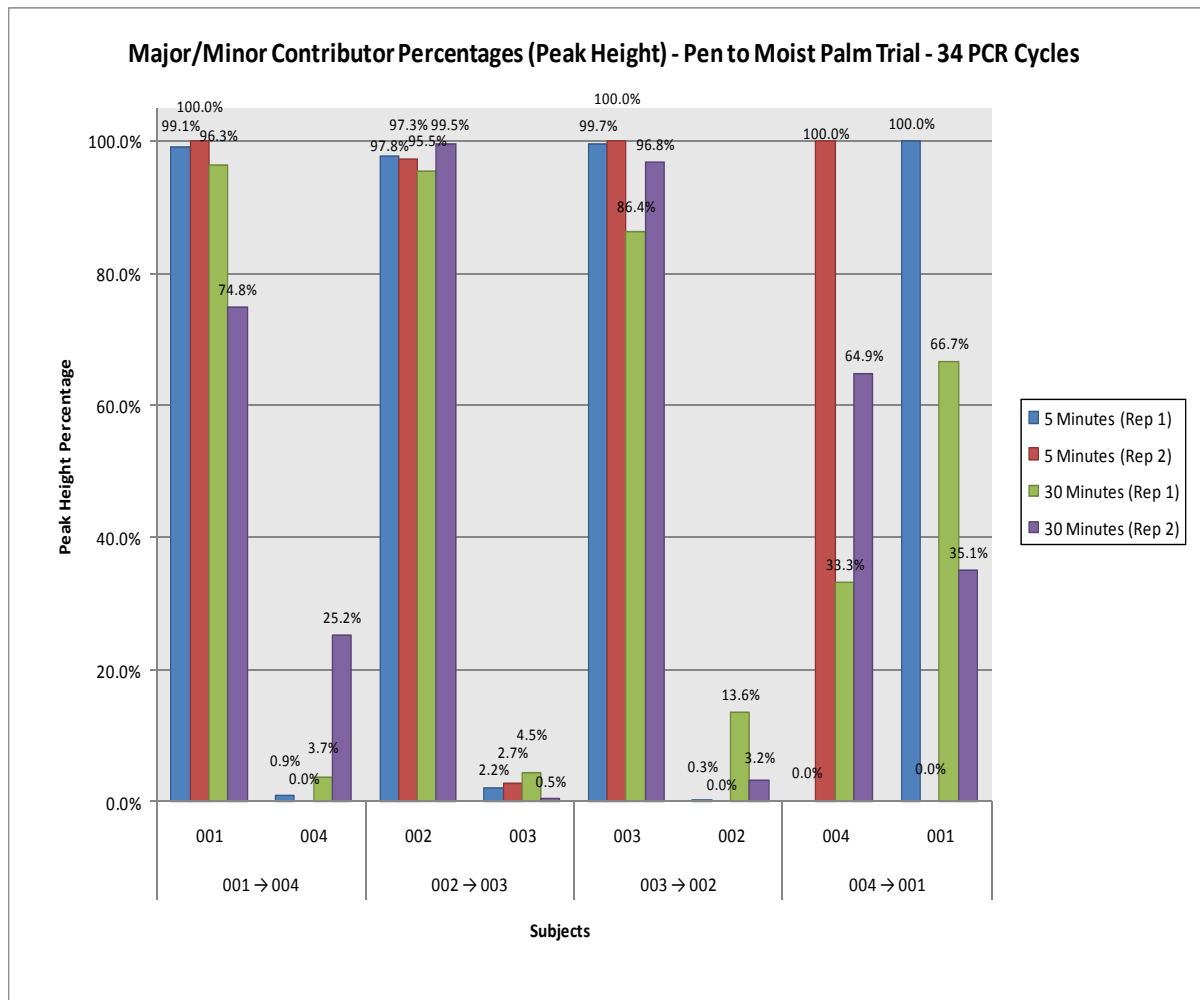


Figure 48. Major/Minor Contributor Percentages (Peak Height) – Pen to Moist Palm Trial – 34 PCR Cycles. In all but the first 5-minute and first 30-minute drying time replicates on the 004→001 trial, the original depositor was the primary DNA contributor. It was possible that lick-to-lick variation or improper grasping of the pens by the recipient subject was responsible for the higher second subject contribution in the first 5- and 30-minute 004→001 replicates.

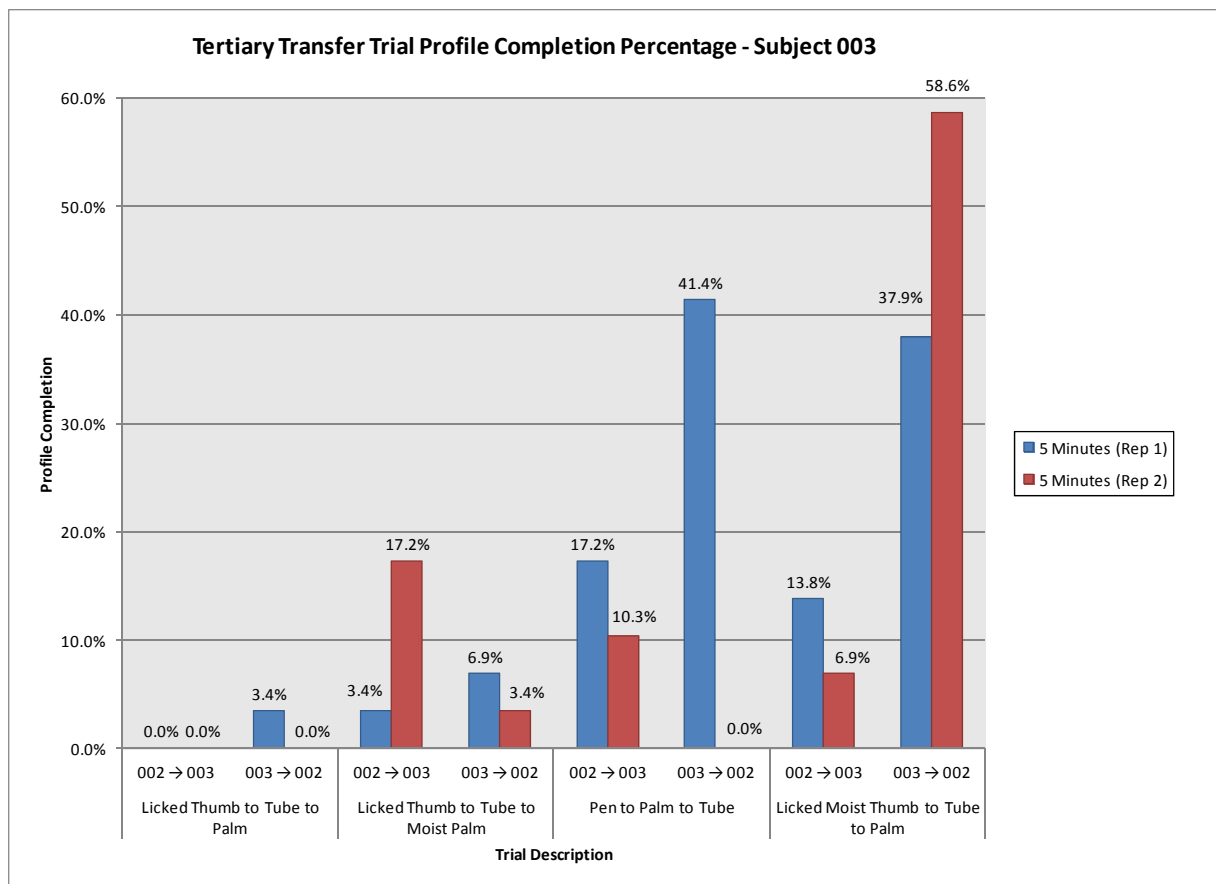


Figure 49. Tertiary Transfer Trial Profile Completion Percentage – Subject 003. Completion percentages for subject 003 were extremely low when this subject was the primary contributor in the Licked Thumb to Tube to Palm trial. When the recipient partner’s palm was moistened prior to gripping the tube, a larger percentage of subject 003’s profile was transferred. When subject 003’s thumb was moistened prior to gripping the tube, an even greater percentage of the profile was transferred.

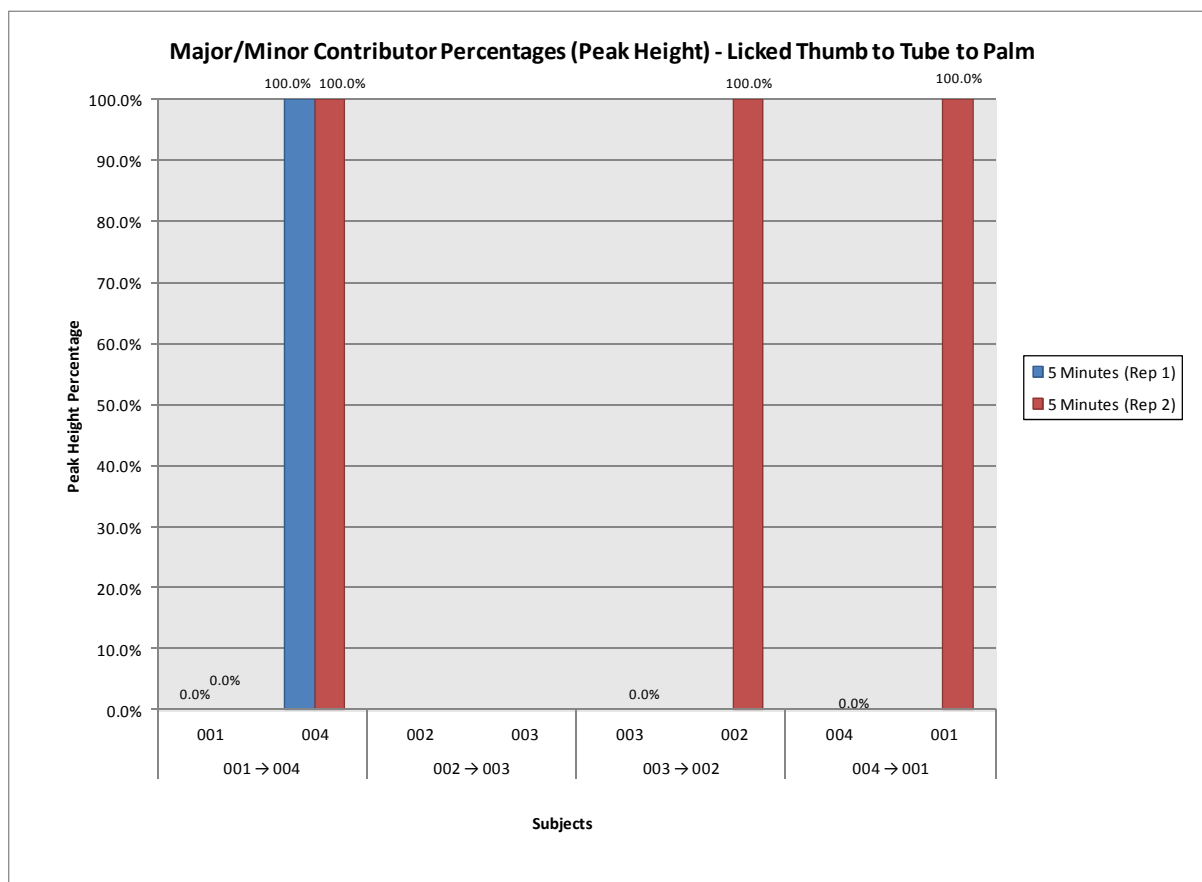


Figure 50. Major/Minor Contributor Percentages (Peak Height) – Licked Thumb to Tube to Palm. In all of the applicable replicates, the secondary contributor was shown to be the source of the majority of DNA in the resulting mixture.

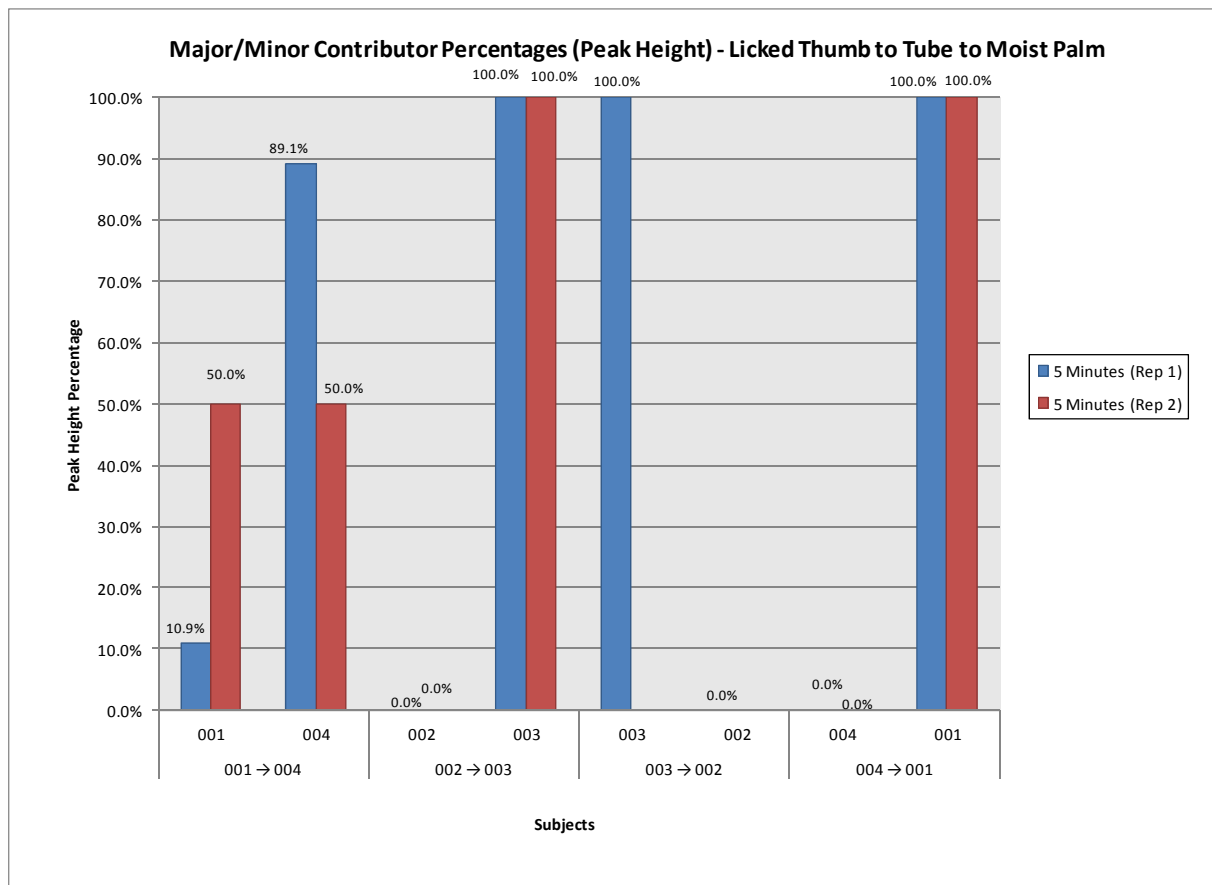


Figure 51. Major/Minor Contributor Percentages (Peak Height) – Licked Thumb to Tube to Moist Palm. In all but two of the applicable replicates, the secondary contributor was shown to be the source of the majority of DNA in the resulting mixture.

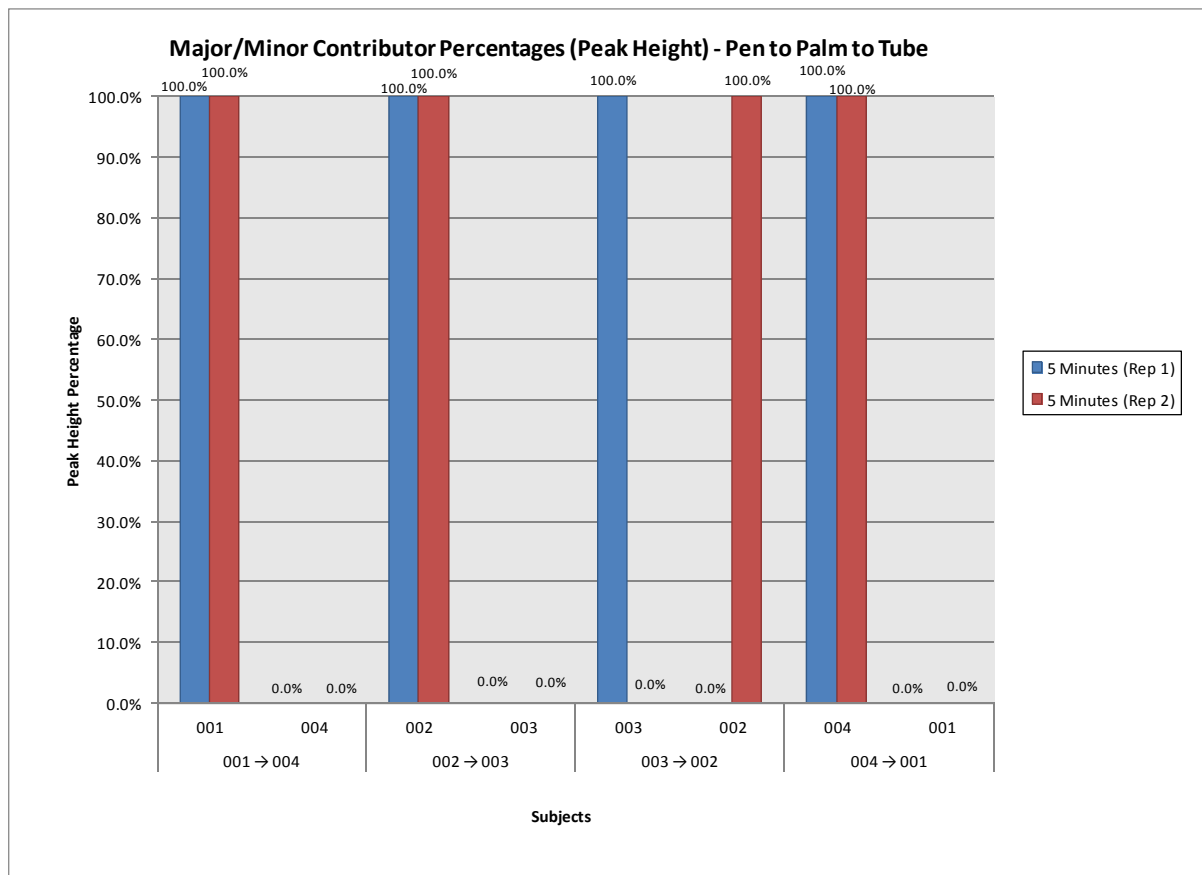


Figure 52. Major/Minor Contributor Percentages (Peak Height) – Pen to Palm to Tube. In all but one of the applicable replicates, the primary contributor was shown to be the source of the majority of DNA in the resulting mixture.

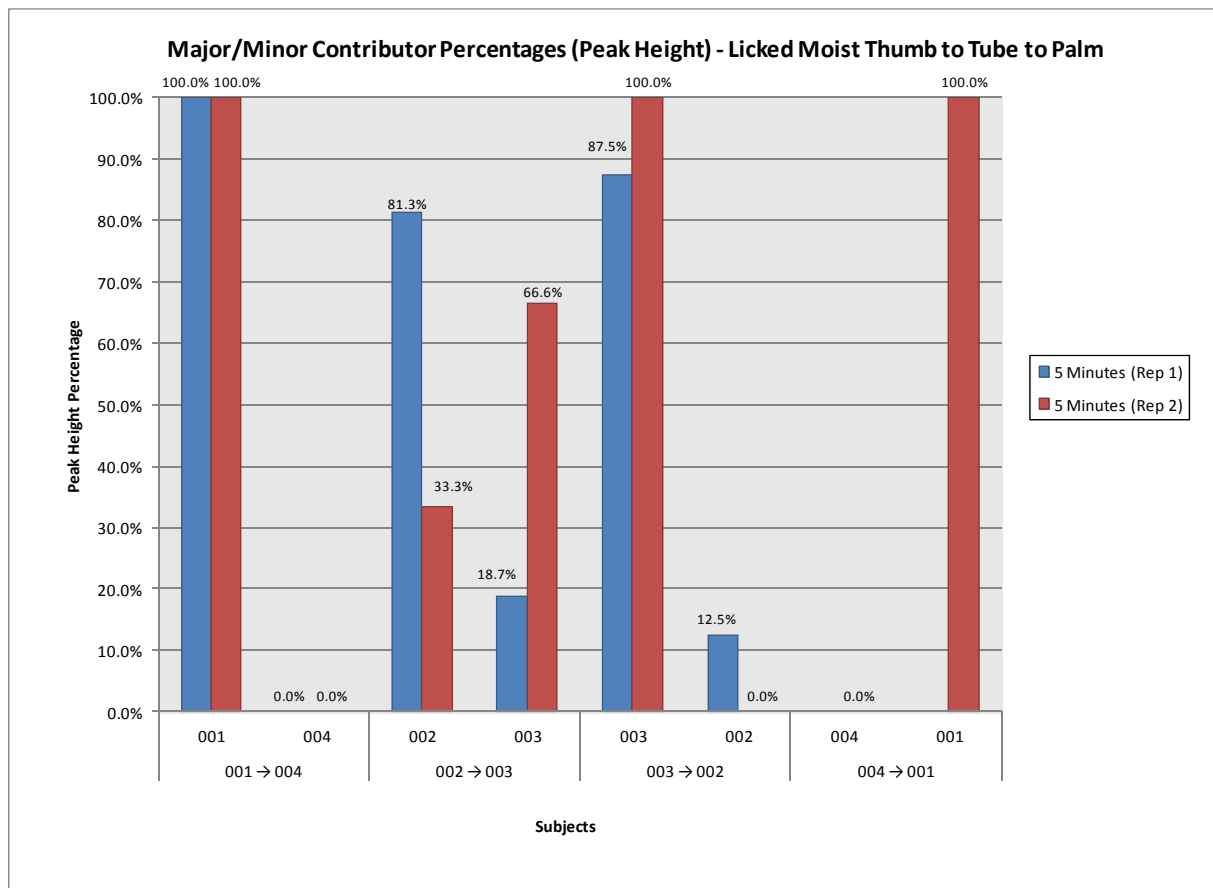


Figure 53. Major/Minor Contributor Percentages (Peak Height) – Licked Moist Thumb to Tube to Palm. In all but two of the applicable replicates, the primary contributor was shown to be the source of the majority of DNA in the resulting mixture.

Table 4. Genotypes of three LCN typing scenarios, each of which has three aliquots.

	Aliquot 1	Aliquot 2	Aliquot 3
(A)	13,15	13,15	13,15
(B)	13,15	15	13
(C)	13,15	15,17	13,19

Figure 54. A diagrammatic representation of the PCR amplification process for STR loci. $A_i(j)$ stands for the number of copies/templates of the allele i at j -th cycle. $A_0(0)$ is the starting copy number of the true allele, $A_2(+1)$ is the copy number of the new allele with one step more than the starting allele at the second cycle and $A_2(-1)$ is the copy number of the new allele with one step less than the starting allele at the second cycle. e_{pcr} is the PCR efficiency, which varies with the cycles. e_{-1} , e_0 , e_{+1} are the proportions of replications with -1, 0, and 1 step slippage mutations, respectively, which can be constant across the cycles. $e_{-1} + e_0 + e_{+1} = e_{pcr}$.

