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Author(s): Kelsey D. Bettex, University of North Texas Health Science Center

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Both unfired and fired ammunition recovered from a crime scene are useful in connecting a crime to a specific firearm, however its use in identifying individuals is less common due to low-level or degraded DNA present. Traditionally, the standard procedure for DNA sampling of ammunition evidence is the use of a swabbing technique. The New York State Police (NYSP) Forensic Investigation Center conducted a study to test the efficacy of an alternative method for obtaining DNA from cartridges and casings, soaking the sample prior to extraction. This method presented a 186% increase in total DNA yield, 169% increase in male DNA yield, and a 44% increase in comparable profiles obtained using GlobalFiler™ and 240% using Yfiler® Plus. Results showed fewer 'no data' determinations using the soaking method, indicating more DNA obtained. Although a statistically significant difference was not observed between the two methods ($p = 0.070$), a qualitative significance was.

KEYWORDS: Trace DNA · Ammunition · DNA Extraction · DNA Inhibition · Soaking Method · Swabbing Method · Cartridges · Casings

ALTERNATIVE PROCEDURE FOR DNA RECOVERY

FROM CARTRIDGES AND CASINGS

Kelsey D. Bettex, B.S.

APPROVED:

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Major Professor

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Committee Member

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Committee Member

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Committee Member

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Chair, Department of Department of Microbiology, Immunology and Genetics

_____

Dean, Graduate School of Biomedical Sciences

ALTERNATIVE PROCEDURE FOR DNA RECOVERY FROM CARTRIDGES AND CASINGS

INTERNSHIP PRACTICUM

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Kelsey D. Bettex, B.S.
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INTRODUCTION

Crimes involving the use of firearms are prevalent, however the associated crime scene evidence is often limited to casings and/or cartridges and not the firearm itself. When examining firearms and ammunition, the markings left on bullets and casings after the firing process are analyzed for identifying characteristics, which are then compared back to those left by known firearms. Although both unfired and fired ammunition recovered from a crime scene are useful in connecting a crime to a specific firearm, its use in identifying individuals is less common due to low-level and/or degraded DNA present. Previous studies have suggested that the copper ions in some cartridges or the high temperatures produced during the firing process may degrade DNA, however recent data suggests this may not be the case. Rather, the DNA present on ammunition could be removed in the firearm chamber as a result of friction, leaving less DNA to be collected from a casing. The recent increase in sensitivity in DNA profiling chemistries coupled with improved collection methods for low-level DNA has made it possible for crime laboratories to obtain DNA from evidence items that may not have been considered for testing previously. Within the categories of murder, robbery, and aggravated assault from the FBI's published data on offenses known to law enforcement, New York state reported 7,761 cases involving the use of a firearm in 2018 alone (FBI.gov, Uniform Crime Reporting). In an effort to increase the recovery of DNA profiles suitable for comparison from unfired and fired cartridges and casings, the laboratory will evaluate a proposed method of soaking ammunition in lysis buffer prior to DNA extraction. To date, several studies [12, 13, 15, 18, 19, 22, 23, 24, 26, 30, 38, 42, 50] have explored methods of obtaining DNA from ammunition, however many report different methods often with variable results.

Trace DNA

Within the forensic field there is a common goal of identifying a sample of evidence and individualizing it, that is, determining whether an association between the evidence and a potential source of the evidence is supported. With the introduction of DNA analysis came the ability to link a biological sample back to the individual who deposited it. However, in its earlier years, DNA analysis was very limited, time consuming, and required a large amount of sample. With the switch from analysis of restriction fragment length polymorphism (RFLP) to short tandem repeat (STR) technology came an increased ability of obtaining DNA profiles from low-level and degraded samples due to the shorter fragment size of STRs and the amplification of the DNA present to measurable levels using polymerase chain reaction (PCR). Over the last 30-35 years, since the introduction of STR analysis, there has been a drive within the forensic community for a higher resolution in DNA identification techniques. Increasingly advanced and sensitive technologies have shifted the amount of biological material needed for DNA analysis. In the 1980's, analysis required biological stains the size of a quarter, by the 1990's it required smaller but still visible stains, to today where samples containing as few as 20 unseen cells may be adequate. Throughout this growth period the importance of DNA evidence tp investigations, and the desire to obtain DNA from touched surfaces, has greatly increased.

Perhaps the most widely known concept within forensic science, Locard's exchange principle expresses that "every contact leaves a trace". That is, the perpetrator of a crime will leave, as well as remove, trace evidence at a crime scene, both of which bear forensically relevant information. Previously, this principle was applied to the use of fingerprints, firearms evidence, and obtaining DNA from visible biological material. However, over time it was discovered that the sweat and oil exchanged during the handling of an object contains DNA bearing cells that are capable of

being used for genetic profiling [4,47]. Trace DNA, often referred to as touch DNA or low-level DNA, is a DNA sample that is typically obtained at a concentration of 100 pg or less from surfaces that have come in contact with an individual [21,26,41,40,47]. Genetic material is discarded from the skin and deposited on a touched surface, for example when a bullet cartridge is inserted into a firearm [21]. Skin cells, which are the most commonly inferred source of touch DNA, contain about 5 picograms of nuclear DNA [47], so as few as 15-20 somatic cells are necessary to obtain a genetic profile [41,47]. In crimes such as burglaries, robberies, and drug crimes where typical biological evidence stains (i.e. blood and semen) are not encountered, touch DNA may be useful in identifying the perpetrator.

Touch DNA technology is the approach to collect and produce viable DNA profiles from areas that have been touched and potentially contain small quantities of DNA bearing cells. However, analysts have identified several issues in interpretation due to DNA transfer and contamination associated with low level samples [26]. There have been several developments that have increased the sensitivity of DNA typing assays and has allowed for the genotyping of very small quantities of DNA. The major contributor to this is arguably the use of polymerase chain reaction (PCR) amplification, which is the enzymatic cycling process used to exponentially generate thousands to millions of copies of particular DNA sites from concentrations below analytical levels [4]. This process involves the heating and cooling, or thermal cycling, of a sample where the DNA is denatured, annealed, and extended. These cycles are repeated between 20-40 times, doubling the DNA present with each cycle. The introduction of multiplex PCR has allowed for the amplification of genetic information from multiple target sites in a single reaction using multiple primer pairs [45]. This technique reduces the sample consumption as well as saves the laboratory considerable amounts of time and effort. Lastly, the use of STRs, having small amplicon sizes typically ranging

from 100 to 400 base pairs (bps) in length, greatly improved the chances of obtaining genetic data from poor quality, such as low-level or degraded, samples [4,45].

In his review of trace DNA [47], Wickenheiser discussed several instances when touch DNA may be obtained as well as several cases where touch DNA aided in finding the perpetrator of a crime. Through his case analyses he drew several key conclusions on trace DNA. First, the last individual to come in contact with an object may be the sole or major contributor to the DNA profile obtained from it. This anomaly is largely due to the fact that the cells containing DNA adhere loosely to those substrates in which they are deposited. This then allows for the next individual who contacts that object to replace the cells present with their own by removing the original cells. Next, Wickenheiser demonstrated the importance of dividing single pieces of evidence into separate sampling areas. In some cases, there may be two key contributors of DNA, for example with a knife involved in a crime, a victim's DNA profile may be found on the blade while the perpetrators DNA may be on the handle. It is important to sample these key areas separately in order to maximize the evidentiary value of the profiles.

Wickenheiser also notes that the amount of DNA transferred to a substrate is dependent on several factors. First, the individual handling the item may be "good shedders" or "bad shedders", meaning they release large or small quantities of epithelial cells. Second, the substrate surface which the epithelial cells are being deposited on affects how well they stick. A non-porous surface, which is typically a good substrate for fingerprinting, may not be conducive to trace DNA analysis, while porous surfaces may allow for better DNA recovery. This then raises the importance of evaluating the item to be analyzed and determine which testing would be more useful (i.e. DNA sampling versus fingerprinting). Although most fingerprint development techniques do not affect subsequent DNA analysis, there is a risk of the quantities being reduced. If it is determined that

both testing methods are necessary, it is then important to determine the best sequence in which to perform them and which methods to utilize. It has been suggested that a good way to obtain an unaltered fingerprint while also maintaining any DNA present would be to utilize the fingerprint fuming process to apply thin layers of acrylic to recover a fingerprint while sealing DNA bearing cells in place for later recovery [46].

Lowe et al [27] originally defined good and bad shedders, as mentioned by Wickenheiser [47], as those who left a full profile after 15 minutes of contact with an object or those who left partial or no profiles, respectively. More recent studies [29,41] attempting to reproduce the results obtained in these initial classifications found that no person can be defined as a good or bad shedder. It was noted that "shedder status" is dependent on several key factors such as time since handwashing and hand dominance. Phipps et al. [29] report that the amount of DNA on skin available for transfer as touch DNA increases with time since hand washing, a finding later confirmed by Templeton et al [41]. Phipps et al. also found that in some cases an individual could be a good shedder one day and a bad shedder the next, amounting to as much DNA variation within themselves as between other individuals. Although there may be significant differences in the amounts of DNA deposited amongst individuals, it was concluded that the distinction between a good shedder and a bad one is too narrow to distinctly classify an individual as one or the other. Further, as interesting as knowing an individual's ability to shed may be, in terms of forensic investigations, a perpetrator's shedder status is unknown and therefore not applicable to the case.

As mentioned, touch DNA samples are known to contain very low quantities of DNA which has unfortunately been proven to be greatly reduced during collection and analysis [40]. Tang et al. found that anywhere from 56-77% of touch DNA is lost during collection and analysis of the sample. The results of their study showed that a majority of DNA in touch samples is lost

to the surface itself and to the collection swab, accounting for more than half of the total DNA loss. It was found that about 16% of deposited DNA is left on the surface, about 24% is retained or trapped in the swab and on average a total of 34% was lost during extraction [40]. With this extreme loss of sample, a touch sample comprising of less than 100pg of DNA may not contain enough genetic material to obtain a useable profile post amplification [41]. Methods have been proposed to resolve this loss, perhaps the most promising being direct PCR.

Direct PCR is an amplification method which bypasses extraction and quantification, therefore eliminating any loss of sample during these steps [8]. During direct PCR, DNA extraction, purification, and quantification are not preformed, and the sample is directly amplified. The goal of utilizing direct PCR methods is ultimately to maximize the amount of DNA that is collected and available for PCR amplification, however extraction methods often remove many inhibitors from a sample and bypassing this step could result in later complications [21]. Although direct PCR allows for the maximum quantity of DNA to be obtained, minimizes potential for error, and reduces processing costs, the results may lead to complexities that result in increased interpretation times [8]. Further, in skipping over the quantification step the quantity of DNA cannot be recorded, eliminating the possibility of uploading the obtained evidence profile to the Combined DNA Index System (CODIS). Additionally, the availability of retained sample is removed, therefore retesting if the sample fails or if requested by other agencies is not possible [8]. Consequently, many crime laboratories cannot incorporate direct PCR into casework analysis as not only is it a requirement per the National Quality Assurance Standards to record the quantity of DNA present, but it is also best practice that some of the sample be retained for future testing (personal communications with NYSP personnel, NRC II recommendation).

As the value of DNA evidence in forensic investigations increases, the desire to obtain genetic information from increasingly lower levels of DNA is rising. Although the sensitivity of profiling chemistries is increasing, and profiles are being obtained from less DNA, it is important to remember that the time since deposition of touch DNA is not yet something that can be determined. Therefore, caution must be taken when analyzing low-level samples when determining their relevance to the crime and crime scene. It is entirely possible for a profile to have been left prior to or after the crime event, making it of no relevance to the crime. The presence of a DNA profile does not signify involvement in a crime; moreover, the absence of a profile cannot exclude a suspect [27]. The interpretation of low-level DNA results is crucial to the outcome of a criminal investigation and care should be taken with final decisions.

Stochastic Effects and Interpretation Difficulties Associated with Trace DNA

With the enhanced sensitivity in DNA typing chemistries, along with the increase in lower quality and quantity (often mixed) trace DNA samples being submitted for analysis, the difficulty in analyzing and interpreting DNA profiles has risen [10]. With lower amounts of DNA and increased detection sensitivities, the likelihood of issues like stochastic effects during PCR amplification, contamination, and allele drop-in are much greater.

Stochastic, or random, effects are a random variation in the results of replicate analyses. This means that if a sample of DNA were to be amplified twice, the resulting alleles of each amplification could differ [6]. With low levels of DNA, the primers used in PCR may not always anneal to all of the DNA present. This unequal sampling at a heterozygous locus, and subsequent preferential amplification of one allele over the other, can result in the failure to detect both alleles. At a heterozygous locus, loss of one allele is termed allele drop-out, furthermore the loss

of both alleles is known as locus drop-out, both of which result in partial DNA profiles [6, 10, 45, 46]. When a sample is degraded or inhibited, having progressively falling peak heights with the increase in amplicon size, there is a greater risk for allele and/or locus drop-out. During the PCR amplification process, the polymerase can lose its place when copying a strand of DNA, slipping forward or backward one repeat unit. This phenomenon results in small peaks, known as stutter peaks, that occur immediately before or after a true allele peak. Dye blobs, which appear as wide rounded peaks on an electropherogram, are the result of excess dyes clumping together. Noise, present in all samples, is the small peaks observed along the baseline. When the analysis software fails to discriminate between two dye colors, peaks observed in one channel can be observed in another. These peaks, known as pull-up or bleed-through peaks, are typically very narrow and can appear very tall compared to the baseline. Another common artifact is known as spikes, which are peaks caused by voltage spikes during analysis that appear very thin and pointed in shape on an electropherogram. Lastly, another very common effect of low-level DNA on analysis is peak height imbalance, or two alleles present at a locus not contributing in equal amounts. When this imbalance is less than 30%, it may indicate the presence of more than one donor, given that the resolution between the peaks is high, potentially denoting they are not a true heterozygous pair. The inherent imbalance of low-level samples due to potential stochastic effects or preferential amplification can lead to imbalance making it difficult to discern between a true mixture and a single source profile. Typically, heterozygote alleles, coming from a single source, are of relatively equal heights, therefore a peak height imbalance indicates the possibility of a mixed profile [4, 10, 45].

Along with the effects of sampling methods on the results of low-level DNA samples, there can also be outside factors that affect the interpretation. Contaminated DNA evidence is a

case sample that contains DNA from an outside source, known as exogenous DNA. Contamination can occur prior to the crime itself, having background DNA where evidentiary profiles are deposited, or at any point after the crime (i.e. during sample collection and packaging or processing in the laboratory) [16]. Although often misrepresented as being synonymous with contamination, allele drop-in is a separate concern. Allele drop-in is an allele that appears on an electropherogram but is not associated with the sample being analyzed [4, 16]. The drop-in allele is not reproducible upon re-amplification, is not attributable to the contributors proposed within the casefile and can be explained by a random event. For example, if during amplification something were to fall into the tube, reamplifying would not reproduce this allele [personal conversation with NYSP personnel]. The main difference between contamination and drop-in is that contamination is reproducible while drop in is not. Contamination can be a single allele in a sample you expect to be single source, such as human remains. If you re-amplify this sample, the same extraneous allele may still be there, therefore the sample itself was contaminated by exogeneous DNA. A drop-in allele will not be there upon re-amplification but would be there if you re-prepped the same amplicon [16, personal conversation with NYSP personnel].

The use of thresholds in DNA analysis allow for an analyst to assess the quality of the data. Being that the peak height of alleles on an electropherogram are representative of the signal intensity (measured in relative fluorescent units, RFUs), a relative confidence can be placed in peaks that meet or exceed an established threshold. Forensic DNA analysts utilize two different thresholds, the analytical and stochastic. An analytical threshold is used to separate signal from noise and is set at the lowest RFU value at which DNA is distinguishable from background. Typically, most labs will establish different analytical thresholds for each dye channel because the noise levels vary, however with increased kit sensitivities and cleaner baselines being

observed a 50 RFU threshold is commonly utilized across all dye channels [36, 45]. Analytical thresholds are established through validation studies where the background noise associated with the chemistry, the specific dye, and the instrument are measured. A signal above the analytical threshold can either be a true allele peak or an artifact, therefore it is important to establish a stochastic threshold to then determine if a locus has been impacted by stochastic effects. Stochastic thresholds are determined based on experimental data obtained within the laboratory, the quantitation and amplification kits, and the detection instrument being used. The Scientific Working Group on DNA Analysis Methods (SWGDAM) suggests establishing a stochastic threshold by "evaluating allelic dropout and peak height ratios across multiple loci in a dilution series of DNA amplified in replicate" [36]. When a locus contains a single peak, it is important to determine if the genotype is that of a true homozygote or if there is a possibility that the heterozygote sister allele has been lost due to dropout. A stochastic threshold is set at the minimum RFU value, typically between 150 and 200 RFU, that a peak must reach to be deemed a true homozygote and not impacted by stochastic effects [2, 36, 45]. Those single peaks that are above the analytical threshold but are below the stochastic threshold cannot be used for comparison as there may be dropout of a sister allele. When analyzing trace DNA, the possibility for stochastic effects to impact the profile are much greater and therefore it is crucial to have proper thresholds so as not to obtain incorrect genotypes [36, 45].

Low Copy Number (LCN) Typing

Another promising approach to analysis of low-level DNA is low copy number (LCN) typing, which is the analysis of any sample containing 200pg of template where the DNA typing results are below the established stochastic threshold [4]. As mentioned, processing small

amounts of DNA increases the chances of stochastic sampling effects, e.g. allele peak imbalance, allelic drop-out or drop-in, and an increase in stutter, making analysis of these samples very challenging. LCN typing methods may include increasing the PCR cycle number, nested PCR, reducing the volume of PCR, utilizing whole genome sequencing prior to PCR, enhancing fluorescent dye signals, post-PCR clean up, and increasing injection times [4].

Although LCN typing helps improve results obtained from low-level DNA samples, it has mainly proven useful for identification of missing persons and human remains as use with evidentiary samples has not been well studied [4]. There are several concerns with the methodology that may make its use unideal for casework. Increasing the PCR cycle number may be useful in increasing DNA yield, but with that comes the risk of increasing any contaminating DNA present [45]. Nested PCR uses two primer sets and two successive PCR reactions in order to improve sensitivity. One set of primers anneals to sequences upstream from the second set in the first PCR and the resulting amplicons are then used as a template in the second PCR. Although useful in increasing sensitivity of the PCR reaction, nested PCR also increases the potential for carryover of contaminating DNA [7]. Reducing the volume of the PCR reaction is done to potentially increase efficiency, however van Oorschott [45] found that it may only be increasing the relative concentration of the template and not the accuracy of the reaction. LCN typing comes with a greater risk for potential error such as allele drop-in and drop-out, peak height imbalance, and large stutter peaks. Primers may not bind proportionately for each allele, leading to preferential amplification and peak imbalance. It was also found that LCN typing can result in peaks that fall far below the analytical threshold and potentially make them too low to even observe [4,45]. Budowle et al. note that caution should be taken in considering this method for evidentiary purposes as LCN typing is not a robust method, and therefore the profiles

generated are not reproducible. They report that the methods are so sensitive that there is increased risk for picking up low levels of DNA from consumables and background DNA during sampling, consequently increasing the potential for a profile obtained having no relevance. Other deterring aspects include the fact there have been no validations for use of LCN typing with mixture profiles and the profiles obtained are not useable for exculpatory purposes.

When working with low-level DNA there are many potentially useful methods for analysis that come with risks and downfalls. It is important for an analyst to consider these methods and be sure the best one for the case is selected. Methods such as LCN typing may show promise for the future of trace DNA analysis, however its use warrants further study and understanding before it can be used for evidentiary purposes.

Mixed DNA Samples

A DNA mixture is a biological sample that originates from two or more donors. When evaluating a casework DNA sample, the presence of a mixture is determined by the visualization of 3 or more allele peaks in at least one tested locus, or peak height ratios outside of the accepted heterozygote peak height ranges observed on an electropherogram. In the earlier years of STR typing most samples were single source and those samples that were mixtures were typically only made up of 2 contributors. With the increase in DNA typing chemistries and submission of low-level DNA samples, the presence of mixture samples observed as well as the number of contributors within them has also increased, leading to what is defined as complex mixtures [2]. The evaluation of casework samples involving low level or degraded DNA evidence poses greater interpretation challenges due to the increased likelihood of a complex DNA mixture and other stochastic effects such as an increased likelihood of dropout, allele sharing between

contributors that leads to allele stacking, and degradation [2, 5]. With the increased number of contributors and the decrease in the quantity and quality of DNA present with low amounts of DNA, the chance of imbalance in allele sampling, preferential amplification, and the loss of some allele targets is greater [5].

Aside from stochastic effects impacting the interpretation of low-level mixtures, there are other challenges such as human error and laboratory differences [10]. There are several decisions that are made by a DNA analyst when interpreting mixture profiles. Perhaps the greatest conclusion for the analyst to make is the number of contributors present in the profile. Using peak heights an analyst determines whether major and minor contributors are distinguishable. If an analyst cannot confidently assign a number of contributors, the analysis of that profile will cease, and therefore an opinion on the inclusion or exclusion of an individual may not be possible. Determining the actual number of contributors, not just a minimum number, is crucial for the interpretation, as the number of contributors directly correlates with the amount of dropout that may have occurred [2]. In their evaluation of DNA mixture interpretation, Bieber et al. propose an example of a mixture profile with exactly four alleles at every locus where two assumptions can be made, leading to very different interpretations. Under the assumption that this profile is comprised of two contributors, there is no dropout occurring, however if the assumption is that of a much larger number of contributors, the probability of dropout is very high [2]. Interpretation becomes even more difficult when low level peaks fall in stutter positions and are discounted as artifact rather than a true allele. When allele sharing occurs among contributors, allele stacking can occur leading to affects in the peak height observed [5]. All of these factors affecting the subjective decisions made by an analyst as well laboratory differences

in analytical and stochastic thresholds, allele frequency databases used, and interpretation protocols, can lead to differences in reported match statistics.

Two of the most widely used statistics in mixture interpretation are the combined probability of inclusion (CPI) and the combined probability of exclusion (CPE). The CPI represents the proportion of a given population that can be included as a potential contributor to the given mixture profile, while the CPE represents the proportion that can be excluded. To calculate the CPI the analyst must assess the profile and determine loci suitable for comparison to a reference sample and compare the evidentiary profile to reference profiles to determine which loci are consistent, including or excluding potential contributors as necessary. Finally, the statistical calculation is done after all interpretation and comparison [2]. However, if the sample is low-level and allele dropout is presumed, this statistic cannot be utilized at those loci. Therefore, in the case of low-level and/or complex mixtures, the use of a likelihood ratio (LR) or random match probability (RMP) is suggested [2, 10]. Coble and Bright noted that the CPI does not determine the genotypes of individuals, and use of a LR is a better approach because it is the only method that can assess stutter and dropout probabilistically [10].It was also noted that while the RMP is the probability of a random unrelated untested individual within a population having a matching DNA profile, the LR is not a probability but rather a ratio of two probabilities given two mutually exclusive hypotheses and is therefore a stronger statistic.

The binary approach, which is the current standard in forensic analysis of mixture profiles, is the probability of an evidentiary profile given a specific proposed genotyped. All of the proposed genotype combinations of the mixture alleles present are considered equally likely and the probability can give one of two results, being the proposed genotype is included or excluded [10]. Although this method can give strong statistical analyses with full profiles, it is

unable to deal with complex low level or mixed DNA [10]. As the number of potential contributors increases, it becomes very difficult to distinguish between how many contributors there are (e.g. five-person mixture versus a six-person mixture) making statistics difficult to calculate [2, 10]. A promising alternative approach to the binary method, known as probabilistic genotyping, has proven much more effective in analysis of complex DNA samples. Probabilistic genotyping, as defined by the SWGDAM [35], "refers to the use of biological modeling, statistical theory, computer algorithms, and probability distributions to calculate likelihood ratios (LRs) and/or infer genotypes for the DNA typing results of forensic samples ("forensic DNA typing results")." Probabilistic genotyping is a useful tool to assist, not replace, the analyst in interpretation of DNA typing results, reducing subjective decisions made by the analyst and therefore improving consistency in interpretations [10, 35].

Probabilistic genotyping can be broken down into two methods, the semi-continuous method and the continuous method. The semi-continuous method focuses on the alleles observed and can incorporate the probability of drop-in and drop-out. This method does not utilize the peak heights or mixture ratios and does not model for artifacts such as stutter when determining the possible genotypes, all of which are parameters useful in determining number of contributors and drop-out [10, 35]. The continuous method utilizes allele and stutter peak heights as well as mixture ratios, taking the quantitative information and models the allelic and stutter peak heights to determine the probability of the peak heights given all possible genotypes for the contributors. Unlike the binary approach which gives either a probability of 0 or 1, the probabilistic genotyping continuous method provides a probability that can fall between 0 and 1. While the binary approach considers all genotype combinations equally likely, probabilistic genotyping provides statistical weight to each possible genotype. By utilizing the probability of drop-out and

drop-in, the probabilistic approach eliminates the need for using a stochastic threshold. When preforming the statistical calculations this approach utilizes more genotyping information which helps to generate stronger likelihood ratios, i.e. higher LR when evaluating an individual who is a true contributor and a lower LR when evaluating someone who is not [35]. Although probabilistic genotyping software does not fully remove interpretation by an analyst, such as determination of number of contributors, it does reduce the level of subjectivity and increases the strength of the statistical analyses.

DNA Transfer

DNA transfer is the exchange of DNA from one source to another and can be broken down into levels, being primary, secondary, and tertiary. Primary transfer is the exchange of DNA, from its original (primary) source, to an object or another individual (secondary source), leaving behind what is known as touch DNA. Secondary transfer follows primary with touch DNA being transferred from an intermediate source (secondary source) to an object or individual (tertiary source). Tertiary transfer then follows secondary where DNA is transferred from the tertiary source to another object or individual.

The reported likelihood of observing secondary and tertiary DNA transfer in casework varies with conflicting results from one study to the next. Wickenheiser [47] reports that secondary transfer (object/person to person) is possible, however it is likely the transferred DNA would be overwhelmed by the DNA of the vector/carrier individual. If the carrier were to transfer the DNA it is likely that a mixture would be observed comprised of the transfer DNA and the carrier's DNA, with the carrier's DNA being the major contributor. Phipps et al. [29] and Ladd et al [32] report similar results stating that secondary transfer is possible but unlikely. In

contrast, when testing shedder status and transfer DNA, Lowe et al. [27] paired known good shedders with bad shedders and found that secondary transfer was a likely possibility. In this study, a good shedder who had not washed their hands held hands with a poor shedder who had just washed their hands for 1 minute. The poor shedder then immediately held a plastic tube for 10 seconds which was then swabbed for DNA. The resulting DNA profiles showed no mixtures with complete transfer of the good shedders full profile. Adding in a 30-minute delay between hand holding and the poor shedder holding the plastic tube still resulted in the transfer of the good shedders full profile, however the results showed mixtures with the 90% of poor shedders alleles present. Going further, a 1-hour delay between hand holding and holding the tube was tested, showing resulting profiles having at most only two of the good shedder's alleles transferred within a mixture with the poor shedder. Goray et al. [17] found that secondary transfer is possible, however the likelihood of occurrence is affected by and depended on the substrates involved in the transfer and the type of biological sample being exchanged. They reported that with porous substrates and/or dry samples transfer was significantly reduced, but with non-porous substrates and/or wet samples transfer was more likely to occur.

Van Oorschot [44] conducted a similar study to Lowe, testing plastic tubes held for short periods by one individual followed by another for DNA profiles. The results of their study showed that a strong DNA profile from the final holder was obtained as well as partial to full profiles from the previous holders. They further tested the hands of the final individual to touch the tube and found the profile of the hand being swabbed as well as the profiles of previous holders of the tube.

Although several studies have focused on the mechanism of DNA transfer and its likelihood in forensic investigations, the results are conflicting and are an area for further

investigation. While some studies have found that DNA transfers independent of time handled, others report that length of contact affects the quantities of DNA transferred. Some report that transfer DNA would not affect forensic investigations, while others caution investigators that it is a very likely occurrence. With that, analysts and investigators who encounter situations of possible DNA transfer should consider all possibilities and factors associated with the evidence and weigh the relevancy of any profiles obtained as they pertain to the case at hand.

Automated Analysis Methods

Since the beginning of its use, DNA evidence and the processes used to analyze it have been extremely useful in solving crime and preventing further incidents. The ever-increasing sensitivity levels and growth in the field surrounding DNA analysis have led to a surge in evidence being submitted for DNA analysis, creating a massive nationwide backlog in evidence that has yet to be analyzed. In an attempt to diminish backlog and increase laboratory efficiency, automation of DNA testing was introduced into many crime laboratories. Much of a DNA analyst's work with an item of evidence is repetitive, which is where automated analysis techniques take effect. Automation can take these repetitive actions, such as mass pipetting or punching stains from a bloodstain card, from the analyst and transfer the responsibility to a robot [11].

When analysts receive an item of evidence, they must first screen it for the presence of biological material, which is not a process that is currently automated and can become very lengthy depending on the complexity of the evidence. Once a stain is identified, a genetic profile is developed to determine a potential source, i.e. begin the analysis process for DNA. First, DNA must be extracted, or removed, from the cells in the biological material and purified, or washed,

from the remaining cellular material. When extracting DNA there is a test tube dedicated to each evidence sample that is frequently opened and closed, so an analyst using manual extraction techniques may only be able to reasonably focus on about a few items of evidence at one time [11]. Manual extraction techniques require laborious amounts of pipetting and, this being the most time-consuming step of DNA analysis, it was the perfect target for automation. The robotic instruments used in automated analysis can employ a 96-well plate format, giving the ability for significantly more samples to be processed at a time and more rapidly, yielding higher quality samples as compared to manual techniques [11].

All laboratories differ in size and amount of cases handled; therefore, universal automated analysis robots would not work for everyone. Some laboratories may not benefit from the cost of a large-scale instrument, but smaller scale robots may help to increase their efficiency at a more reasonable cost. Small scale robots are considered semiautomated because they do require more analyst involvement. Prior to the use of these instruments, the analyst must cut their biological sample and add it to the proper tube, add reagents to every tube, and then incubate the tubes for lysis of the cells. The samples are then transferred to the robot for automated purification of the DNA, followed by the collection of the purified DNA by the analyst [11]. Although this system greatly reduces the responsibility that falls on the analyst, there is still time-consuming involvement and therefore a more automated technique may be more beneficial to larger laboratories. Large-scale robots are capable of handling much of the pipetting, diluting, and transfer steps that the small-scale robots cannot, however there is still a required incubation step preformed manually by the analyst [11].

Following extraction and purification, the next step in DNA analysis is quantification of the DNA obtained to determine the concentration, and if it is of human origin. Quantitation

results are crucial for an analyst to make decisions on how to proceed with the sample. Along with the automation of extraction procedures, quantitation procedures have also been automated, and with ease, due to the extracted samples already being in a 96-well plate [11]. Following quantification, samples are amplified to mass replicate the targeted regions of the DNA present. Amplification is another step that can be semi-automated, having a robot prepare a 96-well amplification plate by adding the necessary PCR reagents and quantity of the DNA extract to their respective wells. The analyst can then take the prepared plate and place it on a thermocycler followed by a capillary electrophoresis instrument for analysis of the samples [11].

DNA analysts are responsible for processing countless amounts of evidence each day, much of this processing being very laborious, repetitive and time-consuming. The introduction of automated analysis methods and robotic systems has allowed analysts to process more samples with an increased efficiency and quality and with a corresponding decrease in time, involvement of the analyst, and number of backlogged evidence items.

Metallic Coextraction and Inhibition on the Generation of a DNA profile

Obtaining appropriate amounts of useable DNA from metallic surfaces, specifically the copper and brass surfaces regularly found on ammunition, has proven to be a challenge in the forensic field. Several studies [1, 9, 12, 25] have reported recovery of DNA being reduced in the presence of copper, however the exact mechanism(s) by which the copper is inhibiting the results is still unclear. The most commonly proposed theory is based on oxidation-reduction reactions and the generation of reactive oxygen species (ROS) that may result in oxidative damage to cells and the DNA contained within them.

Previous thought was that DNA typing results were being negatively affected in the presence of copper ions (Cu (II)) due solely to DNA degradation and PCR amplification inhibition. However, recent studies [25, 48] suggest that copper ions may be affecting all processes associated with DNA typing, e.g. extraction, quantitation, amplification, and capillary electrophoresis. In their 2017 study, Moreno et al. [25] reported that copper ions have negative effects on DNA processing either by interacting and binding directly with the DNA or by affecting its electrophoretic mobility. The suggested change in electrophoretic mobility is likely due to the metal ions irreversibly binding to the DNA, making its separation by capillary electrophoresis difficult. Their results also showed interference with the fluorescent dyes used in quantitative analysis which had negative effects on the resulting values. Several other suggested causes of the inhibited results were that in the presence of copper ions there is a complete inhibition of Taq polymerase during the amplification of a sample, the copper ions are inducing nuclease activity, and/or the binding of the copper to the DNA is preventing the DNA molecule from progressing through the typing mechanisms.

Oxygen derived species, such as hydroxyl (OH) and superoxide (O_2), are produced during normal aerobic metabolism to damage and break down biological materials [1, 9]. In the presence of hydrogen peroxide (H_2O_2), Cu (II) ions bind with DNA and OH radicals are formed. The OH radical initiates the breakdown of DNA by removing a hydrogen atom from along the DNA backbone and due to its small size, it can spread rapidly and cleave molecules at any position [9]. Using ultraviolet spectrophotometry, Cervantes-Cervantes [9] found that a DNA peak was not visible in the presence of Cu (II) and H_2O_2, showing an increased absorption likely due to the formation of complexes. The absence of a peak indicates the breakage of phosphodiester bonds and other chemical changes that may be affecting the DNA base's ability to absorb UV light. The

techniques associated with collecting low levels of DNA may also result in the collection of background metallic ions and when the ions collected are copper, the production of reactive oxygen species (ROS) and free radicals increases, therefore increasing the risk of damage to DNA and essential proteins [9]. These ROS can alter the structure of the DNA double helix, cause strand breaks, change the temperatures at which DNA degrades, as well as result in spectrophotometric changes [9]. Along with affecting the DNA itself, ROS can irreversibly alter proteins causing them to lose their normal function. With an essential part of DNA typing being Taq polymerase, a protein used to amplify target DNA, ROS can result in a decrease in or complete inhibition of results.

Several suggestions have been made on how to prevent and/or reverse the metallic inhibition of DNA typing. Aruoma et al. [1] reported that base damage in DNA by Cu(II)/H_2O_2 can be inhibited by chelating agents such as ethylenediaminetetraacetic acid (EDTA), nitrilotriacetic acid, and catalase. Moreno et al. [25] found that by utilizing purification steps during analysis, the negative effects of Cu (II) ions could be reduced by decreasing the amount of copper carried through analysis. This study also found that choice in extraction method could affect the outcoming results. In the presence of 0.15M or less of copper, full profiles were obtainable with organic extraction, however full profiles were obtainable with solid-phase extraction in the presence of up to 0.75M of copper. In regard to PCR inhibition, Wiley et al. [48] showed that overwhelming the amplification reaction with excess Taq polymerase increased DNA yield. Overall, the mechanism(s) by which copper ions inhibit DNA typing, as well as methods to prevent them, warrants further analysis in order to advance the analysis of forensic evidence containing metallic components.

Temperature Exposure to DNA and the Effects of Heat Generation During the Firing Process of Firearms

Although it is well known that obtaining DNA from fired ammunition is difficult, the exact reasoning why is still unknown. Many studies suggest it is the metallic components of ammunition interfering with DNA typing, others suggest the heat generated during the firing process is degrading any DNA present, some suggest it is a combination of both. While many have speculated that the high temperatures generated during the firing process are degrading DNA, few have actually studied it.

Due to the fact that DNA is stable at temperatures as high as around 100°C, evident in the use of thermal cycling in the PCR amplification process, studying the degradation at higher temperatures is difficult [51]. A study by Karni et al tested the level of degradation under several high temperatures in both aqueous and dry conditions. The results showed that under dry conditions, DNA begins degrading around 130°C with complete degradation around 190°C. Using agarose gel electrophoresis to evaluate the resulting DNA showed that degradation can have several adverse effects on analysis. Typically, intact DNA would appear on a gel as a strong band, however with degradation the band intensity weakens, and the band appears further advanced through the gel, indicating faster migration [51]. In this study, bands began to weaken when the DNA was exposed to temperatures around 130°C and when exposed to temperatures high as 180°C to 190°C the DNA resulted in the complete loss of a representative band on the agarose gel.

Several studies have focused on the effects of temperature on obtaining DNA profiles in cases of burnt human remains and biological samples. Holden et al [20] demonstrated the ability to obtain identifiable DNA profiles from bones that had been exposed to temperatures reaching

1273-1473K (inner cortical bone exposure around 573K (299.9°C)). Garriga et al. [14] found it was possible to amplify DNA profiles comparable to control samples from teeth that had been exposed to temperatures as high as 200°C for up to 5 minutes. Tontarski et al. [39] found that when blood stains were exposed to fire and temperatures reaching as high as 923°C DNA profiles were still obtainable. It was noted that the recovered DNA appeared unaffected by the exposure to heat until temperatures around 800°C or higher were reached.

As mentioned earlier, polymerase chain reaction (PCR) amplification utilizes a thermal cycling technique to generate copies of target DNA sequences. The thermal cycling process consists of 20-40 cycles with consistent temperature changes. The process can be broken down into phases, denaturation, annealing, and elongation. Double stranded DNA is denatured by a high temperature of 95-98°C for a total of 2 minutes. Next, the primers anneal to the target sequences by reducing temperatures to 55-65°C for 2 minutes. Finally, Taq polymerase elongates the target DNA sequences by increasing the temperature to 72-75°C for 2 minutes. At the completion of the cycling process, the temperature is reduced to 4°C until the sample is removed [31].

In order to determine if the temperatures generated during the firing process of a firearm can negatively affect any DNA present on that weapon or the ammunition contained within it, it is important to understand the various temperatures reached. Within the firearm chamber itself temperatures around 1800°C are reached for about 0.5 to 5ms [19]. Using thermal imaging, it was determined that a cartridge casing typically reaches temperatures no higher than 98°C [15]. On average aluminum ammunition reaches higher temperatures than brass, reaching 372K (98.85°C) and 336K (62.85°C) respectively [15].

With the understanding that DNA amplification techniques utilize temperatures as high as 98°C, it is unlikely that the short exposure to heat during the firing process would negatively affect or degrade the DNA present on the ammunition fired. Although the firearm chamber reaches temperatures far beyond those used in PCR reactions, the ammunition is only exposed for a maximum of 5ms. Further, based on previous studies on obtaining DNA from fire scenes and burnt remains, it can be said that DNA can withstand extremely high temperatures for several minutes.

Fingerprint and DNA Recovery from Firearms and Ammunition

The traditional forensic examination of firearms involves comparing the markings left by the firing process on the fired bullets and cartridge casings. When a firearm is apprehended, there are several different ways it may be processed, including photographing the firearm *in situ*, examining for visible fingerprints, processing for latent fingerprints, examination of serial numbers, swabbing for DNA, and test firing to generate test bullets and casings for comparison purposes [26]. During this processing, an important decision must be made in regard to whether the firearm and/or ammunition should be processed for potential DNA or fingerprints first, as the two are not mutually exclusive and may not be conducive to one another [26].

Nunn et al. reports that in 2009 the average turnaround time for latent fingerprint processing was about 43 days, while DNA processing took, on average, 72 days [26]. Although fingerprinting has proven cheaper and less time consuming than DNA testing, several studies [26, 37, 45, 50] have shown that when processing ammunition for latent fingerprints a number of limitations arise, and useable results are not usually obtained. A study by Spear et al. [37], found that when testing the ability to obtain fingerprints from both fired and unfired cartridge cases,

even using eccrine sweat and oily prints, no useable fingerprints could be developed. Typically, fingerprint processing is used on touched surfaces where the fingerprint is the priority over DNA recovery. Most fingerprinting techniques may not directly affect the quality of subsequent DNA that is recovered; however, studies have shown that they may reduce the quantity retrieved [45-47]. It is important to note, though, that fingerprint processing increases the amount the evidence is handled, therefore resulting in a higher risk of contamination. Furthermore, fingerprint brushes may remove and/or transfer DNA between items of evidence and may even retain that DNA for extended periods of time [45]. Wickenheiser and Challoner [46] suggested that rather than risk the potential recovery of DNA bearing cells with traditional fingerprinting techniques, a thin layer of acrylic could be applied using the fingerprint fuming process. This proposed method would, in theory, preserve and seal the cells in place for later recovery through swabbing. With the risk of losing a potential DNA sample, it is important to consider the evidence at hand and determine if obtaining a fingerprint is probable and whether a fingerprint or DNA would be of greater value to the case.

Although DNA processing of firearms is common, the testing of ammunition is not routinely employed in many crime laboratories due to the lack of results observed. Traditionally, the examination of firearms is limited to fingerprinting and DNA sampling of the firearm itself and analyzing the markings left on fired ammunition. Based on the marks left behind, ammunition can be associated with the firearm that fired it as well as with other crimes the firearm is connected to. The inability to obtain DNA from ammunition has been accredited to several factors, including potential metallic inhibition on testing procedures, degradation by high levels of heat exposure during the firing process, friction during the firing process, and simply just low levels of DNA physically present on the evidence. However, improvements to DNA

testing kits, such as the increased resistance to inhibitors, increased sensitivity, and the increased ability to amplify a degraded sample, make sampling both fired and unfired ammunition promising [24].

Several recent studies have investigated the potential to obtain DNA evidence from ammunition, suggesting mechanisms by which DNA testing is being hindered as well as proposing methods for improving standard techniques and new sampling procedures. The most highly suggested inhibiting factor of DNA typing results from ammunition is the presence of copper, preventing amplification and other processes associated with DNA typing. Numerous studies [18, 25] have tested this hypothesis and methods to avoid copper ion inhibition. Holland et al. [18] found that when using massively parallel sequencing (MPS), full haplotypes could be obtained from aluminum cartridges but less than 60% of copper and brass samples produced interpretable profiles. Other researchers proposed the use of direct PCR to improve DNA yields, however contradictory studies [42] found that no difference was observed between results obtained using direct PCR and conventional extraction methods, likely due to the metallic ions normally removed during extraction inhibiting direct PCR results. Others found that by introducing chelating agents, such as EDTA, the inhibiting copper ions may be potentially removed and increase yield as well as reduce degradation [18,25].

Other studies propose that the collection technique may affect the amount of DNA obtained as well as the amount of metallic ions that are collected with it. A proposed collection technique is a tape-lifting method, which is simply applying tape to the item in order to pick up any trace evidence present on the surface without collecting copper ions that are normally collected after exposure to a wet swab [22]. Another collection technique introduced first in 2010 by Dieltjes et al. [12] involves the direct soaking of the evidence item in lysis buffer followed by

swabbing to collect the trace amounts of DNA left on the surface. Although they defined successfully obtaining a reproducible profile as one where only two loci had been detected, the results of their study showed promise and created an area for further research. Montepetit et al. [23] found that when reproducing the methods proposed by Dieltjes et al., while defining success differently, an overall 27.2% of their samples produced interpretable DNA profiles.

In their study, Troy et al. [38] tried incorporating a sonication step, which is simply applying energy to agitate and disrupt particles, to the proposed soaking method for 30 minutes in order to increase overall DNA yield. Unlike the originally proposed method [12], which only soaked the samples for 30 minutes total, this study incubated the samples in lysis buffer overnight. The results showed an increase in yield with their method when compared to the traditional wet:dry double swabbing technique. These researchers also investigated the differences in extraction techniques used for collection of DNA from ammunition and reported higher DNA yields when a manual organic (phenol:chloroform) extraction technique was used as opposed to when an automated EZ1 Advanced XL system was used. They found that the results obtained using the automated analysis were more consistent however demonstrated preferential amplification with lower RFUs and more imbalanced peak heights than did manual extraction methods.

Theoretically, loading a firearm magazine requires a large amount of force and therefore a lot of DNA should be deposited on the ammunition being loaded. Nevertheless, many things can affect the amount of DNA left behind, including the amount of DNA that an individual may shed at that time. Further, the smooth surfaces found on cartridges may not be conducive to retaining any DNA deposited, leaving less DNA to be collected. Although studies have been conducted on creating textured surfaces on cartridges to increase the amount of DNA retained [50], it is likely

not realistic that this advanced ammunition would be mass produced and/or utilized in crimes and therefore it is important for researchers to find methods to obtain the DNA present while decreasing the chances of metallic inhibition, degradation, or loss of the overall sample. Currently, the soaking method shows promise and, with further study and manipulation to the technique, may grow to become the standard procedure for processing ammunition evidence. It is important for analysts to also evaluate the sample at hand and determine whether DNA sampling or fingerprint processing is will yield better results and would be more beneficial to the case, as performing both may not be rational. Ultimately, the processing of ammunition for evidence to associate it with an individual is still an under-studied area with various conflicting results and conclusions and warrants further investigation. In an effort to increase the recovery of DNA profiles suitable for comparison from unfired and fired cartridges and casings, the New York State Police Forensic Investigation Center evaluated the method of soaking ammunition in lysis buffer prior to DNA extraction.

METHODS AND MATERIALS

METHODS AND MATERIALS

Ammunition and Firearms

Ammunition	Case Type	Quantity (per volunteer)
Remington golden saber 9mm Luger +p 124gr	Nickel plated	8
Remington golden saber 40S&W 180gr	Nickel plated	8
Fiocchi 9mm luger 115gr FMJ	Brass	8
Browning 40S&W 165gr FMJ	Brass	8

Table 1. Ammunition used in the study.

The 224 rounds of ammunition for this study consisted of 9mm and .40 (see above) purchased from American Shooter Supply, Albany, NY. Two department provided handguns (Glock 26 and Glock 22) were used in this study. As a control for contaminating DNA, both firearms were cleaned with ultrapure water and 70% ethanol prior to each new volunteer's use.

Recruitment of Volunteers

Seven volunteers were recruited for this study. All shooters were either civilian members or members (sworn officers) of the New York State Police. All volunteers provided a reference DNA sample for data analysis.

Ammunition Exposure/ "Treatment"

In order to reproduce the different lengths of time ammunition may be handled by an individual, ammunition was distributed to volunteers to carry on their person as well as immediately loaded into a firearm from the ammunition box. Each volunteer was given 16 rounds of ammunition (4 of each type listed above) to carry in their pockets for approximately

16-24 hours. For safety reasons, state police members are not authorized to carry ammunition not distributed by the state police during their shifts, therefore, they were instructed to carry their samples after hours over a 48-hour period (on person approximately 16-24 hours). Civilians are not regulated by this restriction and carried the ammunition over the course of one 24-hour period. After the specified timeframe was complete, the ammunition was loaded into the designated firearm. Half of the ammunition was fired (casing collected) while the other half was unloaded (cartridge collected). Each volunteer was then given an additional 16 rounds of ammunition directly from the box and loaded into the firearm. Again, half of the ammunition was fired while the other half was unloaded. All ammunition was collected together in the groups listed in table below.

Ammunition	**Quantity**
9 mm Cartridge – Carried, loaded, unloaded	4
9 mm Cartridge – loaded, unloaded	4
9 mm Casing – Carried, loaded, fired	4
9 mm Casing – loaded, fired	4
.40 Cartridge – Carried, loaded, unloaded	4
.40 Cartridge – loaded, unloaded	4
.40 Casing – Carried, loaded, fired	4
.40 Casing – loaded, fired	4
Total ammunition per volunteer	**32**

Table 2. Ammunition collection groups.

DNA Sampling

Half of the cartridges and half of the casings were sampled using the traditional swabbing method employed at the New York State Police Forensic Investigation Center. Swabbing was done by taking one cotton-tipped swab and pre-wetting it with 1-2 drops of ultrapure water then wiping the surface of the cartridge or casing with the tip. Approximately 90% of the cotton tip was cut from the stick and put into a sterile microcentrifuge tube with 500 µL of lysis buffer (Qiagen Buffer G2 (800 mM guanidine hydrochloride; 30 mM Tris•HCl, pH 8.0; 30 mM EDTA, pH 8.0; 5% Tween 20; 0.5% Triton X-100), Proteinase K, DTT, cRNA) and incubated for 30 minutes. 400 µL of Qiagen Buffer MTL was added to the samples and the swabs were then subjected to DNA extraction/purification. The remaining cartridges and casings were sampled using the proposed soaking method. Soaking was done by placing the cartridge or casing into a 5-mL tube (Laboratory Product Sales, item number L232061), adding 900 µL of lysis buffer (Buffer G2, Proteinase K, DTT, and cRNA), and placed in a 56$_{o}$C incubator for 30 minutes. This incubation period was limited to 30 minutes based on previous research [12] which stated that beyond 30 minutes in lysis buffer oxidation of the ammunition resulted in the surface partially dissolving, potentially damaging useful markings and the analysis of DNA present. The cartridge or casing was removed from the tube and wiped with a single dry cotton-tipped swab to remove any residual buffer and biological material. Approximately 90% of the cotton tip was then cut and deposited into the original 5-mL tube and placed back into the incubator for 90 minutes (a total of 2 hours) at 56$_{o}$C and followed by extraction.

Extraction and purification of all samples was done using the EZ1 DNA Investigator Kit on the EZ1 Advanced XL robot, utilizing the DNA Purification Large Volume Protocol (NYSP

Stains Protocol, Large Volume) with a 50µL elution volume in TE_{-4} buffer. Quantitation was performed in triplicate (four sample sets were run in quadruplicate) using Quantifiler™ Trio DNA Quantification Kit and run on an Applied Biosystems™ 7500 Fast Real-Time PCR System. The samples were then amplified using GlobalFiler™ PCR Amplification Kit according to current NYSP protocols (28 cycles) run on an Applied Biosystems ProFlex™ PCR System or a GeneAmp™ PCR System 9700 Thermal Cycler. All quantitation and GlobalFiler amplification plates were prepared by the JANUS automated platform. Based on the average quantitation values obtained, samples with male DNA concentrations of .0005 ng/µL (approximately 25pg total DNA) and above were dehydrated and concentrated using a Thermo Savant DNA 120 SpeedVac Concentrator and manually amplified using the Yfiler® Plus PCR Amplification Kit (29 cycles). Separation and detection of DNA was done using a 3500xL genetic analyzer at an injection of 1.2 kV for 15 seconds (Yfiler® Plus samples were also injected for 24 seconds, for a total of two injections) and a run of 13.0 kV for 1550 seconds. Raw data was analyzed using GeneMapperID-X v1.5 according to current NYSP protocols.

Data Analysis and Interpretation

In order to compare the recovery of DNA using the proposed soaking method versus the swabbing method, the average quantitation value in the sample sets were compared. Using unpaired two-tail t-tests (α=0.05), the variation among sample groups was measured. First, the proposed soaking method was compared with the traditional swabbing method. If there was a statistically significant difference, this would indicate one method is superior to the other when recovering DNA. Next, ammunition carried for 16-24 hours and ammunition that had not been previously handled was compared to determine if time handled affects the amount of DNA

deposited. Additionally, previous studies have shown that when analyzing brass ammunition, interaction of copper ions present has impeded the extraction, quantitation, amplification, and detection of DNA profiles. In order to asses this, this study compared the amounts of DNA recovered from brass and nickel ammunition. In order to assess reports of DNA loss during the firing process, the final sample groups considered were fired (cartridge casing) and unfired (cartridge) ammunition.

Along with evaluating these methods for the quantity of DNA that is obtained, it was also important to evaluate the resulting profiles to determine if they were suitable for comparison. DNA in an electropherogram (EPG) is visually depicted as peaks whose heights are measured using relative fluorescence units (RFUs). Peak heights can infer the quality of DNA present and can, for example, indicate the presence of degradation. Only alleles with a peak height higher than established NYSP analytical thresholds (Tables 3 and 4) were considered for comparison purposes and the average number of alleles detected overall for both methods, as well as for each sample type, were recorded.

Strong single source DNA profiles will typically exhibit heterozygote peak height ratio (PHR) of at least 60%. For samples that are single source but appear to be degraded, low level, or contain low peak heights, a PHR of less than 60% is possible. Therefore, these imbalanced profiles observed in this study were analyzed as complete and used for comparison purposes. For determination of a homozygous peak, only alleles with a peak height higher than the established NYSP stochastic thresholds were considered for analysis and comparison purposes (Table 3 and 5).

To assess the overall value of performing DNA testing on ammunition for casework purposes, the number of interpretable profiles was evaluated, noting the number of samples

that contain a single source profile, a mixture, insufficient data for analysis, or no DNA present. The total alleles recovered in each profile were quantified then analyzed using current NYSP interpretation protocols to determine suitability for comparison. Profiles considered suitable for comparison are defined as a single source autosomal profile that contains 8 or more loci or a mixture and/or Y profile that contains 12 or more loci (NYSP protocol). Further, the profiles that were deemed suitable for comparison were then compared to reference samples for inclusion or exclusion purposes.

Dye Channel	Analytical Threshold	Stochastic Threshold
Blue	**60**	**120**
Green	**60**	**170**
Yellow	**50**	**90**
Red	**60**	**150**
Purple	**60**	**160**

Table 3. New York State Police Validated GlobalFiler™ Thresholds

Dye Channel	Analytical Threshold (15 & 24 Second Injection)
Blue	**60**
Green	**60**
Yellow	**50**
Red	**60**
Purple	**60**

Table 4. New York State Police Validated Yfiler® Plus Analytical Thresholds

		Stochastic Threshold	
Locus	**Dye Channel**	**15 Second Injection**	**24 Second injection**
DYS385	**Red**	**150**	**170**
DYF387S1	**Purple**	**160**	**180**

Table 5. New York State Police Validated Yfiler® Plus Stochastic Thresholds

Loci Called	Profile
0	No Data
1-8	Insufficient
8-20	Partial Profile
21	Full Profile (Single Source)

Table 6. Classification of profile (GlobalFiler™)

Loci Called	Profile
0	No Data
1-11	Insufficient
12-24	Partial Profile
25	Full Profile (Single Source)

Table 7. Classification of profile (Yfiler® Plus)

RESULTS AND DISCUSSION

DNA Yield Results

As expected, based on previous research and the experience of analysts, unfired ammunition (cartridges) yielded significantly more DNA than did fired ammunition (casings). Overall, cartridges yielded an average of 275pg of DNA (small autosomal) and 190pg of male DNA, while casings yielded only 45pg with both targets (Tables 8 and 9, figures 1 and 2). The cartridges obtained an average of 105pg of DNA using the standard swabbing method, increasing to an average 335pg using the soaking method proposed herein, greater than a 3-fold increase. Similarly, casings that were sampled by swabbing provided an average of 30pg of DNA, while the casings that were soaked gave 55pg. Likewise, the male DNA yields showed a similar increasing trend. Swabbed cartridges provided an average of 95pg of male DNA while soaking increased the yield to 295pg. Swabbed casings provided an average of 30pg of male DNA which increased to 55pg with soaked samples. Overall, the soaking method presents a ~186% increase in average total DNA recovered, yielding almost 3 times as much DNA as swabbed samples, and a ~169% increase in average male DNA yield, with almost 3 times as much DNA obtained compared to swabbed samples (Table 10).

T-tests were performed to determine any statistically significant differences between the two methods, distinguishing between cartridges and casings as well as the overall method. Comparison of soaked casings against soaked cartridges (p =.036) and swabbed casings against swabbed cartridges (p = 0.008) showed statistically significant differences. Comparison of swabbed casings versus soaked casings (p = .070), swabbed cartridges versus soaked cartridges (p = .073), and the overall swabbing method versus soaking method (p = .070) did not show statistically significant differences.

	Casings			Cartridges		
	Overall	**Swabbed**	**Soaked**	**Overall**	**Swabbed**	**Soaked**
Average	0.0009 ng/µL (45pg)	0.0006 ng/µL (30pg)	0.0012 ng/µL (60pg)	0.0044 ng/µL (220pg)	0.0021 ng/µL (105pg)	0.0067 ng/µL (335pg)
Median	0.0005 ng/µL (25pg)	.0004 ng/µL (20pg)	0.0007 ng/ µL (35pg)	0.0016 ng/µL (80pg)	0.0013 ng/µL (65pg)	0.0019 ng/µL (95pg)

Table 8. Average and median quantities of DNA (Small Autosomal) for cartridges and casings

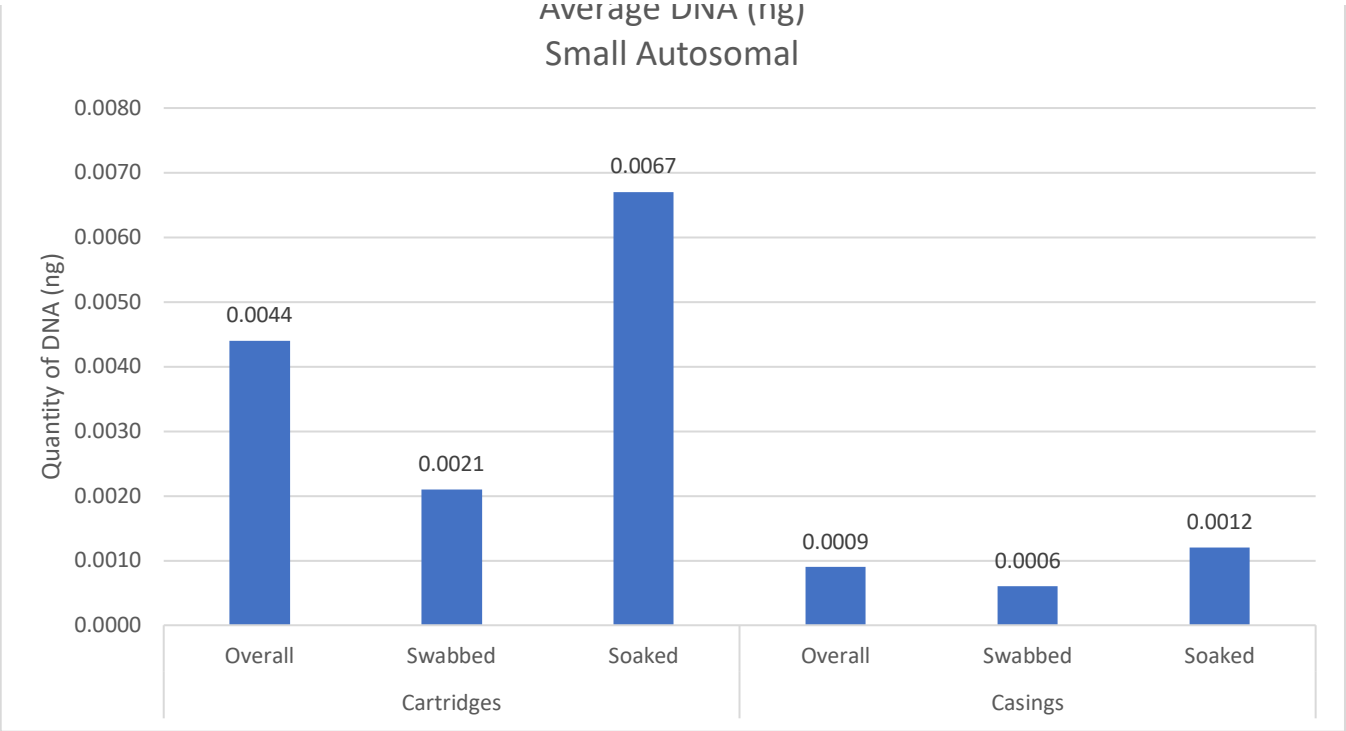

Figure 1. Average quantitation results (Small Autosomal)

	Casings			Cartridges		
	Overall	**Swabbed**	**Soaked**	**Overall**	**Swabbed**	**Soaked**
Average	0.0009 ng/µL (45pg)	0.0006 ng/µL (30pg)	0.0011 ng/µL (55pg)	0.0038 ng/µL (190pg)	0.0019 ng/µL (95pg)	0.0059 ng/µL (295pg)
Median	0.0005 ng/µL (25pg)	0.0003 ng/µL (15pg)	0.0007 ng/µL (35pg)	0.0012 ng/µL (60pg)	0.0010 ng/µL (50pg)	0.0015 ng/µL (75pg)

Table 9. Average and median quantities of DNA (T.Y.) for cartridges and casings

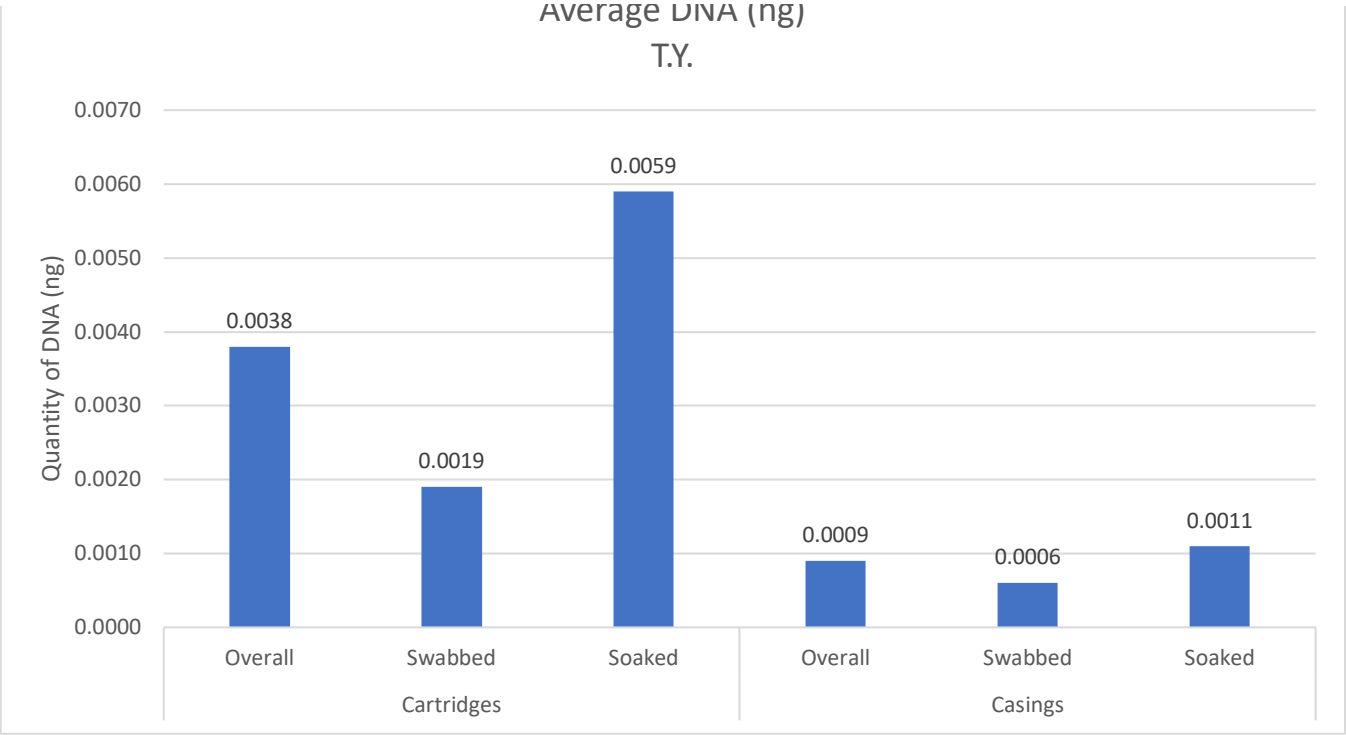

Figure 2. Average quantitation results (T.Y.)

Swabbing Method		Soaking Method	
Small Autosomal	**T.Y.**	**Small Autosomal**	**T.Y.**
0.0014 ng/µL (70pg)	0.0013 ng/µL (65pg)	0.0040 ng/µL (200pg)	0.0035 ng/µL (175pg)

Table 10. Average quantities of DNA obtained with traditional swabbing method and proposed soaking method

Comparison of Brass vs. Nickel

T-tests were performed to determine any statistically significant differences between brass and nickel ammunition, as well as soaking and swabbing both. Comparison of swabbed brass against swabbed nickel ammunition (p = .562), soaked brass against soaked nickel ammunition (p = .920), and overall brass against nickel ammunition (p = .836) did not show statistically significant differences. However, a 25% difference in average total DNA was obtained by swabbing brass and nickel ammunition and an 8% difference obtained using the soaking method. For male DNA, a 55% difference in DNA was observed between swabbed samples and, unexpectedly, a 51% increase in male DNA obtained from soaked brass versus nickel. Overall, more total DNA was obtained from nickel ammunition, however greater amounts of male DNA were obtained from brass ammunition.

Swabbing Method		Soaking Method	
Brass	Nickel	Brass	Nickel
0.0012 ng/µL (60pg)	0.0015 ng/µL (75pg)	0.0038 ng/µL (190pg)	0.0041 ng/µL (205pg)

Table 11. Average quantities of DNA (small autosomal) obtained from brass and nickel ammunition

Swabbing Method		Soaking Method	
Brass	Nickel	Brass	Nickel
0.0009 ng/µL (45pg)	0.0014 ng/µL (70pg)	0.0047 ng/µL (235pg)	0.0023 ng/µL (115pg)

Table 12. Average quantities of DNA (T.Y.) obtained from brass and nickel ammunition

Brass		Nickel	
Small Autosomal	T.Y.	Small Autosomal	T.Y.
0.0026 ng/µL (130pg)	0.0025 ng/µL (125pg)	0.0028 ng/µL (140pg)	0.0018 ng/µL (90pg)

Table 13. Overall average quantities of DNA obtained from brass and nickel ammunition

Profile Interpretation

In this study, DNA typing success (as determined by the New York State Police Forensic Investigation Center, Biological Sciences Section) is defined as those profiles that contain a minimum of 8 loci detected with a single contributor or 12 loci for mixtures using Globalfiler™ and 12 loci for profiles detected using Yfiler® Plus. Alleles detected is defined here as all alleles above the established analytical thresholds, including those at loci not utilized for comparison purposes (i.e. Amelogenin, Yindel, and DYS391). Loci containing a single peak were counted as 2 alleles when above the stochastic thresholds and no peaks were visualized between baseline and analytical thresholds. If a single allele did not pass stochastic thresholds but was called, this could not confidently be deemed homozygous and was therefore only counted as 1 allele detected.

Profile status (i.e. single source (full profile), partial profile, mixture, insufficient data, or no data) was determined based on number of loci detected that were suitable for comparison. Per NYSP interpretation protocols for autosomal DNA, no data profiles contained no called alleles and no useable loci, insufficient profiles consisted of at least 1 allele and 0 to 7 loci, partial profiles had 8 to 20 useable loci, and profiles containing 21 interpretable loci were marked as full single source profiles (Table 6). Those profiles containing loci with 3 or more alleles called and appeared to have more than 1 contributor were marked as mixtures. When interpreting male (Y) DNA the number of loci necessary differs slightly from autosomal DNA interpretation. Here, insufficient profiles consisted of at least 1 allele and 0 to 11 loci, partial profiles contained 12 to 24 useable loci, and profiles containing 25 interpretable loci were marked as full single source haplotypes (Table 7). Chi square test of association between allele recovery of autosomal profiles using the swabbing and soaking methods resulted in a significant difference between the

swabbing and soaking methods for DNA recovery, X_2 (1, N = 112) = 69.3, $p < .00001$ ($\alpha = 0.05$). The chi-square test was calculated using socscistatistics.com ©2020.

Overall, soaking ammunition evidence shows improvement in DNA recovery as opposed to swabbing. While about 82% of swabbed casings provided no data profiles, only about 55% of soaked cartridges yielded no data. Although soaked casings did not show an increase in profiles suitable for comparison, there was an increase in alleles detected indicated by more insufficient profiles obtained (from about 16% to almost 45% of samples), showing promise for further investigation into manipulations of the soaking technique. About 39% of swabbed cartridges provided no data profiles, only about 23% of soaked cartridges yielded no data. Similar to soaked casings, soaked cartridges did not show an increase in useable profiles, however an increase in alleles detected was observed with an overall increase from about 46% of samples to almost 54% of samples yielding insufficient profiles. Overall, the swabbing method provided a 44% increase in comparable profiles obtained using GlobalFiler™, a 240% increase in comparable profiles obtained using Yfiler® Plus and the electropherograms typically displayed more pronounced allele peaks between baseline and threshold using the soaking method. Figure's 6-10 provide an example where a 'no data' profile was obtained using the swabbing method and an 'insufficient' profile was obtained using the soaking method. To further demonstrate the increase in DNA yield using the soaking method, figure's 11-15 demonstrate a situation where 'no data' profiles were obtained with both methods, however the soaking method visibly provided more DNA. These 'no data' profiles demonstrate that although no statistically significant increase in DNA yield was observed based on the quantitation values and profile status, the soaking method has the potential to provide more with further testing.

Of the 224 samples analyzed, 112 (66%) contained detected alleles (insufficient, partial, or full profiles) obtained using GlobalFiler™. Of these 112, 77 were obtained from cartridges and 35 from casings. 8 of these profiles contained at least 1 allele not attributable to the expected donor. Out of the 124 samples analyzed using Yfiler® Plus, 82 (66%) contained detected alleles. Of these 82, 51 were obtained from cartridges and 31 from casings. 6 of these profiles contained at least 1 allele not attributable to the expected donor. Within these unattributable Y profiles, one full profile was obtained that could not be ascribed to any of the donors of the study and therefore assumed to be a result of a contaminated consumable.

	Casings		Cartridges		Overall
Alleles Detected	**Swabbed**	**Soaked**	**Swabbed**	**Soaked**	
0	82.14%	55.36%	39.29%	25.00%	50.45%
1-4	16.07%	26.79%	25.00%	25.00%	23.21%
5-9	0.00%	5.36%	5.36%	14.29%	6.25%
10-14	0.00%	5.36%	10.71%	5.36%	5.36%
15-19	0.00%	1.79%	1.79%	1.79%	1.34%
20-24	0.00%	3.57%	3.57%	5.36%	3.13%
25-29	0.00%	1.79%	0.00%	3.57%	1.34%
≥30	1.79%	0.00%	14.29%	19.64%	8.93%
Total	100%	100%	100%	100%	100%

Table 14. Alleles detected using GlobalFiler™

	Casings		Cartridges	
	Swabbed	**Soaked**	**Swabbed**	**Soaked**
Single Source	1.79%	0.00%	0.00%	8.93%
Partial Profile	0.00%	0.00%	14.29%	14.29%
Mixtures	0.00%	0.00%	0.00%	0.00%
Insufficient	16.07%	44.64%	46.43%	53.57%
No Data	82.14%	55.36%	39.29%	23.21%
Total	100.00%	100.00%	100.00%	100.00%

Table 15. Analysis of profiles obtained using GlobalFiler™

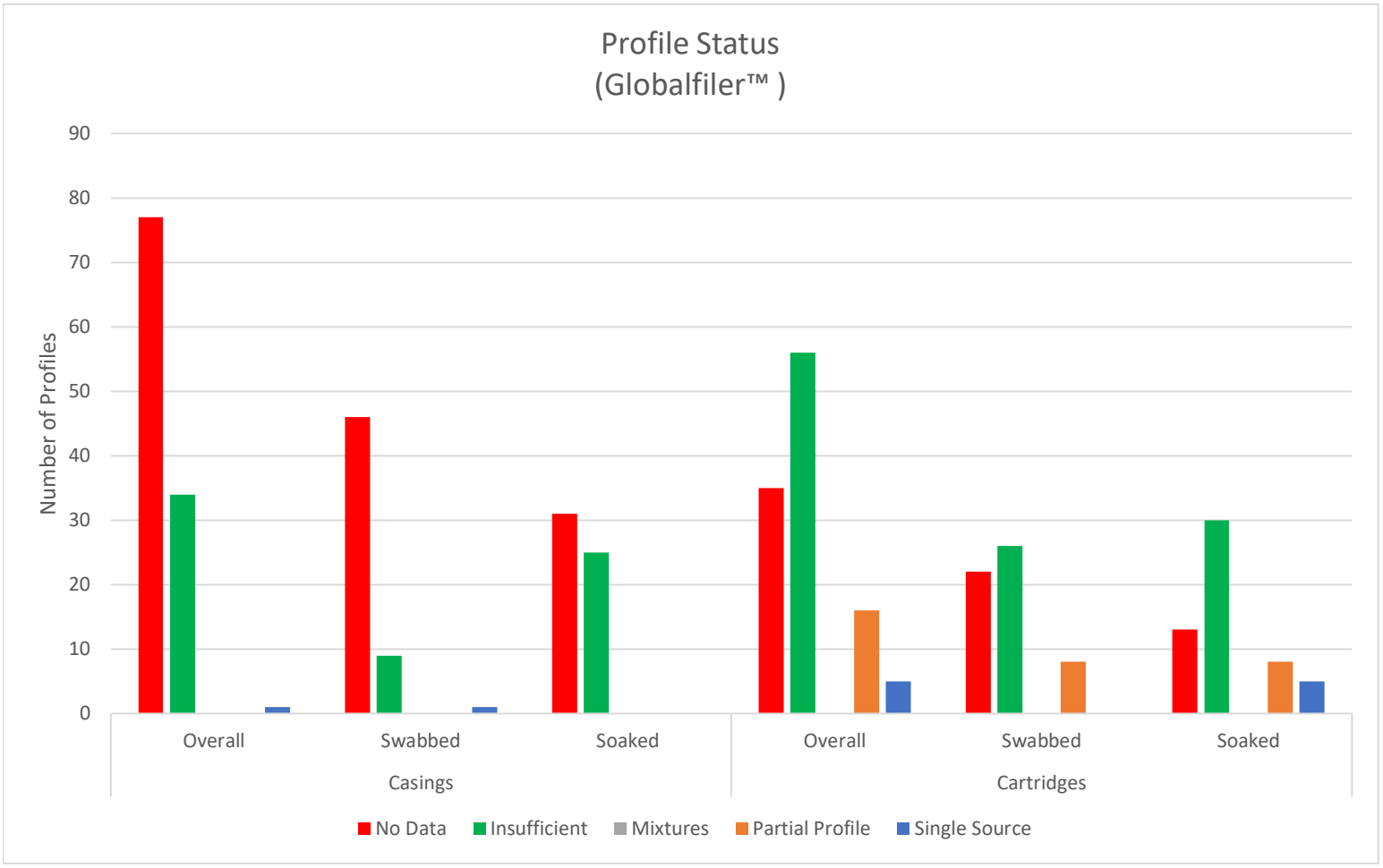

Figure 3. Analysis of profiles obtained using GlobalFiler™

	Casings		Cartridges		Overall
Alleles Detected	**Swabbed**	**Soaked**	**Swabbed**	**Soaked**	
0	70.00%	69.44%	57.14%	18.18%	52.42%
1-4	25.00%	22.22%	20.00%	27.27%	23.39%
5-9	0.00%	5.56%	11.43%	15.15%	8.87%
10-14	5.00%	0.00%	0.00%	9.09%	3.23%
15-19	0.00%	0.00%	5.71%	6.06%	3.23%
20-24	0.00%	2.78%	2.86%	6.06%	3.23%
25-27	0.00%	0.00%	2.86%	18.18%	5.65%
Total	100%	100%	100%	100%	100%

Table 16. Alleles detected using Yfiler® Plus (15 second injection)

	Casings		Cartridges	
	Swabbed	**Soaked**	**Swabbed**	**Soaked**
Single Source	0.00%	0.00%	2.78%	15.15%
Partial Profile	0.00%	2.78%	8.33%	18.18%
Mixtures	0.00%	0.00%	0.00%	0.00%
Insufficient	31.58%	38.46%	30.56%	48.48%
No Data	68.42%	69.44%	58.33%	18.18%
Total	100%	100%	100%	100%

Table 17. Analysis of profiles obtained using Yfiler® Plus (15 second injection)

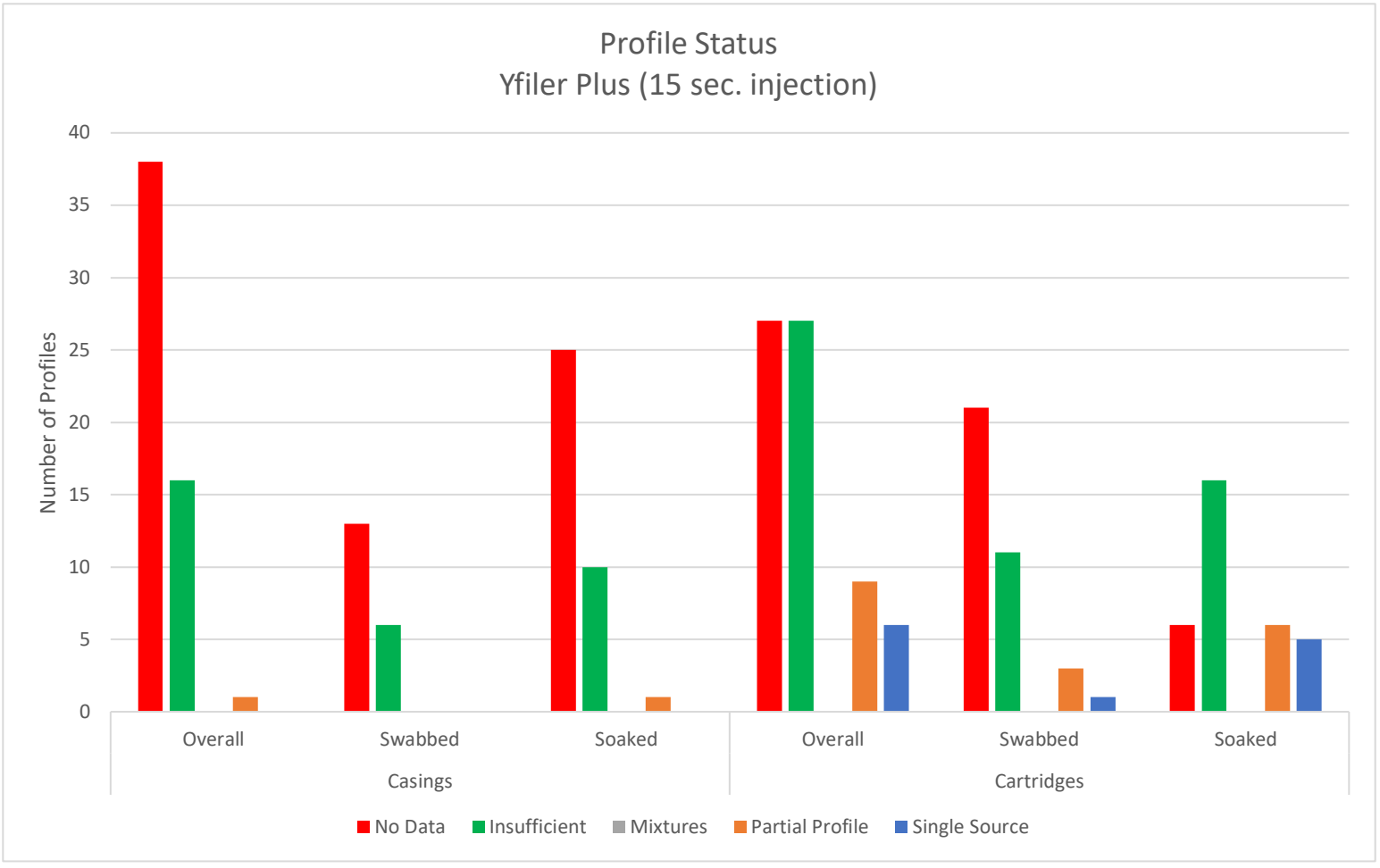

Figure 4. Analysis of profiles obtained using Yfiler® Plus (15 second injection)

	Casings		Cartridges		Overall
Alleles Detected	**Swabbed**	**Soaked**	**Swabbed**	**Soaked**	
0	50.00%	41.67%	40.00%	9.09%	33.87%
1-4	40.00%	38.89%	28.57%	15.15%	29.84%
5-9	5.00%	8.33%	8.57%	21.21%	11.29%
10-14	0.00%	5.56%	11.43%	12.12%	8.06%
15-19	0.00%	2.78%	0.00%	12.12%	4.03%
20-24	5.00%	2.78%	5.71%	12.12%	6.45%
25-27	0.00%	0.00%	5.71%	18.18%	6.45%
Total	100.00%	100.00%	100.00%	100.00%	100.00%

Table 18. Alleles detected using Yfiler® Plus (24 second injection)

	Casings		Cartridges	
	Swabbed	**Soaked**	**Swabbed**	**Soaked**
Single Source	0.00%	0.00%	2.78%	15.15%
Partial Profile	5.26%	5.56%	8.33%	30.30%
Mixtures	0.00%	0.00%	0.00%	0.00%
Insufficient	47.37%	52.78%	47.22%	45.45%
No Data	47.37%	41.67%	41.67%	9.09%

Table 19. Analysis of profiles obtained using Yfiler® Plus (24 second injection)

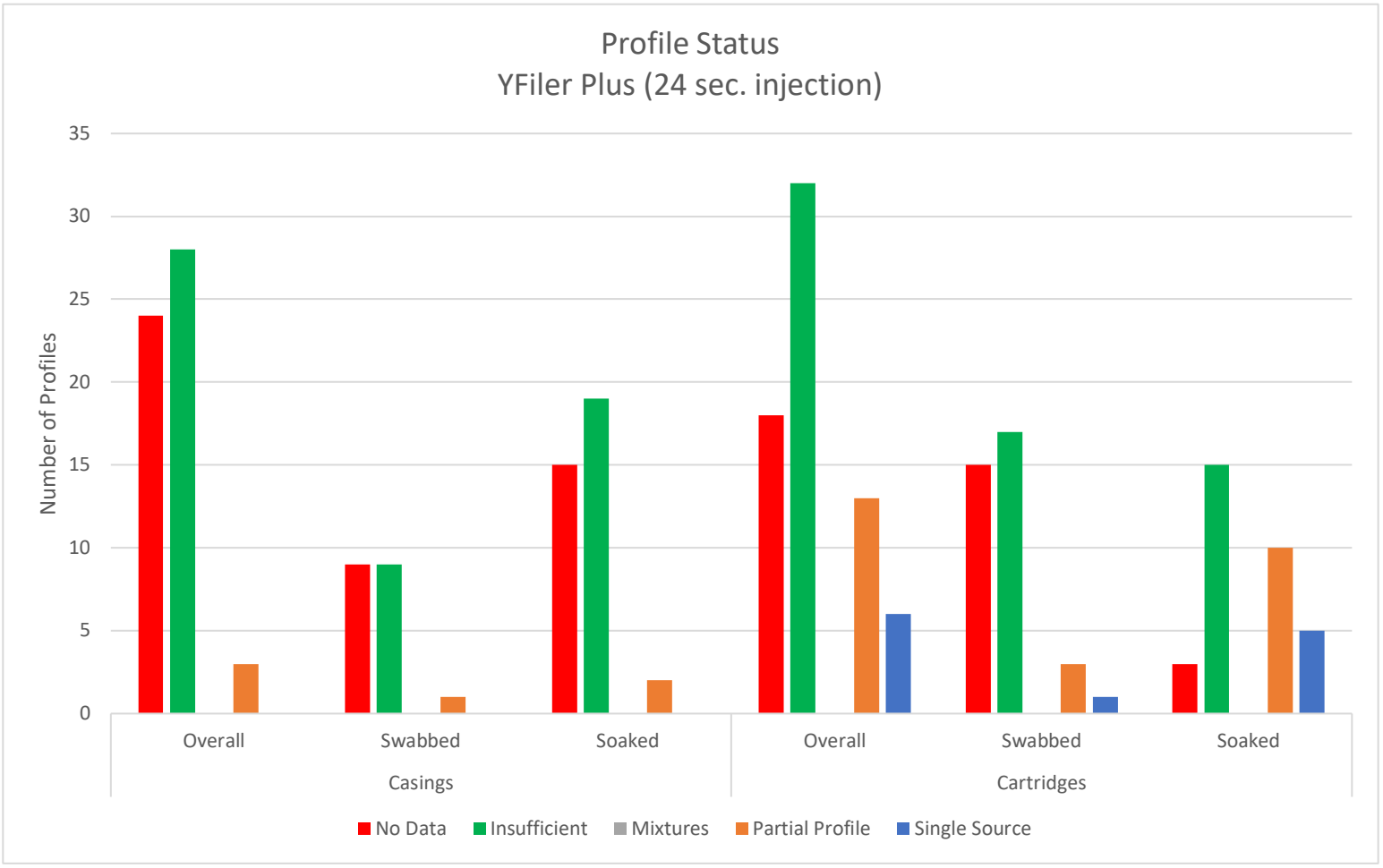

Figure 5. Analysis of profiles obtained using Yfiler® Plus (24 second injection)

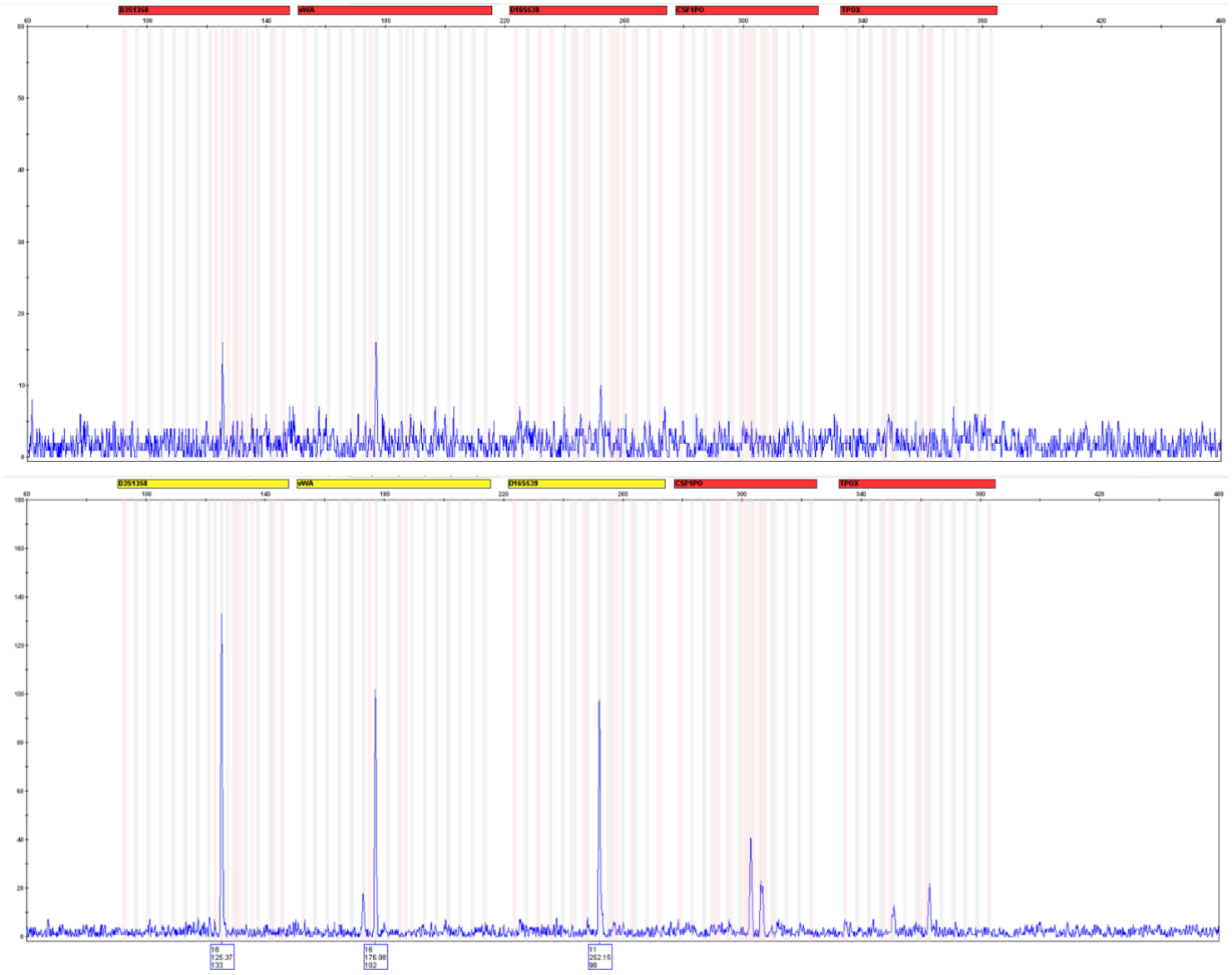

Figure 6. Electropherogram blue dye channel from a 9 mm brass cartridge carried for 24 hours, fired, and sampled using the swabbing method (top) versus the soaking method (bottom) run on GlobalFiler™

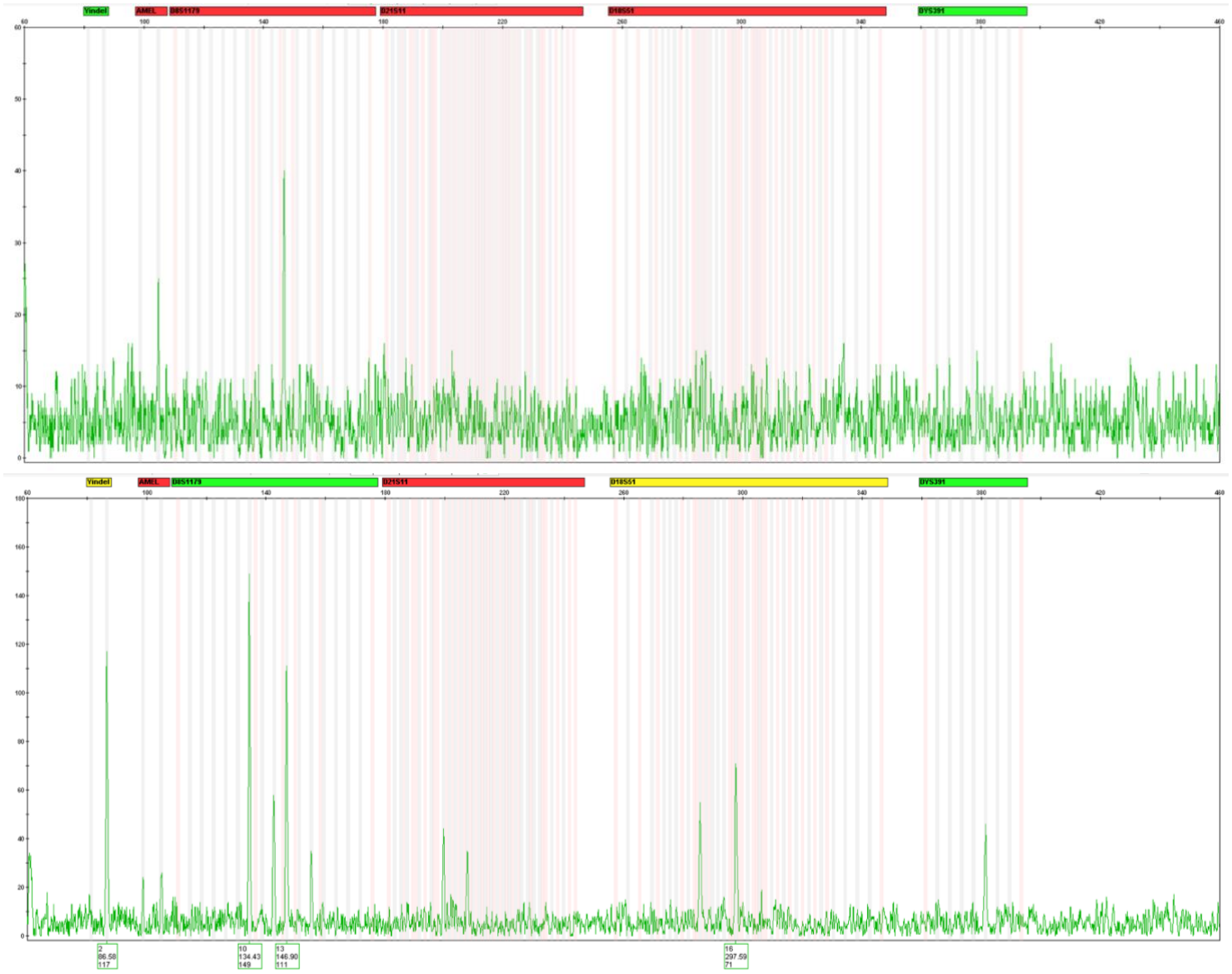

Figure 7. Electropherogram green dye channel from a 9 mm brass cartridge carried for 24 hours, fired, and sampled using the swabbing method (top) versus the soaking method (bottom) run on GlobalFiler™

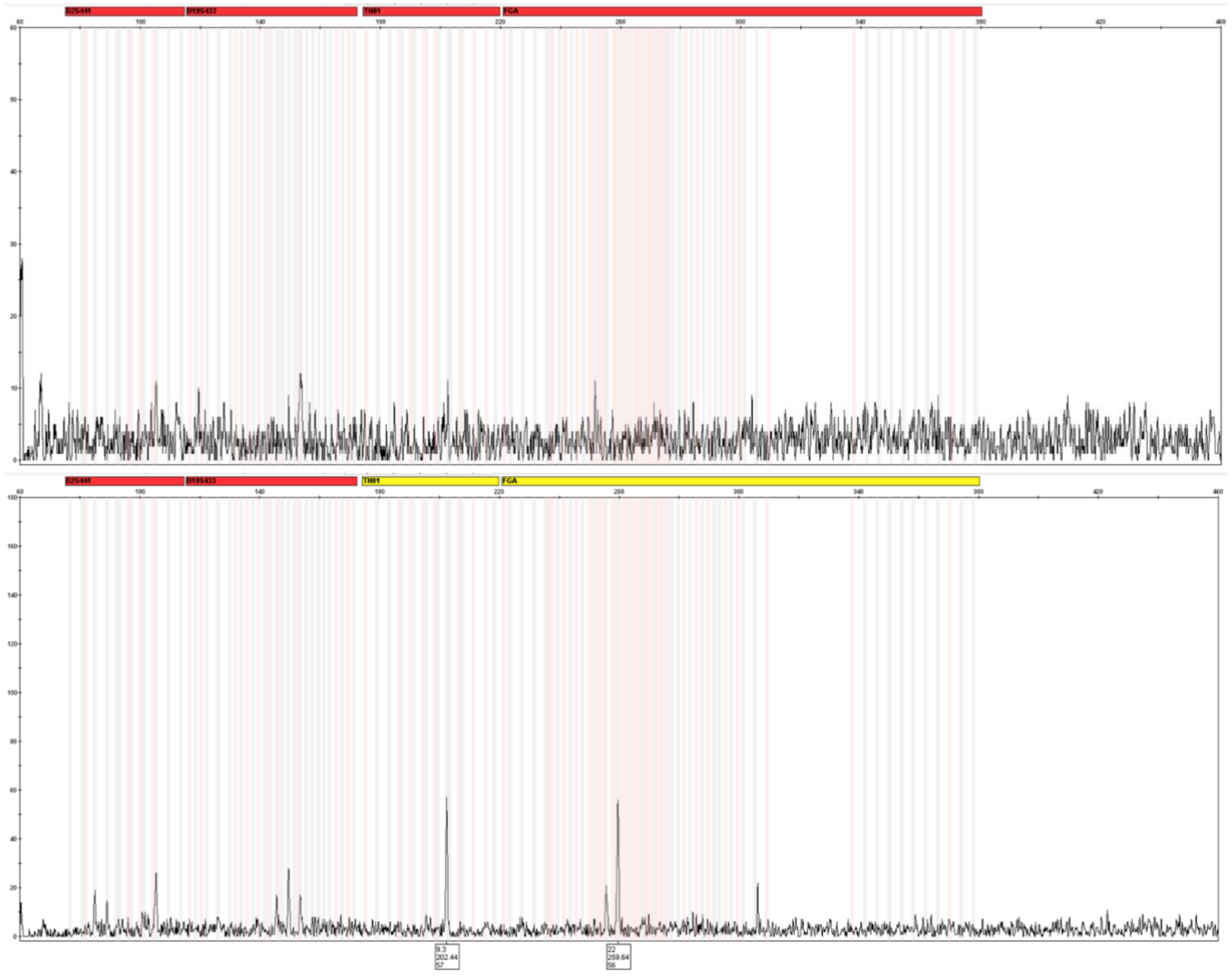

Figure 8. Electropherogram yellow dye channel from a 9 mm brass cartridge carried for 24 hours, fired, and sampled using the swabbing method (top) versus the soaking method (bottom) run on GlobalFiler™

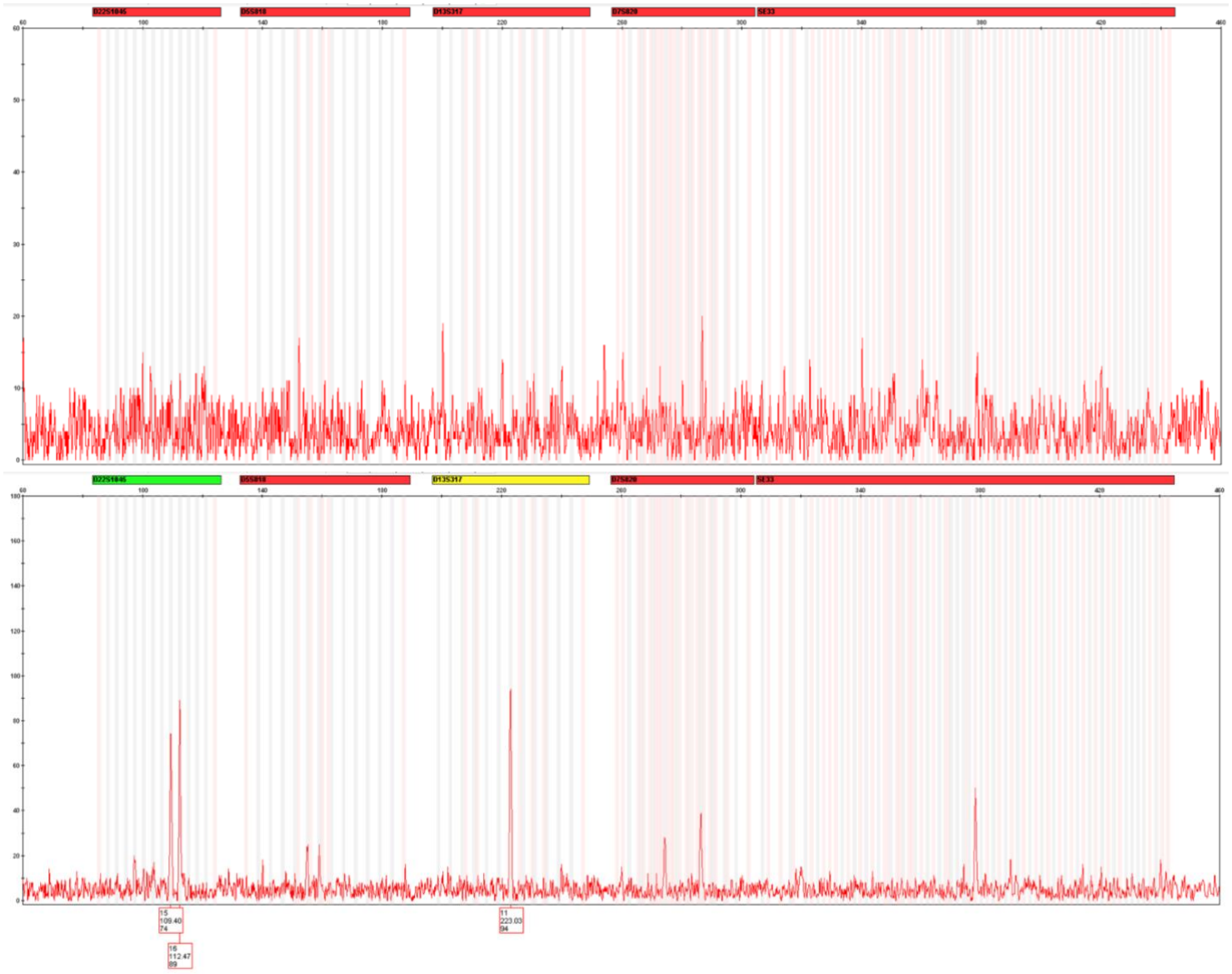

Figure 9. Electropherogram red dye channel from a 9 mm brass cartridge carried for 24 hours, fired, and sampled using the swabbing method (top) versus the soaking method (bottom) run on GlobalFiler™

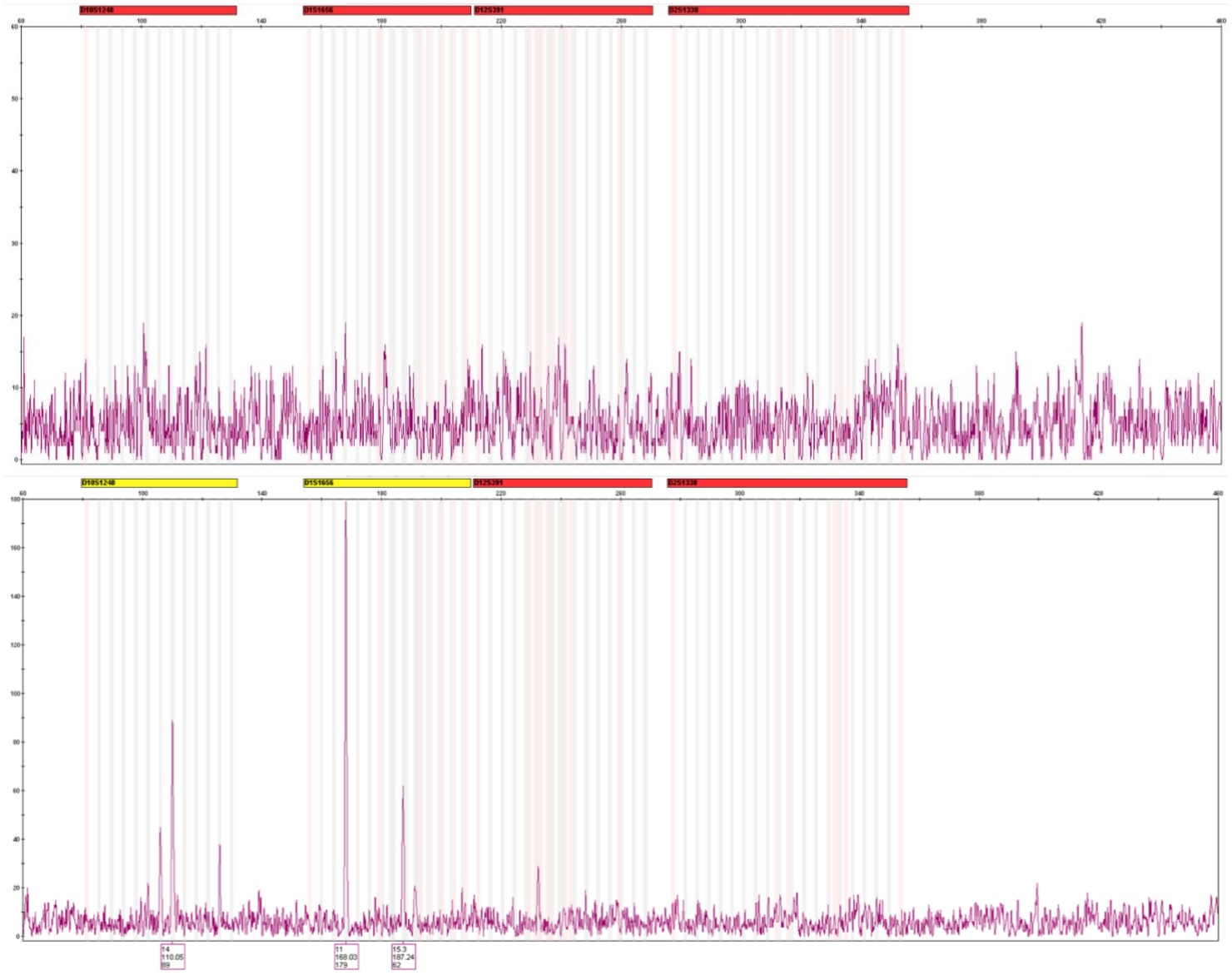

Figure 10. Electropherogram purple dye channel from a 9 mm brass cartridge carried for 24 hours, fired, and sampled using the swabbing method (top) versus the soaking method (bottom) run on GlobalFiler™

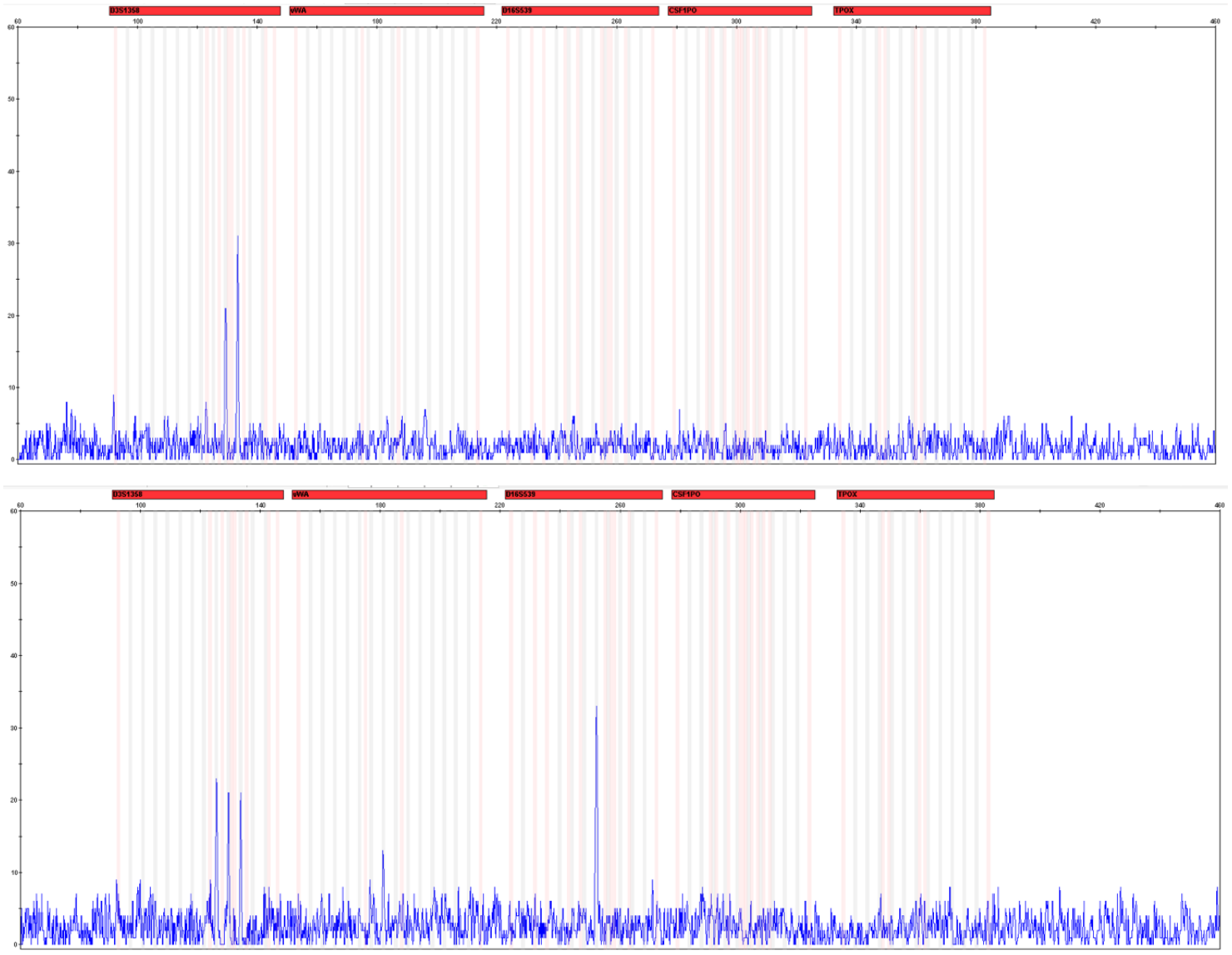

Figure 11. Electropherogram blue dye channel from a 9 mm nickel cartridge carried for 24 hours, fired, and sampled using the swabbing method (top) versus the soaking method (bottom) run on GlobalFiler™

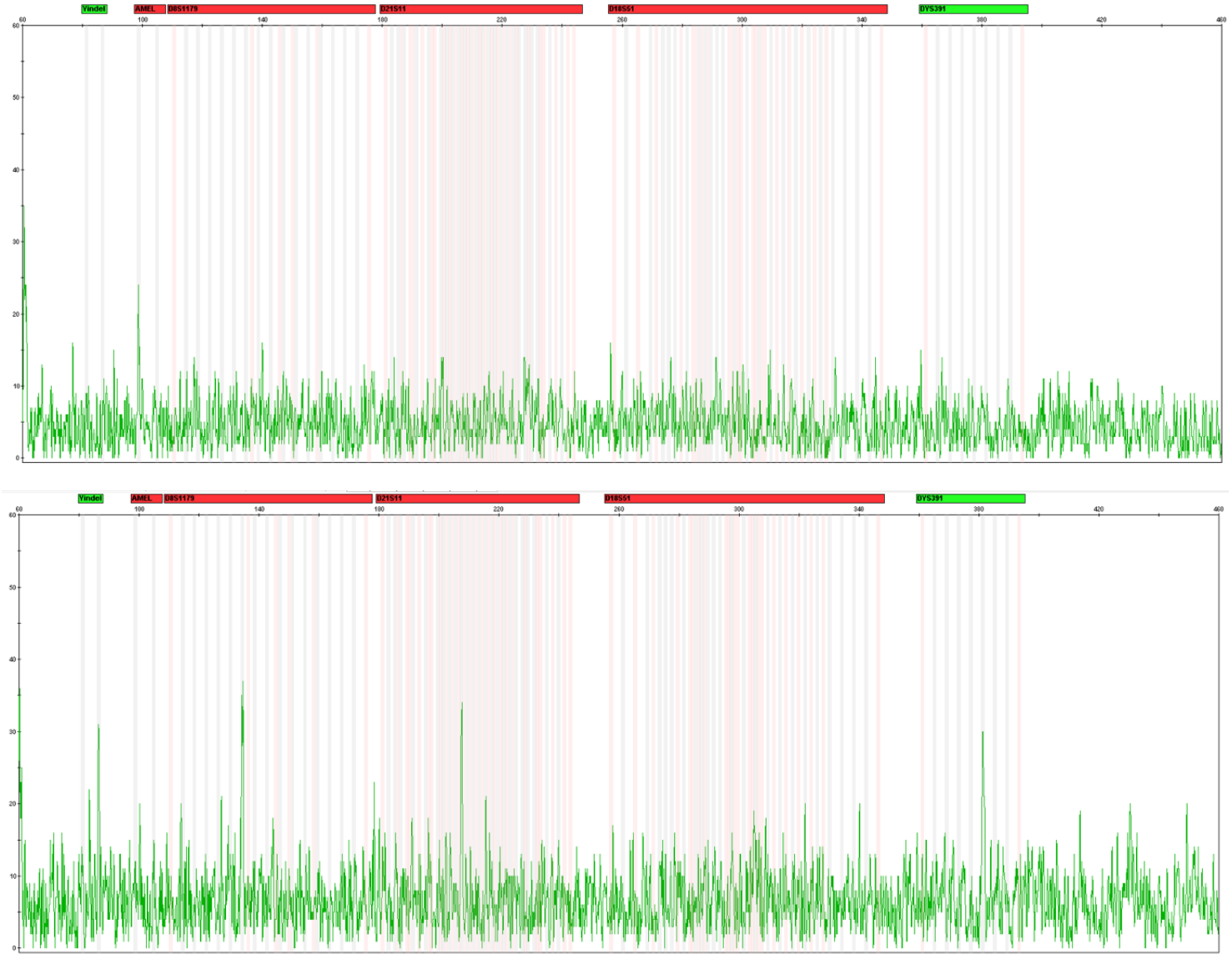

Figure 12. Electropherogram green *dye channel from a 9 mm nickel cartridge carried for 24 hours, fired, and sampled using the swabbing method (top) versus the soaking method (bottom) run on GlobalFiler™*

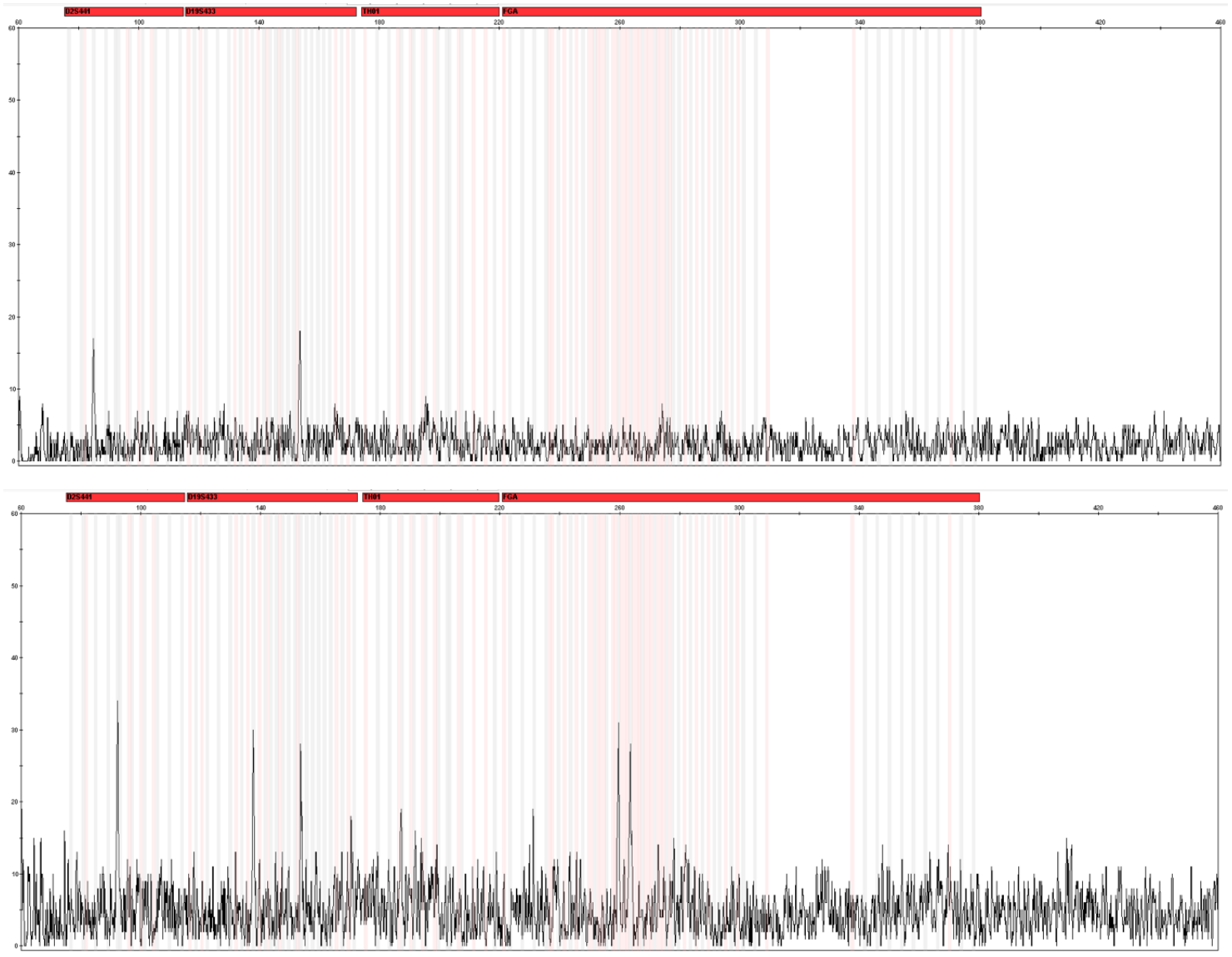

Figure 13. Electropherogram yellow *dye channel from a 9 mm nickel cartridge carried for 24 hours, fired, and sampled using the swabbing method (top) versus the soaking method (bottom) run on GlobalFiler™*

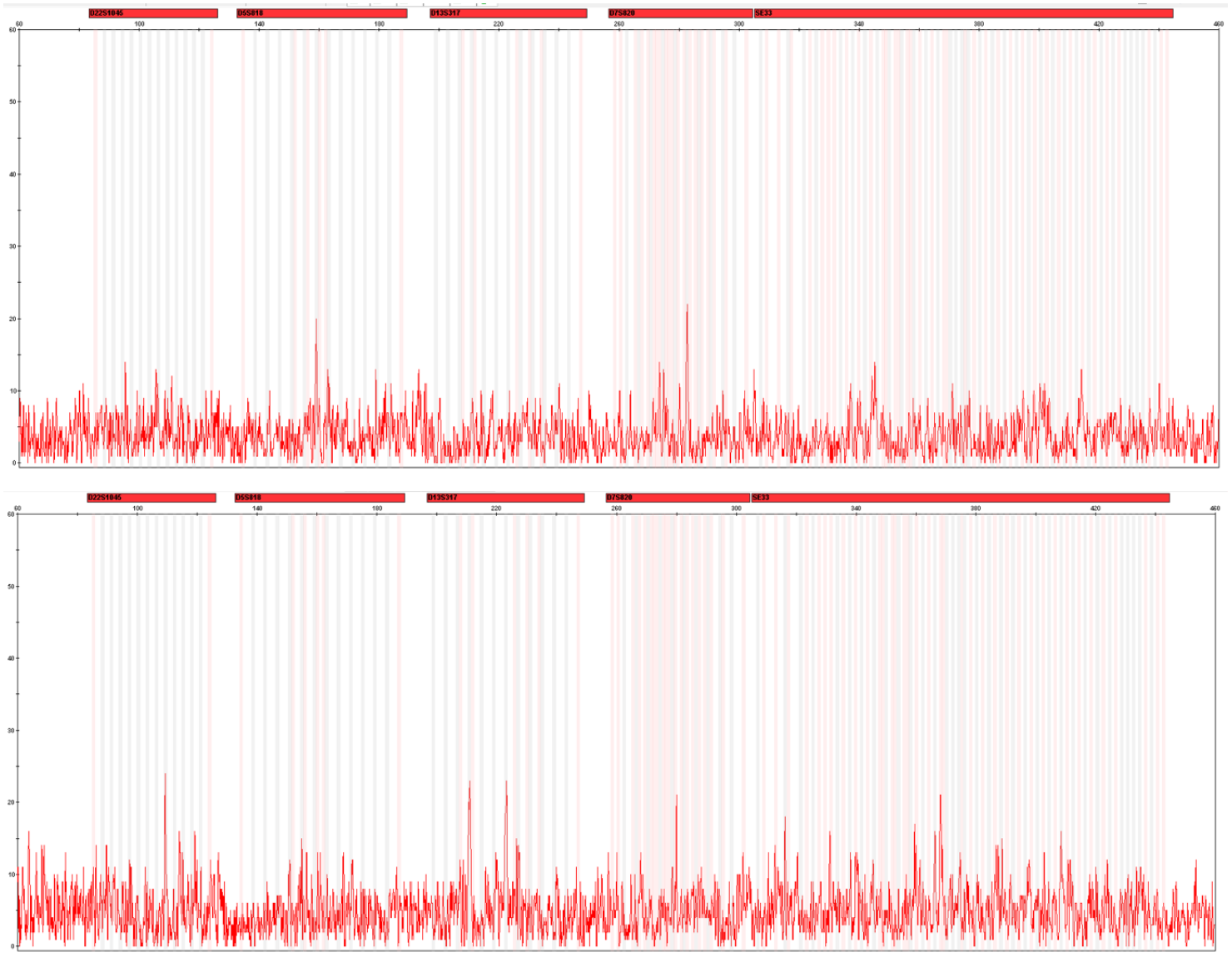

Figure 14. Electropherogram red *dye channel from a 9 mm nickel cartridge carried for 24 hours, fired, and sampled using the swabbing method (top) versus the soaking method (bottom) run on GlobalFiler™*

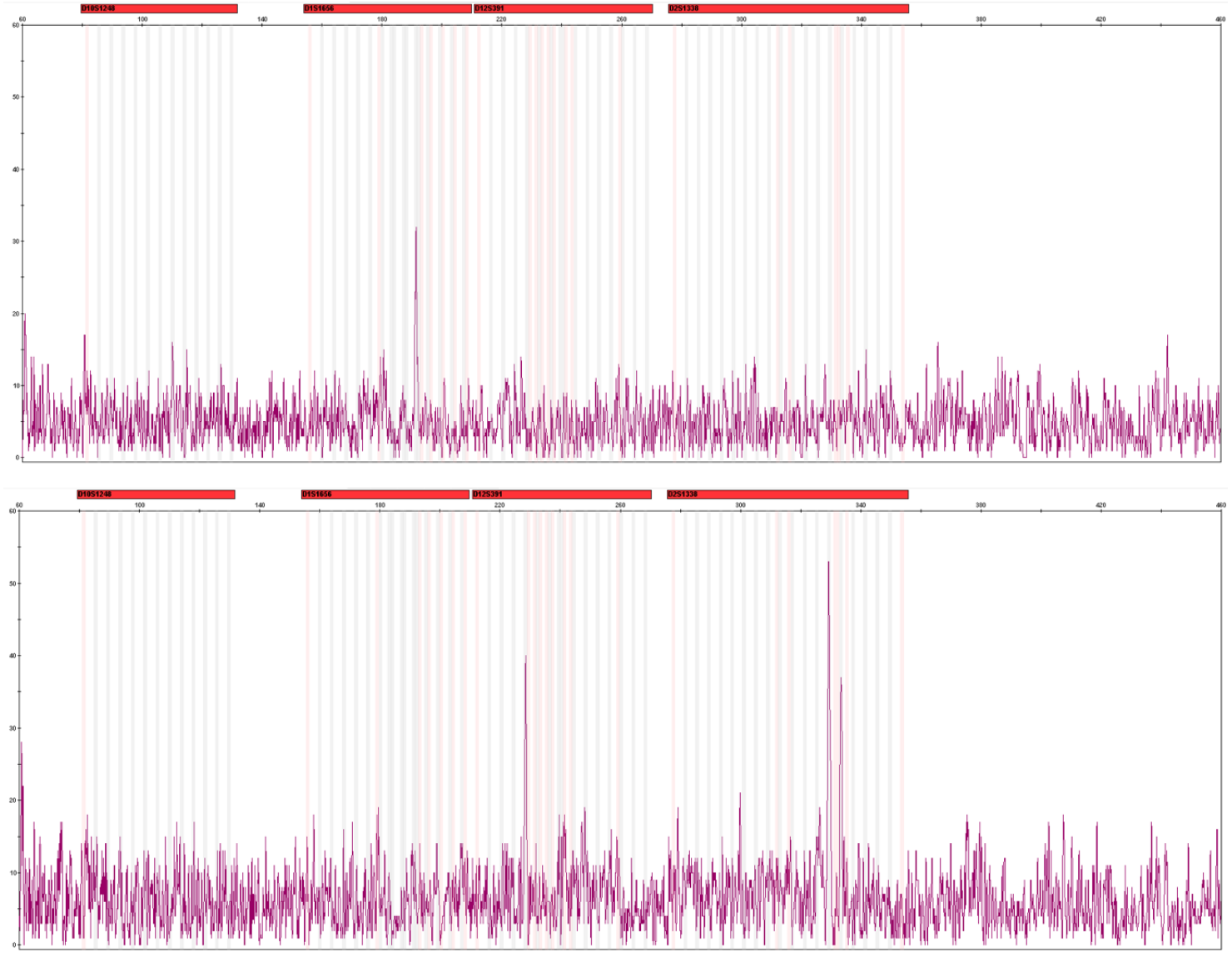

Figure 15. Electropherogram purple *dye channel from a 9 mm nickel cartridge carried for 24 hours, fired, and sampled using the swabbing method (top) versus the soaking method (bottom) run on GlobalFiler™*

DISCUSSION AND CONCLUSIONS

Oftentimes, crime scene evidence from a crime involving a firearm is limited to cartridges and/or casings. The typical analysis of ammunition evidence, i.e. examining striations and markings imparted on casings during the firing process, can be useful for connecting a crime to a specific firearm and consequently one crime to another. To date, however, the use of ammunition to connect a crime to an individual is less common, as fingerprints and DNA deposited on ammunition are often of low quality and quantity. Several studies have proposed mechanisms by which DNA sampling and analysis are inhibited when faced with fired and unfired ammunition evidence, however no definitive conclusions have been established. Some suggest that copper ions present in the composition of the ammunition are coextracted with the DNA and either bind to the DNA or inhibit downstream analysis methods, e.g. extraction, quantitation, amplification, and capillary electrophoresis, and suggests mechanisms to hinder these effects. Other studies report that the high temperatures during the firing process of a firearm are degrading the DNA present, while others stated that there is just simply not enough DNA present and therefore sampling is too difficult. Overall, all studies that focus on obtaining DNA from ammunition evidence are in agreement that there is low-level DNA present and analysis comes with challenges.

Previously, obtaining any DNA from ammunition evidence was unlikely, however with the continuously increasing sensitivity of DNA typing chemistries and improvements to trace DNA collection techniques the ability to obtain partial and even full profiles is a likely possibility. In this study, we investigated the value of soaking ammunition evidence in lysis buffer prior to DNA extraction and compared it with the traditional swabbing method. This method presented a 186% increase in total DNA yield with almost 3 times more DNA obtained,

169% increase in male DNA yield and almost 3 times more DNA obtained, and a 44% increase in comparable profiles obtained using GlobalFiler™ and 240% using Yfiler® Plus. Results showed fewer 'no data' determinations using the soaking method, indicating more DNA obtained. Further, the electropherograms displayed more pronounced allele peaks between baseline and threshold. Although a statistically significant difference was not observed between the two methods ($p = 0.07$), a qualitative significance was.

The evaluated soaking method provided several limitations in this study. Typically, metal tweezers are used for moving small evidence items during processing, however due to their shape and weight it was difficult to maintain a grip on them. Based on the experience of analysts at NYSP, re-concentration of samples followed by amplification using GlobalFiler™ routinely yielded lower to no DNA results. Therefore, re-concentration was limited to samples intended for amplification with Yfiler® Plus. Being that several volunteers were used, there appeared to be significant differences in shedder status among them that could have affected the results obtained, potentially lowering the significance level of the tested method. Also, being that the volunteers were not observed during the 24-hour carrying period, the ammunition may have been handled very differently, potentially leading to low-level samples or stochastic effects. However, it is important to note that the sampling type used here closely reflects that of real case samples, as the handling and treatment of ammunition and the shedder status of the individual handling the evidence in casework is unknown. Finally, it should be noted that the sample size in this study was low with several variables, which is a potential for bias, skewed T-test results, and results that are not normally distributed.

FUTURE DIRECTIONS

With the promising results this study showed in the use of the soaking method for ammunition evidence, many future directions to adapt the method for use in casework and to increase overall DNA yield have been noted. First, a larger sample size would increase the value of the calculated statistics, providing a more accurate representation of the effects of the proposed method. A significant issue noted during the conduction of this study was the difficulty to hold and transfer the ammunition evidence, specifically the unfired cartridges. Due to the reported difficulty in handling unfired ammunition with metal tweezers, it is suggested that a new device or mechanism be proposed for the transfer of this type of evidence from packaging to sampling tube and back. A suggested adjustment to the soaking method tested here is to eliminate the swabbing step after soaking of the ammunition. The suggested method includes soaking and gently shaking the sample for 30 minutes, removing the sample, and spinning down the sample to pellet. Eliminating the cotton swab reduces the potential for loss of DNA to the swab during extraction. Suggested improvements to the overall processing of ammunition evidence are to validate a method for re-concentrating evidence items to be amplified using GlobalFiler™, validate a 24-second injection for GlobalFiler™ samples on capillary electrophoresis, and the introduction of a purification step using products such as MinElute PCR purification columns or Microcon® Centrifugal Filters. The interpretation of ammunition evidence may also benefit from the use of probabilistic genotyping software. If future analysis of this process is desired, the combination of some of the above suggestions could improve the recovery of DNA from ammunition.

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