Final Technical Report

Bait and Capture: Holding on to Molecules of Interest

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ABSTRACT

In this study, we sought to document the efficiency of three DNA bait capture methods by two measures; first, in their ability to retain targeted DNA molecules and secondly in their ability to remove non-target DNA molecules. Efficiencies were estimated by comparing number of "copies in" to "copies out" with quantitative polymerase chain reaction (qPCR).

The first method, "fishing" for DNA, retains only 9.06-3.53% (i.e., loss of 90.94-96.47%) of DNA targets ranging 109-288 base pairs (bps) in length. Minor improvement was achieved by employing a modified fishing protocol (i.e., shortened hybridization time, use of twice the amount of M-270 streptavidin-coated beads, and modified bead washing), resulting in average retention of 31.41-12.08% of the same set of targeted molecules. However, of equal concern was the inability of the method in removing more non-target DNA molecules than targeted molecules. The second method, primer extension capture (PEC), retains 15.88-2.14% (i.e., loss of 84.12-97.86%) of the same target molecules. Experimental modifications of PEC aimed to potentially increase this efficiency were generally of no avail. However, the benefit of PEC, as originally designed, is in its ability to remove most non-target DNA molecules (99.99%). The third method, "mega-probe" capture, increased the count of target molecules from the same DNA standards by 702.46%, an impossible outcome. From our observations of negative controls, bait molecules became counted as captured copies when, in fact, there were no copies to capture. Due to unexpected experimental outcomes, we were not able to estimate the efficiency of this method in its removal of non-target molecules. Nevertheless, our principal concern about mega-probe capture is the possibility that the mega-probe bait becomes counted as captured target molecules. Thus, any attempt to measure its efficiency in retaining targeted molecules will be biased.

Our unique approach to quantifying efficiency allowed us to begin assessing at which step(s) DNA is lost during the protocols tested here. We observed that most (61.35-69.49%) of the molecules are "lost" during the *essential* hybridization step of the fishing protocol. Following the PEC protocol results in the loss of many (56.69%) of the target molecules prior to their immobilization on the streptavidin-coated beads (i.e., following extension by the polymerase and subsequent purification of the extended products). Experiments conducted on DNA retention following hybridization steps of the mega-probe protocol reveal tremendous loss of molecules (up to 83.04%). The losses we documented are attributable to: 1) the inefficiency of retaining molecules by purification using the Qiagen MinElute® PCR Purification Kit, 2) heat degradation of the DNA molecules, making them unavailable for PCR amplification, or 3) both.

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EXECUTIVE SUMMARY

The analysis of DNA extracted and purified from low copy number (LCN), ancient, and/or degraded source materials is largely complicated by: 1) the presence of contaminating "modern" (Kemp and Smith, 2005; Barta et al., 2014b) and "ancient" (Noonan et al., 2005; Poinar et al., 2006) DNA, 2) co-extracted polymerase chain reaction (PCR) inhibitors (Alaeddini, 2011; Kemp et al., 2014a), and 3) the degree to which template molecules have been damaged or chemically modified post-mortem or from the time of deposition of the biological material (Gilbert, 2006; Alaeddini et al., 2010). As these associated problems make the authentication of DNA profiles from such samples particularly problematic, there is continued need for the development and evaluation of methods that increase the yield and purity of genetic material extracted from degraded sources.

High throughput sequencing (HTS) of DNA [also commonly referred to as next generation sequencing (NGS) or massive parallel sequencing (MPS)] holds promise for the continued study of LCN and degraded DNA samples. While forensic DNA practitioners recognize its utility (e.g., Chang et al., 2009; Butler, 2010; Berglund et al., 2011; Parson et al., 2013; Melton, 2014; Yang et al., 2014), it has already been well established as a reliable approach to the study ancient DNA (aDNA).

However, with short tandem repeats (STRs) as the current gold standard for forensic identification, it has been unclear how soon HTS would become widely used by the forensic DNA community, despite its advantages (Holland et al., 2009; Butler, 2010; Yang et al., 2014). One obvious drawback of "standard" HTS is that it is analogous to screening DNA prior to the invention of PCR, in that, it is not directed at molecules of interest, but rather represents a "shot gun" approach. Thus, standard HTS is not well suited for screening specific markers of interest (i.e., with much coverage at any particular site) or mapping repetitive units (Metzker, 2010), such as those represented by the STRs in the Combined DNA Index System (CODIS) panel. However, forensic researchers have recently demonstrated that, following STR multiplex amplification and indexed library preparation of those amplicons, HTS can be leveraged to produce reliable STR profiles of adequate depth (Zeng et al., 2015). Here coverage (or depth) refers to the number of molecules screened. For example, with high throughput sequencing if a site has two or more times coverage, the site has been observed on two or more independent molecules derived from the biological material from which they have been purified. This is vastly different than screening markers with PCR, which theoretically begins from a pool of molecules intact enough to contain both priming sites. In this case, the copy number available [e.g., as assessed by quantitative PCR (qPCR)] from a sample dictates the "coverage", which is observed downstream as an average in the amplicons sequenced using the Sanger method or typed by other means (e.g., RFLP, length variance).

As another means to leverage the power of HTS, aDNA researchers have developed a variety of "DNA capture" methods that allow for pre-selection of genomic regions of interest, which can be later subjected to HTS (e.g., Briggs et al., 2009; Maricic et al., 2010; Carpenter et al., 2013). This is necessary because typical aDNA libraries consist mostly of DNA exogenous to the sample (i.e., microorganismal DNA) (Noonan et al., 2005; Poinar et al., 2006). DNA capture in its simplest form is known as "fishing" for

DNA (Anderung et al., 2008), but is also routinely referred to as “sample enrichment” or “pull down”. Most DNA capture methods, while variable, follow the same general principles. First, the DNA sample is denatured and target molecules are hybridized to synthetic biotinylated DNA molecules (called “probes” or “bait”). Immobilization of this hybridized complex on streptavidin coated magnetic beads effectively “captures” the targets. A strong magnet is used to attract the beads to the side or bottom of the tube. Buffers are used to wash away impurities and non-target DNA (i.e., genomic regions not of interest from the organism under investigation, as well as any exogenous DNA), and then the beads are released from the magnet and the target DNA disassociated from the probes. In the end, there should be enrichment of target over non-target DNA molecules, and it should also be free of impurities (e.g., PCR inhibitors).

While most DNA capture methods have been developed to enrich from aDNA libraries (i.e., already amplified DNA) that typically contain comparatively little endogenous DNA (Carpenter et al., 2013), it is possible that similar methods could be used to capture target molecules from a sample’s total DNA eluate, to prescreen and enrich for DNA markers of interest prior to PCR amplification or even library builds.

Taking this perspective, it is essential to further consider that when targeting CODIS markers (or any set of markers) by traditional PCR, that these reactions contain far more non-target genomic DNA of the individual than target DNA [not to mention the possible complicating factor of non-target exogenous DNA associated with the sample (Noonan et al., 2005; Poinar et al., 2006)]. For example, the sum of the average amplicon lengths targeted in Promega Male 9948 DNA by the Promega PowerPlex 16® System is 3573 base pairs (bps) (Promega, 2008; Kemp et al., 2014b) (see Chapter 1). Given that the human genome is ~3.2 billion bps in length, only ~1.12×10^{-4}% of it need be present in a DNA extract to generate a complete profile. In other words, the *vast majority* of the genome is of no consequence to generating a full CODIS profile. In general, amplification efficiency of targeted DNA is sub-optimal because non-target DNA can interfere between polymerase and targeted DNA molecules and/or compete as binding sites for primers (Wilson, 1997; Nielsen et al., 2008). If “capture extracted” DNA is efficient, it could possibly lead to more reliable DNA profiling.

The goal of our research was to document, by two measures, the efficiency of three DNA capture methods [“fishing for DNA” (following Anderung et al., 2008), “primer extension capture (or PEC)” (following Briggs et al., 2009), and a method we have come to call “mega-probe capture” (following Maricic et al., 2010)] in their abilities to, first, retain targeted DNA molecules (fragments of DNA that contain the Promega PowerPlex 16® System markers) and, secondly, in their ability to remove non-target DNA molecules [Northern fur seal (*Callorhinus ursinus*) mitochondrial DNA (mtDNA) fragments] from a pool containing both. We specifically chose northern fur seal mtDNA as non-target because in our prior experience it served well as a system that could be reliable quantified with quantitative PCR (qPCR) (Winters et al., 2011; Barta et al., 2013; Barta et al., 2014a; Barta et al., 2014b).

To achieve this goal, efficiencies were estimated following a qPCR approach of comparing numbers of “copies in” of DNA standards to those that are retained by the particular method, or “copies out” (Barta et al., 2014b; Kemp et al., 2014b) (see also Chapter 1). Our approach to this matter is rather unique, in that it allows each method to be evaluated relative to 100% efficiency and in direct relation to one another.

The results of these efforts are summarized below and detailed in the four chapters that follow this executive summary.

Chapter 1: How Much DNA is Lost? Measuring DNA Loss of STR Length Fragments Targeted by the PowerPlex 16® System using the Qiagen MinElute Purification Kit

In this chapter we established our methodology of using qPCR to estimate "copies in" and "copies out", which was a critical step for conducting the experiments reported in the remaining chapters. Moreover, we took advantage of his period of the grant to estimate the degree of DNA loss associated with purification with the Qiagen MinElute® PCR Purification Kit, which was found to be particularly useful in later experiments to account for such loss of DNA. In addition, we designed these experiments to fill in some of the gaps in knowledge that remained from experiments conducted during our last NIJ grant ["NIJ Proposal to Enhance Methods for Studying Degraded DNA" 2008-DN-BX-K008] (Barta et al., 2014b).

The success in recovering genetic profiles from aged and degraded biological samples is diminished by fundamental aspects of DNA extraction, as well as its long-term preservation, that are not well understood. While numerous studies have been conducted to determine whether one extraction method performed superior to others, nearly all of them were initiated with no knowledge of the *actual* starting DNA quantity in the samples prior to extraction, so they ultimately compared the outcome of all methods *relative* to the best (Barta et al., 2014b). Using qPCR to estimate the copy count of synthetic standards before (i.e., "copies in") and after (i.e., "copies out") purification (Barta et al., 2014b) by the Qiagen MinElute® PCR Purification Kit, we documented DNA loss within a pool of 16 different sized fragments ranging from 106-409 base pairs (bps) in length, corresponding to those targeted by the Promega PowerPlex 16® System.

Across all standards starting from 10^4 to 10^7 copies/μL, loss averaged 21.75% to 60.56% (mean 39.03%), which is not congruent with Qiagen's (2008) claim that 80% of DNA fragments 70 bp to 4 kb in length are retained using this product (i.e., a 20% loss). Our study also found no clear relationship between either DNA strand length and retention or starting copy number and retention. This suggests that there is no molecule bias across the MinElute® column membrane and highlights the need for manufacturers to clearly and accurately describe how their claims are made, and should also encourage researchers to document DNA retention efficiencies of their own methods and protocols.

As reported in this chapter, understanding how and where to reduce loss of molecules during extraction and purification will serve to generate clearer and more accurate data, which will enhance the utility of ancient and low copy number DNA as a tool for closing forensic cases or in reconstructing the evolutionary history of humans and other organisms.

Chapter 2: Are We Fishing or Catching? Evaluating the Efficiency of Bait Capture of CODIS Fragments

The experiments reported in this chapter were conducted to document the efficiency of fishing for DNA, a method described by Anderung et al (2008), one that forms the basis for the other two capture methods evaluated in this project.

Following the rationale of the fishing method, we converted Promega PowerPlex 16® System primers into biotinylated probes, and subsequently used to them capture target DNA molecules (see Figure 1 in Chapter 2). Retention of DNA targets ranging 109-288 base pairs (bps) in length was only 9.06-3.53% (i.e., loss of 90.94-96.47%) using the fishing protocol as previously described. Minor improvement was achieved by employing a modified fishing protocol (i.e., one with a shortened hybridization time, use of twice the amount of M-270 streptavidin-coated beads, and modified bead washing), resulting in average retention of 31.41-12.08% of the same set of targeted molecules. Of equal concern was the inability of the fishing method in removing more non-target DNA molecules than targeted molecules. In other words, fishing for DNA simply lowered the concentration of DNA in the extract.

We observed that most (61.35-69.49%) of the molecules are "lost" during the *essential* hybridization step of the fishing protocol. A possible explanation for the loss during hybridization is that heat treatment degrades the DNA molecules, making them non-amplifiable, as they no longer contain both priming regions, a requisite for PCR amplification. This rather novel observation will require follow-up studies, ones that could form the basis for a future project funded by the NIJ.

While the fishing method may be useful in the study of DNA compromised by polymerase chain reaction (PCR) inhibitors (Wang and McCord, 2011), the results presented in this chapter will allow others to carefully weigh this possible advantage against the degree of expected DNA loss and the non-selectivity of the method for targeted over non-targeted DNA.

Chapter 3: Evaluating the Efficiency of Primer Extension Capture as a Method to Enrich DNA Extractions

The experiments reported in this chapter were conducted to document the efficiency of primer extension capture (PEC), a method described by Briggs et al. (2009). The PEC method expands on fishing for DNA with the addition of one cycle of annealing and polymerase extension of the biotinylated probes on the target molecules (see Figure 1 in Chapter 3). Following extension, the biotinylated probes become complementary to the length of the target molecule. Thus, the target DNA should be more tightly bound to the probes, in comparison to fishing for DNA with short probes.

Conducted as previously described, PEC retention of DNA targets ranging 109-288 base pairs (bps) in length was only 15.88-2.14% (i.e., loss of 84.12-97.86%). Experimental modifications of PEC aimed to potentially increase this efficiency were generally of no avail. However, the benefit of PEC, as originally designed, is in its ability to remove most non-target DNA molecules (99.99%). This is precisely why it can be of value in focusing the power of HTS on molecules of interest over non-target regions of the genome and also act to exclude DNA exogenous to the individual under study. Regardless of absolute efficiency, PEC is probably more efficient at removing non-target DNA than fishing because, even if the probes were able to anneal to non-target molecules, mismatches at or near the 3' ends of the primers would result in poor extension of the molecule by the polymerase (Palumbi, 1996). Thus, they would be subsequently more difficult to immobilize.

In this set of experiments we also documented the loss of many (56.69%) of the target molecules *prior* to their immobilization on the streptavidin-coated beads, that is, following

extension and purification of the extended products. This loss can be attributed first to degradation of the molecules caused by heat treatment (i.e., causing them to not be countable during qPCR), which is exacerbated by the loss attributed to purification by the Qiagen MinElute® PCR Purification Kit.

The results presented in this chapter will allow researchers to carefully weigh the general inefficiency of PEC in retaining target molecules over its high efficiency in removing non-target molecules. Moreover, these efficiencies are directly comparable to those observed following fishing for DNA (Chapter 2), or any alternative method for DNA enrichment.

Chapter 4: Evaluating the Efficiency of "Mega-Probe" Capture as a Method to Enrich DNA Extractions

The experiments reported in this chapter were conducted to document the efficiency of a DNA capture method described by Maricic et al (2010). Their method converts long DNA fragments into biotinylated probes that are subsequently used to capture other DNA molecules, those complementary to the long probes (see Figure 1 in Chapter 4). We refer to this approach as "mega-probe" capture because it employs biotinylated probes much longer than those used in both fishing and PEC.

Mega-probe capture increased target molecules from standards by 702.46%, an impossible outcome. From our observations of negative controls, 6217.43, 14555.55, and 36621.00 copies/μL of D16s539, D13s317, and amelogenin bait molecules, respectively, became counted as captured copies when, in fact, there were no copies to capture. Due to unexpected experimental outcomes, we were not able to estimate the efficiency of this method in its removal of non-target molecules. Nevertheless, our principal concern about mega-probe capture is the possibility that the mega-probe bait becomes counted as captured target molecules. Thus, any attempt to measure its efficiency in retaining targeted molecules will be biased.

Additional experiments conducted on DNA retention following hybridization steps of the mega-probe protocol reveal tremendous loss of molecules (up to 83.04%) attributable to both purification by the Qiagen MinElute® PCR Purification Kit and heat exposure. These experiments highlight the need for those working with LCN and degraded DNA samples to be mindful of the loss of already limited DNA strands available for analysis due to the experimental treatments used to study them.

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Chapter 1: How Much DNA is Lost? Measuring DNA Loss of STR Length Fragments Targeted by the PowerPlex 16® System using the Qiagen MinElute® Purification Kit

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ABSTRACT

The success in recovering genetic profiles from aged and degraded biological samples is diminished by fundamental aspects of DNA extraction, as well as its long-term preservation, that are not well understood. While numerous studies have been conducted to determine whether one extraction method performed superior to others, nearly all of them were initiated with no knowledge of the *actual* starting DNA quantity in the samples prior to extraction, so they ultimately compared the outcome of all methods *relative* to the best. Using quantitative PCR (qPCR) to estimate the copy count of synthetic standards before (i.e., "copies in") and after (i.e., "copies out") purification by the Qiagen MinElute PCR Purification Kit, we documented DNA loss within a pool of 16 different sized fragments ranging from 106-409 base pairs (bps) in length, corresponding to those targeted by the Promega PowerPlex 16® System. Across all standards starting from 10^4 to 10^7 copies/μL, loss averaged between 21.75% and 60.56% (mean 39.03%), which is not congruent with Qiagen's claim of 80% retention of DNA fragments 70 bp to 4 kb in length (i.e., 20% loss). Our study also found no clear relationship between either DNA strand length and retention or starting copy number and retention. This suggests that there is no molecule bias across the MinElute column membrane and highlights the need for manufacturers to clearly and accurately describe how their claims are made, and should also encourage researchers to document DNA retention efficiencies of their own methods and protocols. Understanding how and where to reduce loss of molecules during extraction and purification will serve to generate clearer and more accurate data, which will enhance the utility of ancient and low copy number DNA as a tool for closing forensic cases or in reconstructing the evolutionary history of humans and other organisms.

It has been a mere 25 years since the first demonstrations that bones can contain preserved DNA even many hundreds to thousands of years following death of the organism (Hagelberg et al., 1989; Horai et al., 1989; Montiel et al., 2007). Shortly afterwards, forensic DNA researchers demonstrated the usefulness of obtaining genetic profiles from skeletal remains (Hagelberg and Clegg, 1991; Hagelberg et al., 1991; Hochmeister et al., 1991; Stoneking et al., 1991; Jeffreys et al., 1992) and today this type of analysis is indispensable to the field (Edson et al., 2004; Milos et al., 2007; Edson et al., 2009; Mundorff et al., 2009; Caputo et al., 2013; Ambers et al., 2014a; Blau et al., 2014; Mameli et al., 2014). Amazingly, DNA has now been recovered from bones dating from 300,000 to 780,000 years old (Dabney et al., 2013; Meyer et al., 2013; Orlando et al., 2013) and complete genomes are being routinely sequenced from ancient specimens (Green et al., 2010; Rasmussen et al., 2010; Reich et al., 2010; Raghavan et al., 2014; Rasmussen et al., 2014; Rasmussen et al., 2015).

However, the success in recovering genetic profiles from aged and degraded biological samples, including bones, needs to be balanced against the sobering reality that there are still fundamental aspects of long-term DNA preservation that are not well understood, aspects that need to be further approached through experiments with simple and clear methodologies. To illustrate this point, one recent study posed the question "DNA in ancient bone – Where is it located and how should we extract it?" (Campos et al., 2012). This seemingly straightforward question had no easy answer, rather, it raised a high degree of speculation over whether the organic or inorganic portions of the bone are superior in their preservation of DNA. As is often the case, this study raised more issues about the mechanics of DNA preservation than it resolved. In addition, despite years of being convinced that DNA preservation is positively correlated with bone density (Parsons and Weedn, 1997; NIJ, 2005; Prinz et al., 2007), two recent studies clearly bring that relationship into question (Mundorff and Davoren, 2013; Barta et al., 2014a). Even more fundamentally, there is no way to know how much DNA exists in bone samples, given that one can only observe how much is retained following the extraction and purification processes that are known to induce loss (e.g., Barta et al., 2014b). This parallels the questioning by van Oorschot and colleagues (2003) of how much DNA is actually available from touched objects, when resulting extracts do not contain all of the DNA molecules that were originally present on the objects. In fact, this reasoning has led to vast improvements in DNA collection swabs (Marshall et al., 2014).

DNA recovered from aged and degraded biological samples has long been observed to be in low copy number (LCN), and is degraded to short strand lengths (Pääbo et al., 1988; Hagelberg et al., 1989; Pääbo, 1989). This is likewise true for many trace DNA or touch DNA samples (Lowe et al., 2002; Hudlow et al., 2010; van Oorschot et al., 2010). As such, it is hardly surprising that numerous efforts have been directed toward determining the best method of DNA extraction (i.e., to retain the most amount of DNA). Many studies have demonstrated that the performance of one extraction method was superior to others tested for some tissue type(s), ranging in age and state of preservation (Cattaneo et al., 1997; Yang et al., 1998; Hoff-Olsen et al., 1999; Castella et al., 2006; Davoren et al., 2007; Loreille et al., 2007; Rohland and Hofreiter, 2007b; Rohland et al., 2010), or compared retention of DNA from the organic portion of bone with that from the inorganic portion (Schwarz et al., 2009; Campos et al., 2012). Yet, these studies began with no knowledge of the *actual* DNA quantity in the samples prior to extraction, so they ultimately compared the outcome of all methods *relative* to the

best. While this approach can result in the identification of a best method, it cannot determine how well the method performs against 100% recovery.

Manipulation of samples containing DNA will result in DNA loss. This is true regardless of whether this loss is due to not swabbing all DNA present on a touched object in the first place, or losing DNA in any of the many subsequent steps used during extraction and purification. Recently, researchers have begun to address these issues by attempting to measure the degree of DNA loss, relative to a standard, associated with used of various DNA collection swabs (Marshall et al., 2014) and DNA extraction methods (Lee et al., 2010; Dabney et al., 2013; Barta et al., 2014b)

Lee and colleagues (2010) artificially degraded human genomic DNA with DNAse I and diluted this to 25 ng standards. They also created non-degraded standards at the same concentration. Standard concentrations were determined via quantitative PCR (qPCR) using the Quantifiler® Human DNA Quantification Kit (Life Technologies, Carlsbad, CA, USA). These standards were then subjected to three extraction methods utilizing Qiagen (Venlo, Limburg, Netherlands) products: 1) QIAamp Mini Kit, which employs QIAamp Mini Spin Columns, 2) QIAquick PCR purification kit , which employs QIAquick Spin Columns, and 3) QIAamp Mini spin columns combined with reagents from the QIAquick PCR purification kit (Buffers PB and PE). While they found that, on average, the third method performed best (retaining 50.8% and 38.9% of the degraded and intact standards, respectively), there was little difference in comparison to the other two methods. Surprisingly, on average 0.7%-11.9% more of the degraded standards were retained in comparison to the intact ones. While it was not possible to assess degradation in strand length caused by Lee and colleagues' (2010) experimental modification with DNAse I treatment (i.e., the authors did not run the degraded samples on a gel to observe the resulting strand lengths), 25 ng of genomic DNA equates to over 7200 copies of each of the ~3.2 billion nucleotides that the nuclear genome comprises (ignoring the collective nucleotide count per cell that the mitochondrial genomes contain). This is equivalent to the amount of nuclear DNA found in 3600 diploid cells.

Regarding copy number, mitochondrial DNA (mtDNA) retrieved from most ancient samples has typically crossed several orders of magnitude from 10 to 10^2 copies/µL (Malmstrom et al., 2005; Poinar et al., 2006; Malmstrom et al., 2007; Schwarz et al., 2009; Winters et al., 2011) with a few mammoths and dogs at 10^3 copies/µL (Malmstrom et al., 2007; Schwarz et al., 2009) and one unusual dog sample that yielded 10^5 copies/µL (Malmstrom et al., 2005). As expected, ancient nuclear DNA has been observed at hundreds to thousands of times fewer copies/µL compared to mtDNA (Schwarz et al., 2009). While forensic researchers may not require a definition for LCN (Gill and Buckleton, 2010) and the threshold may depend, in part, on the technology used to type sample (Budowle et al., 2009), one such description by the National Forensic Science Technology Center (Largo, FL, USA) DNA Analyst Training manual refers to LCN as "the examination of less than 100 picograms of input DNA", further stating that "assuming 3.5 pg of DNA per haploid cell, [this quantity] is equivalent to approximately 15 diploid or 30 haploid cells" (http://www.nfstc.org). Budowle and colleagues (2009) review evidence that less than 200 pg of input DNA should be considered LCN, importantly recognizing that it is really the stochastic threshold of which one should be mindful.

To estimate the degree of DNA loss, Dabney and colleagues (2013) subjected a standard mixture of five NoLimit DNA fragments (ThermoScientific, Waltham, MA, USA)

[35, 50, 75, 100, and 150 base pairs (bps)] at a concentration of 5.7 ng/μL to the extraction method of Rohland and Hofreiter (2007a) and a modified version of that protocol. The modifications included a change to binding buffer composition, buffer volume, and replacement of loose silica for a fixed silica column (Qiagen MinElute spin column). DNA loss was quantified against the standard using a BioAnalyzer with a 1000 DNA chip (Agilent, Santa Clara, CA, USA). This represents a particularly creative approach, as this method can simultaneously estimate DNA loss across various sized fragments. However, the 1000 DNA chip has a low-end detection of ≥0.5 ng/μL. Evaluated against their standard concoction, this equates to ~5.6 billion total copies/μL (or ~1.12 billion copies/μL of each sized fragment) (see discussion by Barta et al., 2014b). This would make it necessary to evaluate loss of very high copy number standards, as they chose to do so, starting at ~64 billion total copies/μL (or ~12.8 billion copies/μL of each sized fragment). In this case, a loss of ≥99.9999% of the standards employed by Dabney and colleagues (2013) would be required to result in $\leq 10^4$ copies/μL, a range typically observed in aDNA studies and those of LCN forensic samples, making it difficult to assess the applicability of their results in these instances. Nevertheless, Dabney and colleagues (2013) observed that the Rohland and Hofreiter (2007a) method was associated with 72% and 22% retention of 150 bp and 35 bp fragments, respectively. Their modified extraction protocol resulted in the opposite relationship, with ~84% and 95% retention of these fragments, respectively [estimated from Figure 1 of Dabney and colleagues (2013)].

Lastly, Barta and colleagues (2014b) used qPCR to estimate DNA loss of a single sized DNA fragment (181 bps) at concentrations of 10^2 to 10^4 copies/μL (~130-50000 copies/μL) associated with common extraction methods, including phenol:chloroform, alcohol precipitation, microconcentration, and silica-based extractions. They determined that methods which employ numerous steps, for example that of Kemp and colleagues (2007), compound DNA loss, which can result in less than 0.5% retention of the molecules. Simple silica based methods [Wizard® PCR Preps Purification System (Promega, Madison, WI, USA) and QIAquick PCR purification kit] were associated with ~36-39% retention of the 181 bp standard. One drawback of the Barta and colleagues' (2014b) study was that their standard contained DNA fragments of only a single size.

Clearly, the differences among the methodological approaches and results obtained in the three studies just described underscores the need for further research directed at understanding DNA retention efficiencies. Thus, the object of the current study is to document DNA loss within a pool of 16 different sized fragments, corresponding to those targeted by the Promega PowerPlex 16® System. Ultimately, we followed the procedure of Barta and colleagues (2014b) in using qPCR to estimate DNA loss by comparing standards before (i.e., "copies in") and after (i.e., "copies out") purification, in this case with the Qiagen MinElute PCR Purification Kit. This permitted us to evaluate the relationship between DNA strand length and retention, and also that of starting copy number and retention.

MATERIALS AND METHODS

System Choices

The Promega PowerPlex 16® System was chosen to create standards because it targets the thirteen Combined DNA Index System (CODIS) markers, in addition to the amelogenin, Penta D, and Penta E markers. The amplicons produced from the

Promega 9948 Male DNA sample range in size from 106 bps of the Amelogenin gene on the X chromosome to 428 bps from the Penta D locus on chromosome 15 (Table 1). Important to this experimental design is that the PowerPlex 16® System is validated for casework and the primer sequences are published (Table 1) (Masibay et al., 2000; Krenke et al., 2002; Butler et al., 2003), which was essential to constructing the standards, as described below.

Qiagen's (2008: pg 8) claim that MinElute columns retain 80% of fragments ranging from 70 bp to 4 kilobases (kb), and that the membrane was made purposely for elution in volumes as small as 10 µL, makes the MinElute PCR Purification Kit a common choice for use in library purification and/or enrichment protocols for high throughput sequencing (HTS) (e.g., Briggs et al., 2009; Maricic et al., 2010; Carpenter et al., 2013; Enk et al., 2013; Warinner et al., 2014). It is being increasingly used in the purification of DNA from ancient samples (Ginolhac et al., 2012; Carpenter et al., 2013; Dabney et al., 2013; Meiri et al., 2013), suboptimal samples [e.g., bones removed from aged owl pellets (Buś et al., 2014)], and also in forensic studies (Coble et al., 2009; Loreille et al., 2010; Ambers et al., 2014a). The columns have also been employed by forensic DNA researchers in modified extraction methods (Marshall et al., 2014).

The components of the Qiagen MinElute PCR Purification Kit reveal that it is based on binding DNA to a fixed silica column [versus using loose silica, such as in the Promega Wizard® PCR Preps Purification System or the Rohland and Hofreiter (2007a) method], from which DNA is eluted after first washing with alcohol. Based on the Material Safety Data Sheets (MSDS), Buffer PB is a mixture of 25-50% guanidinium chloride (or hydrochloride, GuHCl) and 25-50% isopropanol. According to Qiagen, "Buffer PB contains a high concentration of guanidine hydrochloride and isopropanol. The exact composition of Buffer PB is confidential" and "The composition of Buffer PE is confidential" (www.qiagen.com). OpenWetWare (www.openwetware.org) states that Buffer PB is 5 M GuHCl and 30% isopropanol and Buffer PE is 10 mM Tris-HCl pH 7.5 and 80% ethanol. Buffer EB is 10 mM Tris-Cl, pH 8.5 (www.qiagen.com). In general, most silica-based extraction methods, including the Qiagen MinElute PCR Purification Kit, are minor variants of that described by Boom and colleagues (1990).

For these reasons, we thought the Qiagen MinElute PCR Purification Kit represented a well-used product that would benefit researchers when subjected to tests for DNA loss.

Creating Individual Standards and Calculation of qPCR Efficiency (E) for Individual Markers

Each of the sixteen fragments of the genome targeted by the PowerPlex 16® System were individually amplified eight times in 30 µL PCRs containing: 0.32 mM dNTPs, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.24 µL primers (Table 1), 0.6 U Platinum® Taq DNA Polymerase (Life Technologies), and 1.5 µL of male DNA (Promega 9948). PCR negatives accompanied these reactions to monitor for contamination. Cycling was performed with an initial 3 minute hold at 94°C followed by 40 cycles of 15 second holds at 94°C, 60°C, and 72°C, followed by a 3 minute hold at 72°C. Successful amplification was confirmed by separating 4 µL of PCR product on 2% agarose gels, visualized with ethidium bromide staining under UV illumination.

The remaining volumes of each set of eight reactions were pooled and purified with the Qiagen QIAquick PCR Purification Kit following the manufacturer's protocol except that

the pH indicator was not added and the final elution was conducted with molecular grade water. Following purification of the amplicons, standard concentration was determined by taking the average of 2-3 spectrophotometry readings using a Nanodrop (Thermoscientific), from which copy numbers were calculated as follows:

1. The average weight of a base pair (bp) is 650 Daltons. The molecular weight of the amplicons from each of the 16 pools was estimated by taking the product of 650 and their bp length (Table 1). Where the Promega 9948 Male DNA is heterozygous at 11 of the PowerPlex 16® System markers, we used the mean length of the amplicon sizes. The inverse of the molecular weight is the number of moles of template present in one gram of material.

2. Using Avogadro's number of 6.022×10^{23} molecules/mole, the number of molecules of the template per gram can be calculated as: mol/g * molecules/mol = molecules/g

3. Finally, the number of molecules in the purified pool of PCR products can be estimated by multiplying by 1×10^{-9} (g/ng) to convert to ng and then multiplying by the concentration of the template (ng/µL).

This calculation requires the user to input the concentration of the template present in ng/µL determined by spectrophotometry along with the length of the DNA molecules (in bps), and with this information the number of copies of the template can be calculated using the following:

$$\text{number of copies} = [\text{amount (ng)} * 6.022 \times 10^{23}] / [\text{length (bp)} * 1 \times 10^{9} * 650]$$

Following calculation of the number of copies of each pool of amplicons, each was diluted to a volume containing 1×10^9 copies/µL. From these, a series of ten dilutions at 1×10^5, 1×10^4, 5×10^3, 2×10^3, 1×10^3, 5×10^2, 2.5×10^2, 1×10^2, 5×10^1, and 1×10^1 copies/µL were created.

Quantitative PCRs were performed in an Applied Biosystems 7300 Real Time PCR System (Life Technologies). Twenty five µL qPCR reactions for FGA, D8S1179, vWA, and TPOX contained 0.256 mM dNTPs, 0.96X PCR Buffer, 3 mM $MgCl_2$, 0.2X SYBR® Green, 0.5 mM Rox, 0.4uM of each primer, 0.5 U of Platinum® Taq DNA Polymerase, and 5 µL of standard DNA. For the remaining twelve markers, 25 µL qPCR reactions contained: 12.5 µL SYBR® Green Real-Time PCR Master Mix (Life Technologies), 0.5 mM Rox, 0.4uM of each primer, and 5 µL of standard DNA. Cycling conditions were as follows: 50°C for 2 min, 95°C for 3 min, followed by fifty cycles of 95°C for 15 sec, 60°C for 30 sec, 72°C for 30 sec, and then a dissociation step of 95°C for 15 sec and 60°C for 1 min. The efficiency (E) and coefficient of determination (R^2) were determined from standard curves, created from four to six reactions each from the ten dilutions. Four no template controls (NTCs) accompanied each set of reactions to monitor for the presence of contamination.

Creating Pooled Standards and Calculation of qPCR Efficiency (E) for Individual Markers Within the Pool

The sixteen amplicons were pooled and diluted to 1×10^7, 1×10^5, 1×10^4, 5×10^3, 2×10^3, 1×10^3, 5×10^2, 2.5×10^2, 1×10^2, 5×10^1, 1×10^1 copies/µL. For example, the $1 \times$

10^5 dilution contained 1×10^5 copies of each of the sixteen amplicons per µL (totaling 1.6×10^6 total amplicons per µL). Quantitative PCR was conducted using the SYBR® Green Real-Time PCR Master Mix as described above. The E and R^2 for each of the sixteen reactions were determined from standard curves, created from four to six reactions each from ten dilutions ranging 1×10^5 to 1×10^1 copies/µL. Four no template controls (NTCs) accompanied each set of reactions to monitor for the presence of contamination.

Evaluating DNA Loss Associated with the Qiagen MinElute PCR Purification Kit

DNA loss associated with use of the Qiagen MinElute PCR Purification Kit was estimated from the 1×10^7, 1×10^5, and 1×10^4 pooled standards as follows:

1. A 400 µL aliquot of Buffer PB was added to 80 µL of each pooled standard (i.e., copies in) and mixed by inversion.

2. Each mixture was transferred to a MinElute column that was placed over a 2 mL collection tube. The tubes were centrifuged at 16,100 x g for 1 min in a fixed angle Eppendorf 5415D model centrifuge.

3. Flow-though was discarded and the MinElute columns were placed back into the collection tube.

4. A 750 µL aliquot of Buffer PE was added to each MinElute column and the tubes were centrifuged for 1 minute at 16,100 x g.

5. Flow-though was discarded and the MinElute columns were placed back into the collection tube. The tubes were centrifuged for 1 minute at 16,100 x g.

6. The MinElute columns were placed in clean 1.5 mL tubes to which 80 µL of 10mM Tris-HCl, ph 8.5 was added to the center of the column membrane. This was left at room temperature for 1 minute.

7. The tubes were centrifuged for 1 minute at 16,100 x g.

Extraction negatives were conducted in parallel with the standards to monitor the appearance of any contamination. The final 80 µL volumes contained molecules retained from the extraction process (i.e., copies out). Since the Penta D qPCR assay behaved stochastically and at times very poorly, both when analyzed individually, as well as when pooled (Table 1), this fragment was excluded from further analysis. Quantification of the other 15 markers retained in 1×10^7 extracted standards was conducted in duplicate against standard curves, generated from two reactions each of 1×10^7, 1×10^5, 1×10^4, 5×10^3, 2×10^3, 1×10^3, 5×10^2, 2.5×10^2, and 1×10^2. Two additional 1×10^7 standards were quantified as unknowns in order to monitor the concentration of the pre-extracted standard. Quantification of the molecules that remained in the 1×10^5 and 1×10^4 extracted standards was similarly conducted, but against standard curves generated from 1×10^5, 1×10^4, 5×10^3, 2×10^3, 1×10^3, 5×10^2, 2.5×10^2, and 5×10^1 reactions. Two additional 1×10^5 and 1×10^4 standards were also quantified as unknowns in order to monitor the concentration of these pre-extracted

standards. Quantitative PCR reactions were conducted with SYBR® Green Real-Time PCR Master Mix as described above.

Calculating Efficiency of DNA Retention

Subtraction of the number of "copies out" (measured as the average of duplicate qPCR amplifications from the molecules retained following extraction) from "copies in" (measured as the average of duplicate qPCR amplifications of the standards treated as unknowns) divided by "copies in" multiplied by 100 provides the percent efficiency (or percent retention of molecules of each experimental method: [(copies in - copies out)/ copies in] *100=efficiency). One hundred minus efficiency provides a measure of percent loss. Loss of each of the 15 markers was determined in this manner 3-4 times, from which the average loss and its associated uncorrected standard deviation (i.e., the standard deviation of the sample) were calculated.

Linear regression of percent DNA loss against base pair length and starting copy number was conducted in StatPlus. An alpha value of 0.05 was used as the cut-off for statistical significance.

RESULTS

Individually screened, the qPCR efficiencies for 15 markers ranged from 75.81-104.12% (Table 1). The Penta D qPCRs behaved unpredictably, and at best achieved an efficiency of 68.05%. Screened within a pool, qPCR efficiencies for 14 markers ranged from 86.69-100.81%. While the efficiency of the D21S11 reaction was 64.33%, we proceeded to evaluate loss of these amplicons with the intention of omitting the results if the efficiencies did not improve. The efficiency of Penta E was 84.30%, but due to its large fragment size, this was not considered unacceptable, and the copy numbers relevant to the standards were fairly consistent.

The efficiencies of all subsequent qPCRs used to evaluate DNA loss ranged from 82.27-96.53% and the R^2 values from 0.9918-0.9995 (Appendix A). It is notable that the three D21S11 qPCR reactions had efficiencies of 89.89-91.46%, suggesting that quantifications from these reactions are reliable.

Across the trials, average DNA loss of the 1×10^4 standards ranged from 34.68% (SD 5.2%) to 60.56% (SD 1.84%), the 1×10^5 standards ranged from 34.83% (SD 5.25%) to 54.28% (SD 4.72%), and the 1×10^7 standards ranged from 21.75% (SD 2.7%) to 41.17 (SD 1.86%) (Appendix A at the end of this chapter, Table 2). While the shortest DNA fragment (Amelogenin, average 109 bps) was associated with the greatest percentage loss across all of the standards, the slopes between DNA fragment size (106-409 bps) are no different than zero (i.e., p-values are greater than 0.05) (Figure 1). While there is an inverse relationship between starting copy (10^4, 10^5, 10^7) and average loss (see Table 2, 42.95%, 41.72%, 32.44%), the slope is not different from zero (p=0.063).

DISCUSSION

All steps in the extraction and purification of DNA from biological materials will result in some loss of DNA. While the degree of loss associated with various manipulations is largely unknown, it is important to at least have an estimation, which was the goal of this

study. For example, LCN is an expectation for DNA derived from ancient samples. In fact, this is one of numerous characteristics of aDNA that are used for convincing others of the authenticity of one's results (Cooper and Poinar, 2000; Pääbo et al., 2004). However, the potential degree to which researchers may create the condition, then use that condition to authenticate their results has only recently come to light (Barta et al., 2014b). More important than arguing what are acceptable copy numbers for aDNA samples or how LCN is to be defined, is advocating a wide spread recognition that large numbers of precious DNA copies are inadvertently discarded during the extraction and purification processes. This parallels closely with the message conveyed by van Oorschot and colleagues (2003) following their realization that not all of the DNA present on touched objects is recovered. Given that their observation has lead to an improvement in DNA collection swabs (Marshall et al., 2014), we are optimistic that results from our study and other recent studies about DNA loss (Lee et al., 2010; Dabney et al., 2013; Barta et al., 2014b) will encourage researchers to focus attention on potentially solving, or at least minimizing the problem associated with DNA extraction, as was recently done by Dabney and colleagues (2013). It would be very interesting to see if their modified protocol is also useful in retaining lower copy number standards, since that would, presumably, be a more accurate reflection of starting copy numbers in degraded, ancient and/or LCN samples.

During the present study, individual average loss of amplicons ranged from 21.75% to 60.56% (mean 39.03%), which is lower than that observed (~71-74%) of a single 181 bp standard using the Promega Wizard® PCR Preps Purification System and the QIAquick PCR purification kit (Barta et al., 2014b). This might be the result of carrier effect within a pooled standard of 16 different sized fragments. It could also be a product of using the Qiagen MinElute PCR Purification Kit, which employs a different chemistry compared to the Promega Wizard® PCR Preps Purification System, and uses a modified fixed silica column compared to that employed in the QIAquick PCR purification kit.

Our results are inconsistent with Qiagen's (2008) claim that 80% of DNA fragments ranging 70 bp to 4 kb in length are retained (i.e., only a 20% loss) by the MinElute PCR Purification Kit. This might be a product of our deviations from the published protocol (Qiagen, 2008). First, we did not add pH indicator to the Buffer PB to determine if the mixture of this buffer with our DNA standards resulted in suboptimal pH [i.e., indicated when the Buffer PB (with pH indicator) turns from yellow to orange or violet]. Subsequently, we tested whether mixtures of 1×10^7, 1×10^5, and 1×10^4 pooled standards and the Buffer PB (with pH indicator) resulted in suboptimal pH; they did not. Secondly, we eluted DNA in the final step with 10mM Tris-HCl, pH 8.5 instead of using Buffer EB. Yet, as described in the Qiagen (2008) manual and at their website (www.qiagen.com), Buffer EB is 10mM Tris-HCl, pH 8.5. In either case, we do not feel that these deviations from protocol explain much, if any, of the deviation between Qiagen's claim of 20% loss and our observed loss of 21.75% to 60.56% (mean 39.03%). Since Qiagen (2008) does not report on the variance of their observed loss, it is impossible to know if it overlaps sufficiently with ours to warrant no statistical difference between our observed means and theirs.

As highlighted by Barta and colleagues (2014b), manufacturers' methods that lead to claims of extraction efficiencies are typically not described, which is true for the Qiagen MinElute PCR Purification Kit (Qiagen, 2008). While the Qiagen (2008) manual describes visual estimation of DNA loss of a 5.5 kb fragment on an agarose gel, it is not clear how this relates to their estimation of the efficiency of the MinElute PCR

Purification Kit. It should be the responsibility of manufacturers to clearly and more accurately describe how their observations were made and/or to produce peer reviewed reports that could be scrutinized by members of the scientific community.

On a related note to manufacturers' claims, it is incredible that Dabney and colleagues (2013) were able to retain ~95% of 35 bp fragments (estimated from their Fig 1), given Qiagen's (2008) claims that the Qiagen MinElute PCR Purification Kit will specifically remove fragments ≤40 bps. Further understanding of what specific aspect of their modified protocol led to this unexpected recovery is needed, because at present the cause of this effect is not clear (i.e., buffer composition or volume, or perhaps both modifications are required).

Our experiments to evaluate the relationship between DNA strand length and retention, and also that of starting copy number and retention, revealed no clear linear relationships. With regard to the former, DNA binding efficiency to silica should be unrelated to molecule length (Melzak et al., 1996), yet silica methods are used specifically to remove short fragments of DNA (e.g., Qiagen, 2008) and have been demonstrated empirically to do so, at least with the method of Rohland and Hofreiter (2007a) conducted by Dabney and colleagues (2013). It is interesting that we observed no relationship between starting concentration and DNA loss, which suggests that we did not reach a saturation point of DNA on the silica column. Yet this saturation point does not appear to have been reached even by Dabney and colleagues (2013) with a much higher copy number standard (1×10^{10}). This begs an important question regarding the mechanism of DNA loss that has yet to be addressed—is the DNA not binding efficiently to the silica, or is it not being efficiently released from the silica once it is bound? Additional experiments to resolve this question could lead to some intriguing insights.

Models are, by their nature, inaccurate representations of reality, built to be simple, and to test specific aspects of reality. We are well aware that our "naked" DNA standards mimic *only* the sizes, and possible concentrations, of DNA typically recovered from degraded and ancient samples. It would be ideal to be able to generate synthetic DNA standards that exhibit, for example, a known degree of cytosine deamination and/or crosslinking to other biomolecules (e.g., that which forms Maillard products), and/or are in association with known quantities of PCR inhibitors. While some of these associated variables could lead to better retention of DNA during the extraction and purification processes, the mechanism(s) by which they would work are presently not clear. The experiments of Lee and colleagues (2010) demonstrate that the efficiency of silica based extraction in retaining 50 ng of genomic DNA is largely *unaffected* by the presence of hematin (12-60 nmol) or humic acid (1.5-15.0 µg). However, simply mixing some quantity of DNA with some concentration of PCR inhibitors may also not be a good reflection of reality. For example, even with the potential of losing a tremendous amount of DNA each time they are conducted, repeated silica extractions have proven very useful in the retrieval of DNA from ancient specimens associated with high amounts of PCR inhibitors (Kemp et al., 2006; Grier et al., 2013; Kemp et al., 2014; Moss et al., 2014).

These observations make it obvious to us that reality is more complex than any model being proposed. Others have noted this as well. For example, in recognition that previous assessments of PreCR™ DNA repair treatment were conducted on naked DNA controls, Ambers and colleagues (2014b) sought to determined how well this treatment

would repair experimentally degraded “native” DNA. Interestingly, they first noted that damaging native nuclear DNA was more difficult than damaging naked DNA, presumably because the former is found tightly coiled and complexed to various proteins within the cell nucleus. Secondly, they demonstrated that DNA repair was more efficient on bleach-treated bloodstains in comparison to environmentally exposed bloodstains and bone samples. Following the rationale of Ambers and colleagues (2014b), it is important to draw caution over the experimental results reported here based on naked DNA. It is possible that degraded native DNA will behave drastically different when subjected to the extraction method tested here. However, it is currently impossible to determine how much DNA is present in a degraded bone sample without first extracting and purifying to its naked state. That is, one cannot determine how much DNA is present in a bone sample, only the amount that is retained following extraction and purification (Barta et al., 2014b), which according to our experiments using naked DNA standards could be associated with tremendous loss of genetic material. Moreover, DNA preserved across single elements may be too variable to assume that one piece of a bone will contain equivalent DNA copy number to another piece (Barta et al., 2014a). Future experiments could be designed to investigate the behavior of damaged native DNA across various extraction and purification methods.

Given that there are about as many extraction protocols as there are labs working with aDNA (Anderung et al., 2008), we do not claim to know the best method for reducing loss. We have only tested one such protocol here and previously we evaluated a few others (Barta et al., 2014b). However, researchers using *any method* can adopt the rationale and methodological outline we provide for testing DNA loss against a standard. We strongly encourage others to follow our lead [or similar approach (Lee et al., 2010; Dabney et al., 2013)]. By comparing extraction results against a standard, testing the efficiency of DNA retention of *any and every* method is possible.

Twenty-five years following initial demonstrations that ancient bones contain preserved DNA, even hundreds to thousands of years after death, we are still trying to resolve the many difficulties and unique obstacles generated by the study of degraded and LCN DNA. The power to derive even partial profiles from skeletal elements is indispensable to the forensic sciences and the aDNA field. However, it is advisable to remain humble to the notion that there are still fundamental aspects of DNA preservation and its extraction that are poorly understood. Our study and those of others now collectively suggest that there may be appreciably more DNA preserved in ancient and degraded bone samples, and demonstrate that the mechanisms for retaining DNA in extracts may be highly variable. Any additional amount of DNA that can be retained through the extraction process would only serve to improve the ability to close forensic cases and develop more accurate reconstructions of the evolutionary history of humans and other organisms.

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TABLE 1. Primers used in the PowerPlex 16® System, genotype and ampicon lengths of Promega 9948 Male . Primer sequences taken from Krenke et al. (2002) and Male 9948 genotypes are as reported in the PowerPlex 16® System technical manual, from which amplicon lengths were calculated.

		Promega 9948 Male DNA		Individual qPCR Reactions		Pooled qPCR Reactions	
Locus	**Primer Sequence 5' to 3'**	**Genotype**	**Amplicon Sizes (bps)**	**Efficiency (%)**	**R^2**	**Efficiency (%)**	**R^2**
Amelogenin	CCCTGGGCTCTGTAAAGAA ATCAGAGCTTAAACTGGGAAGCTG	X, Y	106, 112	96.85	0.9991	100.03	0.9982
D3S1358	ACTGCAGTCCAATCTGGGT ATGAAATCAACAGAGGCTTGC	15, 17	127, 135	95.03	0.9981	94.12 95.91§	0.9971 0.9940§
D5S818	GGTGATTTTCCTCTTTGGTATCC AGCCACAGTTTACAACATTTGTATCT	11, 13	135, 143	95.60	0.9981	95.82	0.9924
vWA	GCCCTAGTGGATGATAAGAATAATCAGTATGTG GGACAGATGATAAATACATAGGATGGATGG	17, 17	151	104.12	0.9979	97.13	0.9946
TH01	GTGATTCCCATTGGCCTGTTC ATTCCTGTGGGCTGAAAAGCTC	6, 9.3	164, 184	93.08	0.9967	96.78	0.9921
D13S317	ATTACAGAAGTCTGGGATGTGGAGGA GGCAGCCCAAAAAGACAGA	11, 11	192	92.29	0.9994	100.81	0.9970
D21S11	ATATGTGAGTCAATTCCCCAAG TGTATTAGTCAATGTTCTCCAGAGAC	29, 30	223, 227	92.75 89.41†	0.9966 0.9989†	64.33	0.9974
D8S1179	ATTGCAACTTATATGTATTTTTGTATTTCATG ACCAAATTGTGTTCATGAGTATAGTTTC	12, 13	223, 227	97.74	0.9961	89.96	0.9912
D7S820	ATGTTGGTCAGGCTGACTATG GATTCCACATTTATCCTCATTGAC	11, 11	235	90.66	0.9988	95.72	0.9936
TPOX	GCACAGAACAGGCACTTAGG CGCTCAAACGTGAGGTTG	8, 9	270, 274	98.82	0.9953	95.83 86.69§	0.9921 0.9933§
D16S539	GGGGGTCTAAGAGCTTGTAAAAAG GTTTGTGTGTGCATCTGTAAGCATGTATC	11, 11	288	96.77	0.9989	96.07	0.9982
D18S51	TTCTTGAGCCCAGAAGGTTA ATTCTACCAGCAACAACACAAATAAAC	15, 18	318, 330	91.08 86.79§	0.9929 0.9962§	89.22	0.9949

CSF1PO	CCGGAGGTAAAGGTGTCTTAAAGT ATTTCCTGTGTCAGACCCTGTT	10, 11, 12	337, 341, 345	91.10	0.9992	90.50	0.9968
FGA	GGCTGCAGGGCATAACATTA ATTCTATGACTTTGCGCTTCAGGA	24, 26	354, 362	98.68	0.9965	100	0.9930
Penta E	ATTACCAACATGAAAGGGTACCAATA TGGGTTATTAATTGAGAAAACTCCTTACAATTT	11, 11	409	77.25 87.19§ 75.81†	0.9960 0.9957§ 0.9983†	84.30	0.9947
Penta D	GAAGGTCGAAGCTGAAGTG ATTAGAATTCTTTAATCTGGACACAAG	8, 12	408, 428	614.3 42.00§ 49.74* 68.05†	0.0876 0.9350§ 0.9871* 0.9919†	96.73	0.9872

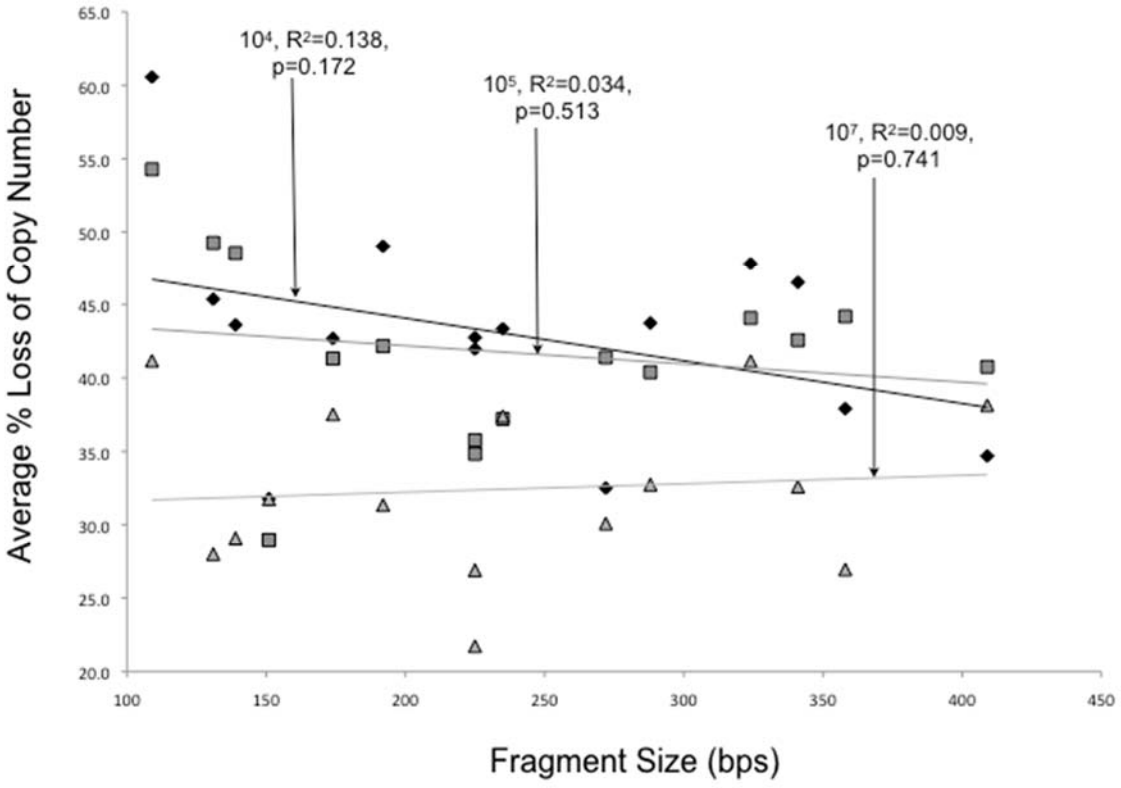
* from a second dilution series created from the original amplification
† from a second set of amplifications and dilution series created from those reactions
§ repeat qPCR from original dilutions

TABLE 2. Summary of percent DNA loss across all fifteen markers evaluated from three standards. See Appendix A for details.

		Percentage loss of 1 × 10^4 standard						Percentage loss of 1 × 10^5 standard						Percentage loss of 1 × 10^7 standard					
Locus	**Size***	**1**	**2**	**3**	**4**	**Mean**	**SD**	**1**	**2**	**3**	**4**	**Mean**	**SD**	**1**	**2**	**3**	**4**	**Mean**	**SD**
Amelogenin	109	60.21	62.99	58.54	60.51	60.56	1.84	59.70	48.25	55.20	53.96	54.28	4.72	43.11	39.39	41.00	n/a	41.17	1.86
D3s1358	131	31.72	44.92	54.04	50.79	45.37	9.85	48.76	47.02	49.78	51.49	49.26	1.87	29.09	27.24	30.39	25.27	28.00	2.23
D5s818	139	25.35	34.74	57.99	56.38	43.61	16.14	42.62	54.18	47.42	50.05	48.57	4.84	31.62	26.96	28.63	29.09	29.07	1.93
vWA	151	25.90	22.99	37.70	40.41	31.75	8.59	27.54	25.64	31.81	30.84	28.96	2.87	41.78	22.79	37.57	24.87	31.75	9.35
TH01	174	43.08	36.30	44.95	46.40	42.68	4.47	46.56	39.27	37.04	42.40	41.32	4.13	46.55	32.89	38.31	32.35	37.53	6.59
D13s317	192	49.52	49.17	51.51	45.93	49.03	2.31	53.00	39.30	37.61	38.76	42.17	7.26	28.40	32.16	33.47	n/a	31.34	2.63
D21s11	225	49.45	45.66	42.97	32.96	42.76	7.05	42.41	33.75	32.80	30.35	34.83	5.25	27.30	25.42	27.96	n/a	26.90	1.32
D8s1179	225	36.06	46.11	40.68	45.02	41.97	4.58	39.83	39.06	35.05	29.11	35.76	4.90	21.15	25.49	21.31	19.03	21.75	2.70
D7s820	235	29.33	48.16	48.95	46.98	43.35	9.39	37.63	35.38	40.91	34.91	37.21	2.74	39.58	36.56	38.34	35.12	37.40	1.96
TPOX	272	34.56	25.32	30.18	39.92	32.49	6.22	50.57	42.70	30.11	42.20	41.39	8.44	34.62	27.24	33.99	24.48	30.08	5.01
D16s539	288	44.96	45.14	41.97	42.89	43.74	1.56	47.26	34.60	39.70	40.00	40.39	5.21	32.17	29.60	36.42	n/a	32.73	3.44
D18s51	324	51.65	56.36	47.62	35.67	47.82	8.85	50.70	50.80	38.09	36.73	44.08	7.72	39.26	40.74	43.44	n/a	41.14	2.12
CSF1PO	341	48.79	49.30	45.38	42.64	46.53	3.12	48.30	47.64	36.59	37.79	42.58	6.25	31.81	32.26	33.72	n/a	32.60	1.00
FGA	358	27.15	33.49	46.60	44.40	37.91	9.18	40.30	53.52	42.36	40.58	44.19	6.28	27.35	27.67	27.01	25.74	26.94	0.85
Penta E	409	33.93	41.76	29.24	33.80	34.68	5.20	41.61	38.89	43.90	38.63	40.76	2.49	36.49	36.60	40.52	38.93	38.13	1.95
				Average loss:		**42.95**	**7.17**			**Average loss:**		**41.72**	**6.23**			**Average loss:**		**32.44**	**8.7**

*Average amplicon size based on genotype of Promega Male 9948 (Table 1)

Figure 1. Regression of average percent DNA loss against DNA fragment size, taken from data presented in Table 2. Black diamonds represent standards at 10^4, dark gray squares represent standards at 10^5, and light gray triangles represent standards at 10^7. R squared and p-values for each slope are indicated.



Appendix A. Quantitative PCR results based on 10^4 standards. These data are summarized in Table 2.

Locus	Copies In	SD	Copies Out	SD	% Efficiency	% Loss	qPCR Efficiency (%)	R squared
Amel	11,407.70	97.40	4,539.61	136.00	39.79	60.21	94.37	0.9958
			4,221.82	273.00	37.01	62.99		
	12,188.30	730.00	5,053.05	100.00	41.46	58.54	90.69	0.9935
			4,812.73	18.90	39.49	60.51		
D3s1358	9,207.84	266.00	6,287.37	10.60	68.28	31.72	92.87	0.9952
			5,071.84	278.00	55.08	44.92		
	13,190.60	163.00	6,062.74	43.70	45.96	54.04	90.75	0.9957
			6,491.43	161.00	49.21	50.79		
D5s818	8,909.34	126.00	6,650.87	258.00	74.65	25.35	91.56	0.9952
			5,813.79	330.00	65.26	34.74		
	12,962.40	320.00	5,445.88	113.00	42.01	57.99	89.79	0.9987
			5,654.46	49.90	43.62	56.38		
vWA	10,188.40	n/a	7,549.30	162.00	74.10	25.90	92.33	0.9921
			7,845.78	n/a	77.01	22.99		
	9,449.84	n/a	5,887.43	9.84	62.30	37.70	89.09	0.9964
			5,631.29	233.00	59.59	40.41		
TH01	12,477.20	73.90	7,102.63	356.00	56.92	43.08	91.93	0.9960
			7,948.24	n/a	63.70	36.30		
	12,116.70	129.00	6,670.38	93.10	55.05	44.95	92.14	0.9972
			6,494.83	212.00	53.60	46.40		
D13s317	9,053.71	n/a	4,570.52	69.00	50.48	49.52	96.53	0.9948
			4,602.02	156.00	50.83	49.17		
	11,017.10	n/a	5,342.73	243.00	48.49	51.51	93.46	0.9924
			5,956.74	163.00	54.07	45.93		
D21s11	8,012.87	n/a	4,050.85	480.00	50.55	49.45	89.89	0.9953
			4,354.53	74.60	54.34	45.66		
	10,259.40	n/a	5,850.77	523.00	57.03	42.97	91.46	0.9928
			6,877.92	139.00	67.04	32.96		
D8s1179	12,932.20	393.00	8,268.43	256.00	63.94	36.06	90.76	0.9919
			6,969.63	291.00	53.89	46.11		
	12,470.60	3,017.41	7,397.35	569.00	59.32	40.68	89.38	0.9926
			6,856.80	222.00	54.98	45.02		
D7s820	10,926.50	n/a	7,722.17	114.00	70.67	29.33	93.68	0.9921
			5,664.74	5.76	51.84	48.16		
	11,924.50	n/a	6,087.71	2.70	51.05	48.95	90.24	0.9928
			6,322.89	95.90	53.02	46.98		
TPOX	10,653.30	1,197.39	6,971.65	415.00	65.44	34.56	92.73	0.9930
			7,956.00	n/a	74.68	25.32		
	10,101.90	686.00	7,052.93	231.00	69.82	30.18	92.05	0.9935
			6,069.62	499.00	60.08	39.92		
D16s539	10,119.70	633.00	5,569.75	162.00	55.04	44.96	91.04	0.9962
			5,551.62	73.40	54.86	45.14		
	12,253.30	99.00	7,110.13	72.20	58.03	41.97	90.62	0.9965
			6,998.46	139.00	57.11	42.89		
D18s51	9,990.00	292.00	4,830.00	212.00	48.35	51.65	82.27	0.9930
			4,360.00	153.00	43.64	56.36		
	9,341.07	2,607.00	4,893.19	198.00	52.38	47.62	84.65	0.9930
			6,008.97	677.00	64.33	35.67		
CSF1PO	11,306.10	70.10	5,789.47	42.10	51.21	48.79	90.56	0.9959
			5,732.42	509.00	50.70	49.30		
	11,921.10	269.00	6,511.68	236.00	54.62	45.38	90.33	0.9925
			6,838.42	70.70	57.36	42.64		
FGA	10,021.00	1,637.93	7,299.89	244.00	72.85	27.15	90.75	0.9957
			6,665.05	271.00	66.51	33.49		
	10,899.80	261.00	5,821.01	1,144.08	53.40	46.60	87.75	0.9954
			6,060.78	49.70	55.60	44.40		
Penta E	10,974.20	n/a	7,250.37	514.00	66.07	33.93	87.18	0.9918
			6,390.89	14.50	58.24	41.76		
	8,940.39	n/a	6,326.48	434.00	70.76	29.24	84.24	0.9984
			5,918.48	527.00	66.20	33.80		

Appendix A (Continued). Quantitative PCR results based on 10^5 standards. . These data are summarized in Table 2.

Locus	Copies In	SD	Copies Out	SD	% Efficiency	% Loss	qPCR Efficiency (%)	R squared
Amel	107,676.00	4,689.83	43,393.00	1,845.41	40.30	59.70	94.37	0.9958
			55,726.30	1,144.58	51.75	48.25		
	102,449.00	303.00	45,893.40	2,675.53	44.80	55.20	90.69	0.9935
			47,169.90	165.00	46.04	53.96		
D3S1358	104,515.00	227.00	53,548.90	1,259.45	51.24	48.76	92.87	0.9952
			55,370.20	97.50	52.98	47.02		
	120,669.00	711.00	60,600.40	518.00	50.22	49.78	90.75	0.9957
			58,533.40	182.00	48.51	51.49		
D5S818	98,494.40	28.20	56,513.70	1,681.48	57.38	42.62	91.56	0.9952
			45,132.10	15,195.50	45.82	54.18		
	106,248.00	640.00	55,869.50	1,042.35	52.58	47.42	89.79	0.9987
			53,071.90	n/a	49.95	50.05		
vWA	101,770.00	1,784.77	73,743.50	405.00	72.46	27.54	92.33	0.9921
			75,671.90	5,895.69	74.36	25.64		
	82,630.90	9,939.58	56,345.90	3,511.98	68.19	31.81	89.09	0.9964
			57,149.60	469.00	69.16	30.84		
TH01	130,294.00	860.00	69,631.80	2,613.91	53.44	46.56	91.93	0.9960
			79,121.50	1,038.34	60.73	39.27		
	106,937.00	8,627.97	67,330.90	2,251.38	62.96	37.04	92.14	0.9972
			61,591.90	7,251.53	57.60	42.40		
D13s317	101,197.00	2,291.97	47,564.70	3,157.44	47.00	53.00	96.53	0.9948
			61,425.40	1,558.34	60.70	39.30		
	103,444.00	2,196.96	64,543.50	2,603.42	62.39	37.61	93.46	0.9924
			63,353.00	1,756.17	61.24	38.76		
D21s11	81,731.50	479.00	47,072.30	1,156.72	57.59	42.41	89.89	0.9953
			54,144.90	511.00	66.25	33.75		
	101,655.00	2,239.30	68,309.70	1,816.18	67.20	32.80	91.46	0.9928
			70,807.10	3,112.21	69.65	30.35		
D8s1179	131,040.00	20,286.40	78,850.40	474.00	60.17	39.83	90.76	0.9919
			79,856.20	8,964.77	60.94	39.06		
	111,082.00	n/a	72,149.60	1,058.41	64.95	35.05	89.38	0.9926
			78,746.00	n/a	70.89	29.11		
D7s820	114,899.00	2,838.81	71,658.90	3,455.92	62.37	37.63	93.68	0.9921
			74,244.00	7,568.27	64.62	35.38		
	114,404.00	1,315.17	67,599.20	3,157.22	59.09	40.91	90.24	0.9928
			74,463.40	1,249.15	65.09	34.91		
TPOX	149,917.00	60,809.90	74,105.30	3,307.90	49.43	50.57	92.73	0.9930
			85,906.20	2,249.70	57.30	42.70		
	105,177.00	4,465.83	73,507.90	2,572.62	69.89	30.11	92.05	0.9935
			60,791.70	n/a	57.80	42.20		
D16s539	106,939.00	16,019.30	56,401.30	4,239.40	52.74	47.26	91.04	0.9962
			69,939.00	687.00	65.40	34.60		
	127,137.00	2,781.69	76,659.40	479.00	60.30	39.70	90.62	0.9965
			76,279.00	847.00	60.00	40.00		
D18s51	93,500.00	6,670.00	46,100.00	797.00	49.30	50.70	82.27	0.9930
			46,000.00	15,700.00	49.20	50.80		
	106,462.00	4,275.45	65,905.70	5,193.11	61.91	38.09	84.65	0.9930
			67,363.80	n/a	63.27	36.73		
CSF1PO	105,457.00	5,625.98	54,524.90	2,181.08	51.70	48.30	90.56	0.9959
			55,221.50	20,935.80	52.36	47.64		
	109,893.00	393.00	69,687.00	222.00	63.41	36.59	90.33	0.9925
			68,367.00	n/a	62.21	37.79		
FGA	114,129.00	3,179.14	68,135.30	495.00	59.70	40.30	90.75	0.9957
			53,051.70	11,296.10	46.48	53.52		
	105,021.00	1,305.38	60,534.70	77.20	57.64	42.36	87.75	0.9954
			62,400.50	n/a	59.42	40.58		
Penta E	126,296.00	253.00	73,748.30	1,376.85	58.39	41.61	87.18	0.9918
			77,182.40	991.00	61.11	38.89		
	98,700.00	4,855.34	55,368.50	5,792.51	56.10	43.90	84.24	0.9984
			60,573.80	346.00	61.37	38.63		

Appendix A (Continued). Quantitative PCR results based on 10^7 standards. . These data are summarized in Table 2.

Locus	Copies In	SD	Copies Out	SD	% Efficiency	% Loss	qPCR Efficiency (%)	R squared
Amel	11,859,700.00	214,118.00	6,747,380.00	362,077.00	56.89	43.11	94.15	0.9983
			7,188,130.00	147,727.00	60.61	39.39		
			6,996,940.00	354,779.00	59.00	41.00		
D3s1358	10,511,400.00	1,131,750.00	7,453,530.00	44,245.10	70.91	29.09	93.46	0.9995
			7,648,330.00	262,303.00	72.76	27.24		
			7,316,650.00	172,932.00	69.61	30.39		
			7,854,990.00	32,772.60	74.73	25.27		
D5s818	11,045,100.00	100,172.00	7,552,780.00	89,505.20	68.38	31.62	91.55	0.9990
			8,067,740.00	88,051.80	73.04	26.96		
			7,882,890.00	80,585.80	71.37	28.63		
			7,831,700.00	270,933.00	70.91	29.09		
vWA	10,986,500.00	692,725.00	6,396,400.00	507,972.00	58.22	41.78	91.96	0.9984
			8,482,530.00	521,759.00	77.21	22.79		
			6,858,930.00	528,361.00	62.43	37.57		
			8,253,770.00	315,314.00	75.13	24.87		
TH01	11,455,800.00	265,142.00	6,122,870.00	330,153.00	53.45	46.55	92.07	0.9995
			7,687,470.00	451,292.00	67.11	32.89		
			7,067,390.00	66,602.70	61.69	38.31		
			7,749,460.00	370,801.00	67.65	32.35		
D13s317	11,228,600.00	608,076.00	8,039,560.00	176,326.00	71.60	28.40	94.53	0.9964
			7,617,780.00	787,037.00	67.84	32.16		
			7,469,860.00	50,788.70	66.53	33.47		
D21s11	11,705,200.00	1,394,300.00	8,509,310.00	629,650.00	72.70	27.30	90.54	0.9954
			8,729,460.00	680,621.00	74.58	25.42		
			8,432,280.00	36,969.70	72.04	27.96		
D8s1179	10,220,200.00	3,629,130.00	8,058,150.00	243,418.00	78.85	21.15	89.47	0.9994
			7,615,040.00	308,690.00	74.51	25.49		
			8,041,960.00	421,844.00	78.69	21.31		
			8,275,160.00	39,615.70	80.97	19.03		
D7s820	12,319,100.00	344,323.00	7,442,620.00	224,313.00	60.42	39.58	90.75	0.9988
			7,815,150.00	138,855.00	63.44	36.56		
			7,596,330.00	62,360.50	61.66	38.34		
			7,992,860.00	347,930.00	64.88	35.12		
TPOX	9,919,070.00	350,929.00	6,485,310.00	114,279.00	65.38	34.62	90.65	0.9992
			7,217,250.00	21,475.90	72.76	27.24		
			6,547,370.00	60,658.80	66.01	33.99		
			7,491,280.00	119,035.00	75.52	24.48		
D16s539	12,663,900.00	317,588.00	8,589,490.00	290,997.00	67.83	32.17	90.89	0.9991
			8,915,300.00	27,290.10	70.40	29.60		
			8,052,100.00	168,036.00	63.58	36.42		
D18s51	11,861,600.00	306,053.00	7,204,480.00	87,174.60	60.74	39.26	83.27	0.9962
			7,029,610.00	119,257.00	59.26	40.74		
			6,709,420.00	23,538.90	56.56	43.44		
CSF1PO	10,844,200.00	421,729.00	7,394,370.00	279,203.00	68.19	31.81	90.36	0.9985
			7,345,780.00	42,382.60	67.74	32.26		
			7,187,670.00	520,241.00	66.28	33.72		
FGA	10,393,300.00	2,010,230.00	7,551,120.00	115,882.00	72.65	27.35	89.07	0.9992
			7,517,140.00	373,381.00	72.33	27.67		
			7,586,300.00	19,905.40	72.99	27.01		
			7,717,930.00	172,097.00	74.26	25.74		
Penta E	12,006,700.00	1,195,770.00	7,625,540.00	250,236.00	63.51	36.49	85.95	0.9983
			7,612,260.00	240,306.00	63.40	36.60		
			7,142,110.00	361,882.00	59.48	40.52		
			7,332,700.00	44,364.20	61.07	38.93		

Chapter 2: Are We Fishing or Catching? Evaluating the Efficiency of Bait Capture of CODIS Fragments

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ABSTRACT

This study sought to document the efficiency of DNA bait capture (i.e., “fishing”) methods by two measures: 1) its ability to retain targeted DNA molecules, and 2) its ability to remove non-target DNA molecules from a pool containing both. Efficiencies were estimated by comparing the number of “copies in” to “copies out” with quantitative polymerase chain reaction (qPCR). Retention of target DNA molecules, ranging 109-288 base pairs (bps) in length, averaged just 9.06-3.53% (i.e., loss of 90.94-96.47%) using the fishing protocol as previously described. Some improvement was achieved by employing a modified protocol (i.e., with a shortened hybridization time, use of twice the amount of M-270 streptavidin-coated beads, and modified bead washing), resulting in average retention of 31.41-12.08% of the same set of targeted molecules. Noted was the lack of efficacy in removing non-target DNA molecules as opposed to targeted molecules. It was also observed that most of the molecules (61.35-69.49%) are “lost” during the *essential* hybridization step of the fishing protocol, suggesting its suitability for high copy number samples only. While the bait capture method may be useful in the study of DNA compromised by polymerase chain reaction (PCR) inhibitors as previously suggested, it is necessary to carefully weigh this possible advantage against the degree of expected DNA loss and the non-selectivity of the method for targeted over non-targeted DNA.

INTRODUCTION

The analysis of DNA extracted and purified from low copy number (LCN), ancient, and/or degraded source materials is largely complicated by: 1) the presence of contaminating "modern" (Kemp and Smith, 2005; Barta et al., 2014) and "ancient" (Noonan et al., 2005; Poinar et al., 2006) DNA, 2) co-extracted polymerase chain reaction (PCR) inhibitors (Alaeddini, 2011; Kemp et al., 2014a), and 3) the degree to which template molecules have been damaged or chemically modified post-mortem or from the time of deposition of the biological material (Gilbert, 2006; Alaeddini et al., 2010). As these associated problems make the authentication of DNA profiles from such samples particularly problematic, there is continued need for the development and evaluation of methods that increase the yield and purity of genetic material extracted from degraded sources.

Anderung et al (2008) developed a method of "fishing" for DNA, building on the "target-hooking" method described by Tofanelli and Nencioni (1999). The method works by denaturing DNA and hybridizing target molecules to synthetic biotinylated DNA primers (i.e., "probes" or "bait") (Figure 1). Immobilization of this hybridized complex onto streptavidin coated magnetic beads effectively "captures" the targets. Application of a strong magnet attracts the DNA-bound beads to the side or bottom of the tube while buffers are used to wash away impurities and non-target DNA. Once washed, the beads are released from the magnet and targeted DNA is disassociated from the probes in preparation for downstream applications. In direct comparison to a more widely-practiced silica-based extraction method (Yang et al., 1998), Anderung et al (2008) found the performance of the fishing method to be at least equivalent in retrieving DNA originating from ancient cattle bones and teeth (ranging in age from the Neolithic to the Mediaeval period). It is notable, however, that for two of twelve samples they analyzed, only the fishing method yielded amplifiable DNA. Anderung et al (2008) argued that fishing for DNA should produce volumes of template DNA that are free of PCR inhibitors. They also suggested, but did not demonstrate, that serial rounds of fishing for DNA could be used to concentrate molecules of interest from a pool of target and non-target molecules. At a minimum, Anderung et al (2008) were the first to demonstrate the successful use of a capture-based method directly on degraded, LCN extracts.

With the goal of disentangling mixtures of human and non-human DNA, Nandineni and Vedanayagam (2009) used a fishing approach to capture Combined DNA Index System (CODIS) short tandem repeats (STRs), plus the D2s1338, D19s433, and Amelogenin markers. In total, these are the markers genotyped by the AmpF*l*STR® Identifiler® PCR Amplification Kit (Life Technologies™). They designed 16 biotinylated primers that probe a few nucleotides upstream from where the proprietary AmpF/STR® primers anneal (Collins et al., 2004), and captured these markers from human DNA that had been sonically fragmented to resemble the state of typically degraded DNA samples. Successful genotyping was achieved for all loci, albeit on an unreported number of samples. Furthermore, Nandineni and Vedanayagam (2009) noted, but did not describe in any detail, that capture was unsuccessfully performed with non-biotinylated primers. Since hybridization to biotinylated probes is required for immobilization (Figure 1), this was an expected experimental result. It is peculiar that Nandineni and Vedanayagam (2009) did not assess the efficiency of capturing human DNA from a pool that contains non-human DNA, as that was one of the stated foci of their study.

Building directly off the study of Nandineni and Vedanayagam (2009), Wang and McCord (2011) demonstrated that allelic drop out could be minimized, and low intensity amplification of large-fragment STRs improved, from experimentally degraded DNA samples after fishing with biotinylated primers. Interestingly, while determining that fishing for DNA would result in the removal of PCR inhibitors, they also found that the presence of hematin at 0.8 mM or humic acid at 0.24 mg/mL (i.e., PCR inhibitors) might actually interfere with the essential hybridization step.

None of the studies reviewed above documented the efficiency of fishing for target DNA or that of non-target DNA removal by measuring against a standard of known concentration. In the case of the Anderung et al (2008) study, comparison to the silica-based method of Yang et al. (1998) is useful, but the comparison is a *relative* one; neither method was tested for its efficiency against a known quantity of DNA. Subsequently, it has been demonstrated that the Qiaquick PCR Purification kit, as employed by Yang et al. (1998), is associated with the loss of 71.25% (SD 30.56%) of a known standard containing naked DNA fragments 181 base pairs (bps) in length (Barta et al., 2014). Yet, measured efficiency against a known quantity of DNA has not been established for the fishing method. Likewise, the efficiency of capturing CODIS markers against a standard of known quantity was not established by either Nandineni and Vedanayagam (2009) or Wang and McCord (2011). While the latter study compared the results relative to genotyping from a pool of degraded, non-captured DNA, this simply demonstrated that the method works, not how efficient it is.

In this study, we sought to document the efficiency of fishing for CODIS STRs sized fragments following the approach of comparing “copies in” to “copies out”, as estimated by quantitative PCR (qPCR) (Barta et al., 2014; Kemp et al., 2014b) (Chapter 1). This general approach allowed us to measure efficiency of capturing target DNA molecules while simultaneously measuring the efficiency of removing non-target DNA molecules from a common pool containing both (Figure 2).

MATERIALS AND METHODS

Creation of the Pooled Standards

The Promega PowerPlex 16® System was chosen to create DNA standards because it targets the thirteen CODIS STRs, in addition to the amelogenin, Penta D, and Penta E markers. The amplicons produced from the Promega 9948 Male DNA sample range in size from 106 bps of the amelogenin gene on the X chromosome to 428 bps from the Penta D locus on chromosome 15 (Kemp et al., 2014b) (Chapter 1). Critical to our experimental design choice were the published PowerPlex 16® System primer sequences and casework validations (Masibay et al., 2000; Krenke et al., 2002; Butler et al., 2003), as knowing the primer sequences was essential for constructing the standards, as described below.

Each of the sixteen fragments of the human genome targeted by PowerPlex 16® System were individually amplified eight times in 30 µL PCRs containing: 0.32 mM dNTPs, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.24 µL primers (described by Krenke et al., 2002), 0.6 U Platinum® Taq DNA Polymerase (Life Technologies), and 1.5 µL of Promega 9948 Male (10 ng/µL) DNA. These were used as target DNA in the capture experiments described below. PCR negatives accompanied these reactions to monitor for contamination.

Cycling was performed with an initial 3 minute hold at 94°C followed by 40 cycles of 15 second holds at 94°C, 60°C, and 72°C, followed by a 3 minute hold at 72°C.

A 181 base pair (bp) portion of the northern fur seal (*Callorhinus ursinus)* mitochondrial cytochrome B gene was PCR amplified eight times using primers NFS-F5-CtB and NFS-R185-CtB (Moss et al., 2006). This was used as non-target DNA in the capture experiments described below. These amplicons will henceforth be referred to as "NFS mtDNA". Each 30 µL PCR contained: 0.32 mM dNTPs, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.24 µM primers, 0.6 U Platinum® Taq DNA Polymerase, and 1.5 µL of northern fur seal DNA. PCR negatives accompanied these reactions to monitor for contamination. Cycling was performed with an initial 3 minute hold at 94°C followed by 40 cycles of 15 second holds at 94°C, 55°C, and 72°C, followed by a 3 minute hold at 72°C.

Successful amplification was confirmed by separating 4 µL of PCR products using 2% agarose gel electrophoresis, and visualizing with ethidium bromide staining under ultraviolet (UV) illumination. The remaining volumes of each set of eight reactions were pooled and purified with the Qiagen QIAquick PCR Purification Kit following the manufacturer's protocol except that the pH indicator was not added and the final elution was conducted with molecular grade water. Following purification of the amplicons, standard concentration was determined by taking the average of 2-3 spectrophotometry readings using a Nanodrop (Thermoscientific), from which copy numbers were calculated as follows (Kemp et al., 2014b) (Chapter 1):

$$\text{number of copies} = ([\text{amount (ng)}] \times [6.022 \times 10^{23}])/ ([\text{length (bp)}] \times [1 \times 10^{9}] \times [650])$$

The seventeen amplicons were pooled and diluted to final concentrations of 1×10^5, 1×10^4, and 1×10^3 copies/µL. For example, the 1×10^5 dilution contained 100,000 copies of each of the sixteen PowerPlex 16® System amplicons and 100,000 copies of northern fur seal mtDNA per µL.

Indirect Fishing Protocol (largely following Anderung et al., 2008) (Figure 1)

This fishing protocol is referred to as "indirect", in contrast to what we describe as the "direct" method below, because this protocol hybridizes the target DNA to the biotinylated primers (through the remainder of this paper, these will be referred to as "probes") and then immobilizes the hybridized product to the streptavidin-coated beads. In contrast, the "direct" method employs immobilization of the probes to the streptavidin-coated beads prior to hybridization of the target DNA (Figure 3).

Sample hybridization

A 50 µL volume of the pooled standard was added to 1000 µL of 1X Binding and Wash (B&W) buffer (5 mM Tris-HCl ph 7.5, 0.5 mM EDTA, 1 M NaCl) and then mixed. Ten µL of PowerPlex 16® System biotinylated probes (at a concentration of 0.005 pmol/µL each) were added to each tube and mixed. Tubes were incubated at 100°C for 15 minutes, immediately placed on ice, transferred to a -20°C freezer for 15 minutes, and incubated at 50°C for 30 minutes. Tubes were then allowed to return to room temperature.

Bead Preparation

Forty microliters of Dynabeads® M-280 Streptavidin-coated beads were prepared by first adding 1000 µL of 1X B&W buffer. This solution was vortexed and placed on the magnet (Dynamag™) for 2 min, followed by discarding the supernatant. An additional 200 µL volume of 1X B&W buffer was added, the tube vortexed, and placed on the magnet for 2 minutes, followed by discarding the supernatant. This was repeated two additional times for a total of three washes with 200 µL of 1X B&W. A final addition of 600 µL of 2X B&W buffer brought the concentration of the beads to 0.067 mg/mL.

Immobilization

A 60 µL volume of the prepared bead solution (containing a total of 0.04 mg beads) was added to the 1060 µL of the hybridized solution, resulting in a solution containing 1X B&W buffer. This results in a 1M NaCl concentration, the optimal binding condition, as described by Invitrogen (2012). This mixture was gently rocked for 30 min at room temperature. The samples were then placed on the magnet for 2 min, followed by discarding the supernatant. The beads were twice washed with 200 µL of 1X B&W buffer as described above, followed by two washes with PCR buffer (10mM Tris-HCl pH 8.0, 50mM KCl). Finally, the immobilized template was re-suspended in 40 µL of dH_2O.

Modified Indirect Fishing Protocol #1 (M-270 beads)

The indirect fishing protocol described above was followed with the substitution of Dynabeads® M-270 streptavidin-coated beads for M-280 beads.

Modified Indirect Fishing Protocol #2 (modified hybridization, 2X M-270 beads, modified bead washing)

The indirect fishing protocol described above was executed with the following modifications: 1) during hybridization, tubes were incubated at 100°C for 15 minutes, immediately placed on ice for 15 minutes, then allowed to return to room temperature (i.e., bypassing the 30 min hold at 50°C), 2) twice the amount of Dynabeads® M-270 streptavidin-coated beads were substituted for M-280 beads, and 3) following immobilization, the beads were thrice washed with 200 µL of 1X B&W buffer, followed by resuspension in 50 µL of dH_2O.

Direct Fishing Protocol (Figure 3)

Bead Preparation and Immobilization of Primers

Eighty microliters of Dynabeads® M-270 Streptavidin-coated beads were prepared as described above for the indirect fishing protocol. The final elution with 600 µL of 2X B&W buffer resulted in the final concentration of beads at 0.134 mg/mL.

To 60 µL of prepared beads was added a 10 µL volume of PowerPlex 16® System biotinylated probes (at a concentration of 0.005 pmol/µL each) and the tubes were gently shaken for 30 min at room temperature.

Sample hybridization

A volume of 50 µL of pooled standard was added to the tubes containing beads and probes. This resulted in 120 µL of bead-immobilized beads in a 1 X B&W buffer. Tubes

were incubated at 100°C for 15 minutes, placed on ice 15 minutes, and allowed to return to room temperature. The tubes were placed on the magnet for 2 min and the supernatant removed. The beads were thrice washed with 200 µL of 1X B&W buffer and finally resuspended in 50 µL of dH_2O.

Hybridization Only (from Indirect Fishing Protocol)

The effect of hybridization alone (following sample hybridization described under the indirect fishing protocol and depicted as steps 1-2 in Figure 1) on the DNA standards was assessed. Since B&W buffer inhibits qPCR, the final products had to be purified prior to quantification (described below).

A volume of 1000 µL of 1X B&W buffer was added to 50 µL of the pooled standard, along with 10 µL of PowerPlex 16® System biotinylated probes (at a concentration of 0.005 pmol/µL each) and subsequently mixed. Tubes were then incubated at 100°C for 15 minutes, immediately placed on ice, and incubated at 50°C for 30 minutes. Tubes were then allowed to return to room temperature.

Aliquots of 132.5 µL of the hybridized solutions were purified using the Qiagen MinElute PCR Purification Kit. The manufacturer's protocol was followed except that 132.5 µL of 10mM Tris-HCl, ph 8.5 was added to the center of the column membrane and left at room temperature for 1 min prior to centrifugation into clean 1.5 mL tubes. As this volume of DNA is 1/8 the initial concentration (i.e., 132.5/1000), final values from qPCR results were corrected by multiplying by eight.

Modified Hybridization Only (from Modified Indirect Fishing Protocol #2)

To 50 µL of the pooled standard was added a 1000 µL volume of 1 X B&W buffer and 10 µL volume of PowerPlex 16® System biotinylated primers (at a concentration of 0.005 pmol/µL each) and the tubes mixed. Tubes were incubated at 100°C for 15 minutes and immediately placed on ice. Tubes were then allowed to return to room temperature. Samples were purified with the Qiagen MinElute PCR Purification Kit and final values adjusted as described above.

Quantitative PCR (qPCR) and Calculating Percent Retention and Loss

The amount of amelogenin, D13s317, D16s539 DNA remaining in the standard following capture with the various fishing protocols described above or following speficial subjection to the hybridization steps (i.e., "copies out") was determined by qPCR following Kemp et al. (2014b) (Chapter 1). Quantitative PCR reactions of 25 µL contained: 12.5 µL SYBR® Green Real-Time PCR Master Mix (Life Technologies), 0.5 mM Rox, 0.4 µM of each primer, and 5 µL of template DNA. Cycling conditions were as follows: 50°C for 2 min, 95°C for 3 min, followed by 50 cycles of holds at 95°C for 15 sec, 60°C for 30 sec, 72°C for 30 sec, and then a dissociation step of 95°C for 15 sec and 60°C for 1 min.

These three markers were chosen as representatives of the 16 fragments targeted during hybridization. The average Amelogenin, D13s317, D16s539 fragment lengths are 109, 192, and 288 bps. Moreover, the efficiencies (E, 96.07-100.81%) and coefficient of determination (R^2, 0.9970-0.9982) of these reactions were previously determined to be acceptable when screened in a pool (Kemp et al., 2014b) (Chapter 1).

The amount of NFS mtDNA remaining in the standards was also determined via qPCR. This represents non-target DNA retained following subjection to capture or hybridization only. Quantitative PCR reactions were conducted as described above.

Quantification of each marker was conducted in duplicate against standard curves generated from two reactions each of standard pools at 1×10^5, 1×10^4, 5×10^3, 2×10^3, 1×10^3, 5×10^2, 2.5×10^2, 1×10^2 and 50 copies/μL. Two additional wells of each untreated 1×10^5, 1×10^4, and 1×10^3 copies/μL standards were quantified as unknowns. These provided estimates of "copies in".

Subtraction of the number of "copies out" (measured as the average of duplicate qPCR amplifications) from "copies in" (measured as the average of duplicate qPCR amplifications of the standards treated as unknowns) divided by "copies in" multiplied by 100 provides the percent retention:

[(copies in - copies out)/ copies in] × 100

One hundred minus retention provides a measure of percent loss. Retention and loss of targeted amelogenin, D13s317, D16s539, and non-targeted NFS mtDNA was determined in this manner three times, from which the average loss and the uncorrected sample standard deviation (i.e., the standard deviation of the sample) were calculated.

Statistical Analyses

Two-tailed t-tests were used to statistically evaluate differences in mean percentage retention at the 0.05 level of probability. Statistical analyses were conducted in StatPlus (AnalystSoft Inc.).

RESULTS

Quantitative PCR efficiencies for amelogenin, D13s317, and D16s539 ranged from 91.33-98.91%, with R^2 values ranging 0.9983-0.9996 (Tables 1-6), which are comparable values to those previously observed (Kemp et al., 2014b) (Chapter 1). The NSF mtDNA qPCR efficiencies ranged from 90.20-92.96% with R^2 values from 0.9963-0.9994.

Across all three standards (1×10^5, 1×10^4, and 1×10^3 copies/μL), average DNA retention of the targeted amelogenin, D13s317, and D16s539 by the indirect fishing method ranged from 9.06-3.53% (i.e., loss of 90.94-96.47%; see Table 1). The retention of the non-targeted NFS mtDNA was similar at 4.02-6.64% (two-tailed t-test, p=0.633). Substitution of M-270 streptavidin-coated beads for M-280 beads resulted in an average retention of 13.33-7.82% for amelogenin, D13s317, D16s539, and 17.43-10.10% for NFS mtDNA (Table 2). The mean difference between percent retention of the targeted markers was not statistically different from that of the non-targeted NSF mtDNA (p=0.102). The second modified indirect fishing protocol (i.e., modified hybridization, 2X M-270 beads, modified bead washing) resulted in average retention of 31.41-12.08% for amelogenin, D13s317, D16s539, which was not statistically different from the 24.65-22.07% retention of the NFS mtDNA (p=0.623) (Table 3). The average retention for the three targeted markers by the direct fishing method (0.16-0.02%) was not statistically

different from that observed for the non-targeted NFS mtDNA (0.25-0.12%, p=0.129) (Table 4).

The mean retention of targeted molecules by the second modified indirect method (21.70%,Table 3) was larger than that of the means of the indirect method (5.79%, $p=2.4 \times 10^{-7}$, Table 1), the first modified indirect method (11.37%, $p=5.3 \times 10^{-5}$, Table 2), and the direct method (0.10%, $p=1.7 \times 10^{-9}$, Table 3).

Standards subjected to only the hybridization step from the indirect method witnessed losses ranging from 75.52-90.07% (Table 5). Standards subjected only to the hybridization step from the second modified indirect fishing protocol resulted in a loss of 81.01-93.08% (Table 6). The average loss of the latter (87.36%) exceeds that of the former (83.15%) (p=0.032).

DISCUSSION

In this study we sought to document the efficiency of fishing for DNA two measures, first in its ability to retain targeted DNA molecules and, secondly, in its ability to remove non-target DNA molecules from a pool containing both. Following the indirect protocol of Anderung et al (2008) results in losses exceeding 90% across the three targeted markers (amelogenin, D13s317, and D16s539) (Table 1). Using the direct fishing method, the average loss was further exacerbated (Table 4). The average loss for direct fishing in comparison to the indirect fishing method was reduced with the substitution of M-270 streptavidin-coated beads for M-280 beads (Table 2). The difference between beads is that the latter are hydrophilic, whereas the former are hydrophobic (Invitrogen, 2010). Further manipulation of the indirect fishing method, including a reduced time of hybridization, use of twice the amount of M-270 beads, and modified washing of the beads, resulted in the best retention values of target molecules at >20% (Table 3).

While it is likely that further manipulation of the basic indirect fishing protocol could lead to increased efficiency of retaining targeted molecules, one must still contend with the fact that the method binds both target and non-target DNA rather than selectively hybridizing only to the target DNA molecules. Across all experimental trials, the amount of the non-targeted NFS mtDNA retained was statistically indistinguishable from the amount of targeted DNA retained (Tables 1-4). These observations clearly show that fishing for DNA is inefficient for both metrics considered in this study.

Our unique approach to quantifying efficiency by comparing “copies in” to “copies out” estimated from quantitative PCR (Barta et al., 2014; Kemp et al., 2014b) (Chapter 1) permitted us to begin assessing where DNA is lost in the fishing protocol. Specifically, by observing the effects of the hybridization conditions alone on the DNA standards, we noticed that over 80% of the molecules are lost in this step (Tables 5 and 6). However, meaningful correction can be made to that estimate. In pursuit of estimating DNA loss during hybridization, it was necessary to purify the DNA prior to quantification because the B&W Buffer inhibits PCR. Since estimates of percentage loss of Promega PowerPlex 16® System amplicons by Qiagen MinElute PCR Purification Kit purification has been established (Kemp et al., 2014b) (Chapter 1), a correction factor for loss of amelogenin, D13s317, and D16s539 of 52%, 40.85%, 38.95% (average loss across pooled standards starting at 1×10^7, 1×10^5, and 1×10^4 copies/µL) can be employed. Once corrected, the values still demonstrate that over 60% of the molecules are lost

during hybridization (see last column in Tables 5 and 6 for corrected values). Similar loss of the NFS mtDNA has not been documented with the Qiagen MinElute PCR Purification Kit. A possible explanation for the loss following hybridization is that the heat treatment degrades the DNA molecules, making them non amplifiable as they no longer contain both priming regions, a requisite for PCR amplification. The rate of DNA strand breakage is related directly to temperature (Lindahl and Andersson, 1972; Lindahl, 1993) and it has been demonstrated that the "thermal age" of an ancient specimen is more meaningful in predicting DNA recovery than is calendrical age (Smith et al., 2003). Worthy of consideration is that in order to create degraded DNA conditions in their study of fishing for DNA, Wang and McCord (2011) exposed full genomic DNA in deionized water to 95°C temperature for 10 minutes. This implies that genomic DNA will readily degrade at high temperature in a short time. Admittedly, our results showing that hybridization conducted at 100°C for 15 minutes followed by 50°C for 30 minutes results in less DNA loss (Table 5) than simply heating at 100°C for 15 minutes (Table 6) is difficult to reconcile, given that DNA in the first treatment was exposed to heat for a greater period of time. Nevertheless, the magnitude of DNA loss observed in this study is particularly troublesome, as hybridization of targeted molecules to the biotinylated probes is *essential* to the fishing method. Even when considering this loss, one can conclude that, of the intact markers that are present following hybridization, only about half of them are retained during the best of our fishing methods. For example, if 65% of the molecules are lost during hybridization (estimated from Tables 5 and 6), of the 35% remaining, approximately 60% of them are retained (considering the observed endpoint average of ~21% retention, Table 3).

If the impact of this heat treatment damage is verified by future studies, it has implications for improving approaches for working with degraded DNA samples. For example, polymerases that required extended hot starts (e.g., a 12 minute activation period at 95ºC for Amplitaq® Gold) might be less preferable to those that require shorter periods of hot start (e.g., 94ºC for three minutes to activate Platinum Taq). In fact, an informative experiment would be to compare PCR yields wherein DNA is added to the reactions following hot start to those wherein the DNA is added prior to the hot start activation. If our presumptions are correct, former will yield less DNA than the latter based on less template molecules available for amplification.

It is worth emphasizing that the outcome of the experiments described here were not compared with standard methods of DNA extraction and genotyping, which would have pointed to the strength and weakness of fishing for DNA over standard methods. Nevertheless, as detailed in the introduction, our study is the first to evaluate the efficiency of fishing for DNA against 100% recovery of molecules sized exactly for amplification with the Promega PowerPlex 16® System. A possible future direction would be to experimentally degrade native DNA (e.g., Ambers et al., 2014) and evaluate the efficiency of fishing methods for capture of target molecules from total genomic DNA that is degraded and chemically modified.

Another variable that we did not consider, but one that could form the basis for an informative follow-up study, is the hybridization conditions employed in our study. Hybridization temperatures that are too stringent (i.e., too high) would decrease the efficiency of targeted bait molecules to be captured. Alternatively, hybridization temperatures that are less stringent (i.e., too low) would permit for more non-target molecules to be captured. Exploration of variable hybridization temperatures and possibly even a touch-down hybridization step would be particularly illuminating.

Fishing for DNA may still be a useful method when there is a need for purifying high copy number DNA associated with PCR inhibitors (Wang and McCord, 2011), however far less expensive and time consuming methods suitable for high and low copy samples have been described (Kemp et al., 2006; Kemp et al., 2014a). Therefore, the possible advantage of PCR inhibitor removal by fishing must be carefully weighed against the degree of expected DNA loss when targets are not caught, as well as the non-selectivity of the method for targeted over non-targeted DNA.

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Table 1. Retention of Amelogenin, D13s317, D16s539, and northern fur seal mitochondrial DNA (NSF mtDNA) molecules using the Indirect Fishing Protocol

					Hybridization 1		Hybridization 2		Hybridization 3		Average			
		R^2	Copies In	SD	Copies Out	SD	Copies Out	SD	Copies Out	SD	Copies Out	SD	% Retention	% Loss
Amelogenin	98.91	0.9991	129978.00	9748.87	5696.24	810.06	5726.00	1237.82	8087.27	1129.38	6503.17	1371.95	5.00	95.00
			12091.30	571.00	645.58	190.97	730.96	177.38	863.50	42.13	746.68	109.81	6.18	93.82
			996.02	149.00	55.82	3.54	51.57	n/a	36.43	2.06	47.94	10.19	4.81	95.19
D13s317	93.78	0.9987	83525.20	9335.61	6999.06	n/a	7203.15	279.10	8501.16	1055.72	7567.79	814.74	9.06	90.94
			9194.53	368.00	558.34	58.04	628.98	33.17	659.53	51.83	615.62	51.90	6.70	93.30
			760.69	36.90	76.58	17.53	44.43	6.20	39.41	7.28	53.47	20.17	7.03	92.97
D16s539	96.68	0.9996	134781.00	2328.16	4748.40	825.81	5888.48	324.18	6231.40	196.67	5622.76	776.39	4.17	95.83
			11599.60	855.00	440.30	29.62	740.86	76.29	777.91	53.10	653.02	185.15	5.63	94.37
			1057.39	139.00	64.15	0.75	33.60	0.16	14.13	1.29	37.29	25.21	3.53	96.47
												Average Targeted DNA	5.79	94.21
NFS mtDNA	92.96	0.9975	91048.80	12218.40	5878.23	n/a	5830.70	739.43	6435.91	1018.81	6048.28	336.54	6.64	93.36
			10348.20	59.10	489.62	3.88	412.84	19.37	344.11	11.98	415.52	72.79	4.02	95.98
			811.66	2.82	50.10	16.64	50.82	9.40	23.79	0.06	41.57	15.40	5.12	94.88
												Average Non-Targeted DNA	5.26	94.74

Table 2. Retention of Amelogenin, D13s317, D16s539, and northern fur seal mitochondrial DNA (NSF mtDNA) molecules using the Modified Indirect Fishing Protocol #1 (M-270 beads)

					Hybridization 1		Hybridization 2		Hybridization 3		Average			
	E	R^2	Copies In	SD	Copies Out	SD	Copies Out	SD	Copies Out	SD	Copies Out	SD	% Retention	% Loss
Amelogenin	96.44	0.9993	106588.00	4247.28	8862.16	641.49	6098.58	438.77	10045.44	724.19	8335.39	2025.47	7.82	92.18
			10237.80	n/a	1275.28	117.86	1014.10	39.63	1204.60	326.14	1164.66	135.09	11.38	88.62
			1025.67	19.00	142.47	11.73	115.78	0.58	83.60	0.67	113.95	29.48	11.11	88.89
D13s317	94.56	0.9983	77891.20	n/a	7870.15	30.52	6300.68	567.26	9447.96	297.27	7872.93	1573.64	10.11	89.89
			9111.40	n/a	1100.10	327.54	1056.42	n/a	1149.91	n/a	1102.15	46.78	12.10	87.90
			852.42	8.51	107.06	24.56	122.81	14.59	79.29	19.14	103.05	22.03	12.09	87.91
D16s539	96.12	0.9995	120297.00	3919.65	14000.16	1223.01	8679.44	392.36	13013.40	249.07	11897.67	2830.40	9.89	90.11
			11841.90	76.10	1871.50	97.13	1219.60	45.35	1643.34	1.02	1578.15	330.80	13.33	86.67
			1131.67	56.10	172.27	9.78	196.11	7.80	124.13	1.52	164.17	36.66	14.51	85.49
Average Targeted DNA													11.37	88.63
NFS mtDNA	92.41	0.9969	81232.10	n/a	5730.84	78.66	8416.19	735.35	10454.52	780.82	8200.52	2369.21	10.10	89.90
			7764.42	731.00	1304.40	211.85	1224.56	n/a	1531.58	n/a	1353.51	159.29	17.43	82.57
			905.77	5.11	187.49	n/a	144.34	2.08	85.86	23.86	139.23	51.01	15.37	84.63
Average Non-Targeted DNA													14.30	85.70

Table 3. Retention of Amelogenin, D13s317, D16s539, and northern fur seal mitochondrial DNA (NSF mtDNA) molecules using the Modified Indirect Fishing Protocol #2 (modified hybridization, 2X M-270 beads, modified bead washing)

					Hybridization 1		Hybridization 2		Hybridization 3		Average			
	E	R^2	Copies In	SD	Copies Out	SD	Copies Out	SD	Copies Out	SD	Copies Out	SD	% Retention	% Loss
Amelogenin	95.75	0.9994	115459.00	1497.85	17120.90	876.00	23768.20	4069.11	19377.40	1992.22	20088.83	3380.27	17.40	82.60
			10080.00	909.00	2889.33	457.00	1470.43	556.00	1967.21	191.00	2108.99	720.00	20.92	79.08
			1077.60	4.19	190.12	13.50	292.50	22.50	305.62	13.40	262.74	63.24	24.38	75.62
D13s317	95.89	0.9983	78569.90	n/a	16636.10	1785.21	19084.40	1619.12	21092.00	n/a	18937.50	2231.58	24.10	75.90
			8877.80	691.00	1856.82	427.00	1849.81	n/a	1449.64	131.00	1718.76	233.09	19.36	80.64
			876.08	24.40	228.69	8.80	271.11	51.10	325.75	47.80	275.18	48.66	31.41	68.59
D16s539	96.41	0.9993	129002.00	2567.76	13249.20	2868.09	17747.30	669.00	15741.60	4450.15	15579.37	2253.43	12.08	87.92
			11557.90	620.00	2588.96	1321.11	2432.83	551.00	2717.58	850.00	2579.79	142.60	22.32	77.68
			1232.71	39.70	231.26	49.80	292.16	65.20	339.60	145.00	287.67	54.31	23.34	76.66
Average Targeted DNA													21.70	78.30
NFS mtDNA	92.68	0.9982	79168.20	9571.68	14529.50	2191.49	17877.50	6353.05	20538.80	4448.40	17648.60	3011.18	22.29	77.71
			8902.93	1319.44	2548.53	1534.00	1526.77	240.00	1820.20	n/a	1965.17	526.08	22.07	77.93
			941.08	6.35	224.65	14.30	235.59	15.00	235.71	43.20	231.98	6.35	24.65	75.35
Average Non-Targeted DNA													23.01	76.99

Table 4. Retention of Amelogenin, D13s317, D16s539, and northern fur seal mitochondrial DNA (NSF mtDNA) molecules using the Direct Fishing Protocol

					Hybridization 1		Hybridization 2		Hybridization 3		Average			
	E	R^2	Copies In	SD	Copies Out	SD	Copies Out	SD	Copies Out	SD	Copies Out	SD	% Retention	% Loss
Amelogenin	94.43	0.9983	106797.00	929.00	20.33	1.84	25.56	0.59	73.39	4.05	39.76	29.24	0.04	99.96
			10847.50	316.00	2.51	0.09	1.18	0.39	2.11	0.58	1.93	0.68	0.02	99.98
			954.01	33.90	0.25	0.00	0.09	0.04	0.16	0.02	0.17	0.08	0.02	99.98
D13s317	91.33	0.9983	88762.00	5997.40	86.03	3.63	137.90	5.92	246.48	n/a	156.80	81.88	0.18	99.82
			8814.22	145.00	17.33	n/a	5.24	n/a	10.70	n/a	11.09	6.05	0.13	99.87
			865.28	24.30	0.67	0.01	1.42	0.94	1.61	0.29	1.23	0.50	0.14	99.86
D16s539	95.15	0.9984	124962.00	5160.58	164.32	0.94	253.47	0.15	170.51	19.30	196.10	49.78	0.16	99.84
			12957.70	59.10	12.19	0.50	8.04	0.51	22.24	0.95	14.16	7.30	0.11	99.89
			1096.78	57.30	1.33	0.37	0.90	0.42	1.88	0.01	1.37	0.49	0.13	99.87
Average Targeted DNA													0.10	94.21
NFS mtDNA	90.20	0.9994	86532.50	1260.36	88.64	1.09	131.21	11.40	148.18	2.55	122.68	30.67	0.14	99.86
			8683.78	109.00	7.73	0.71	7.69	n/a	16.06	2.22	10.49	4.82	0.12	99.88
			750.00	30.40	0.82	n/a	0.64	0.05	4.09	4.18	1.85	1.94	0.25	99.75
Average Non-Targeted DNA													0.17	94.74

Table 5. Loss of Amelogenin, D13s317, D16s539, and northern fur seal mitochondrial DNA (NSF mtDNA) molecules attributable to the Hybridization Only from Indirect Fishing Method

					Hybridization 1		Hybridization 2		Average		%	
	E	R^2	Copies In	SD	Copies Out	SD	Copies Out	SD	Copies Out	SD	Loss	% Loss (corrected)
Amelogenin	97.51	0.9992	122537.00	1599.63	10034.12	233.91	6920.24	437.29	8477.18	2201.84	89.38	79.58
			11140.70	222.00	1248.69	26.07	706.03	22.15	977.36	383.72	90.34	81.41
			1065.88	41.30	90.73	8.33	128.73	8.72	109.73	26.87	90.07	80.91
D13s317	94.14	0.9987	79142.40	n/a	13692.40	1422.70	10753.80	999.06	12223.10	2077.90	75.52	40.08
			8685.10	748.00	2091.01	45.23	1208.34	59.67	1649.67	624.14	78.30	46.88
			915.41	3.75	127.64	6.29	148.20	26.85	137.92	14.54	77.87	45.83
D16s539	95.02	0.9991	136766.00	3856.13	16992.44	1271.72	15931.84	307.05	16462.14	749.96	84.03	58.99
			11638.90	78.60	2334.66	64.39	1207.10	131.75	1770.88	797.30	84.93	61.31
			1281.71	5.06	149.02	12.12	184.80	9.07	166.91	25.29	83.31	57.16
NFS mtDNA	90.61	0.9964	102430.00	n/a	11319.88	1474.69	9368.60	3728.48	10344.24	1379.76	74.90	n/a
			10111.60	1353.64	1735.15	162.87	944.52	3.69	1339.83	559.06	84.53	n/a
			1046.37	126.00	116.66	8.68	146.43	43.44	131.55	21.05	84.56	n/a
										Average	83.15	61.35

Table 6. Loss of Amelogenin, D13s317, D16s539, and northern fur seal mitochondrial DNA (NSF mtDNA) molecules attributable to the Hybridization Only from Modified Indirect Fishing Protocol #2

					Hybridization 1		Hybridization 2		Average			
	E	R^2	Copies In	SD	Copies Out	SD	Copies Out	SD	Copies Out	SD	% Loss	% Loss (corrected)
Amelogenin	97.51	0.9992	122537.00	1599.63	10034.12	233.91	6920.24	437.29	8477.18	2201.84	93.08	86.70
			11140.70	222.00	1248.69	26.07	706.03	22.15	977.36	383.72	91.23	83.13
			1065.88	41.30	90.73	8.33	128.73	8.72	109.73	26.87	89.71	80.20
D13s317	94.14	0.9987	79142.40	n/a	13692.40	1422.70	10753.80	999.06	12223.10	2077.90	84.56	62.19
			8685.10	748.00	2091.01	45.23	1208.34	59.67	1649.67	624.14	81.01	53.50
			915.41	3.75	127.64	6.29	148.20	26.85	137.92	14.54	84.93	63.12
D16s539	95.02	0.9991	136766.00	3856.13	16992.44	1271.72	15931.84	307.05	16462.14	749.96	87.96	69.10
			11638.90	78.60	2334.66	64.39	1207.10	131.75	1770.88	797.30	84.78	60.94
			1281.71	5.06	149.02	12.12	184.80	9.07	166.91	25.29	86.98	66.57
NFS mtDNA	90.61	0.9964	102430.00	n/a	11319.88	1474.69	9368.60	3728.48	10344.24	1379.76	89.90	n/a
			10111.60	1353.64	1735.15	162.87	944.52	3.69	1339.83	559.06	86.75	n/a
			1046.37	126.00	116.66	8.68	146.43	43.44	131.55	21.05	87.43	n/a
										Average	87.36	69.49

Figure 1. Depiction of the Indirect Fishing Protocol (largely following Anderung et al (2008). Target molecules are green (in this study, representing the 16 fragments targeted by PowerPlex 16® System), non-target molecules are red (in this study, representing NFS mtDNA), biotinylated probes are blue, and the streptavidin coated magnetic beads are white.

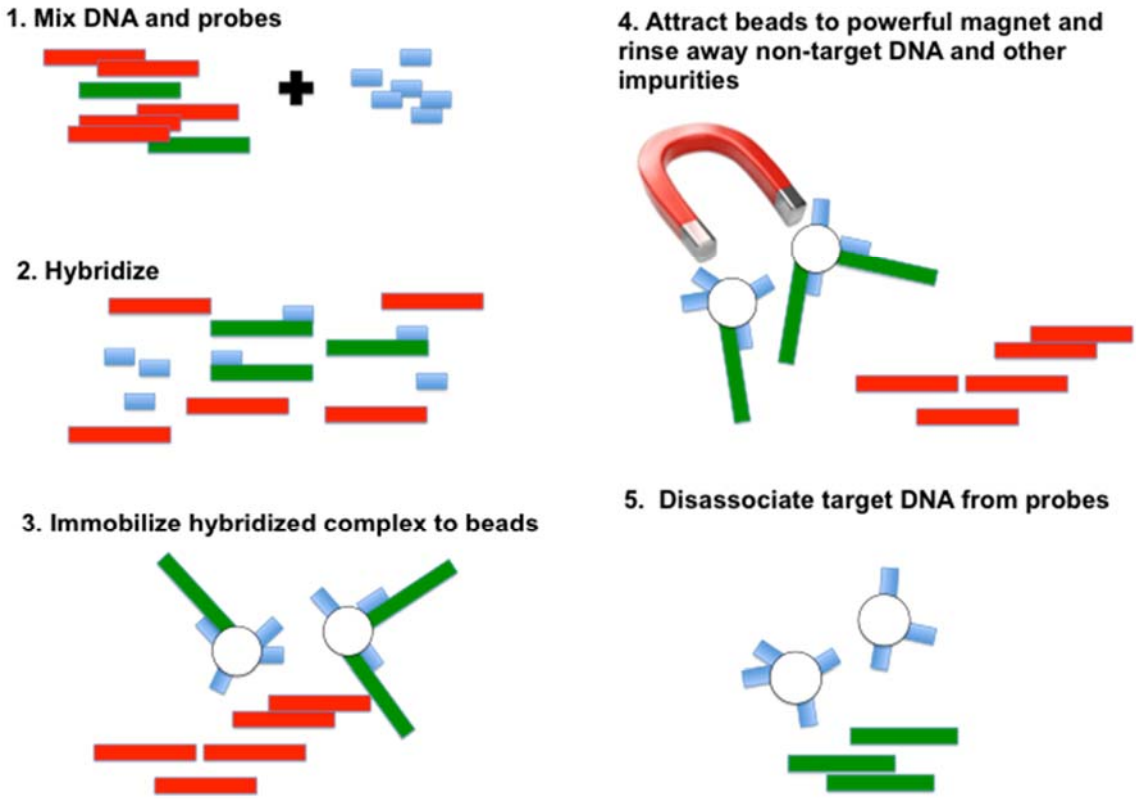


Figure 2. Schematic of methods used to quantify percent retention and loss of DNA standards subject to fishing protocol.

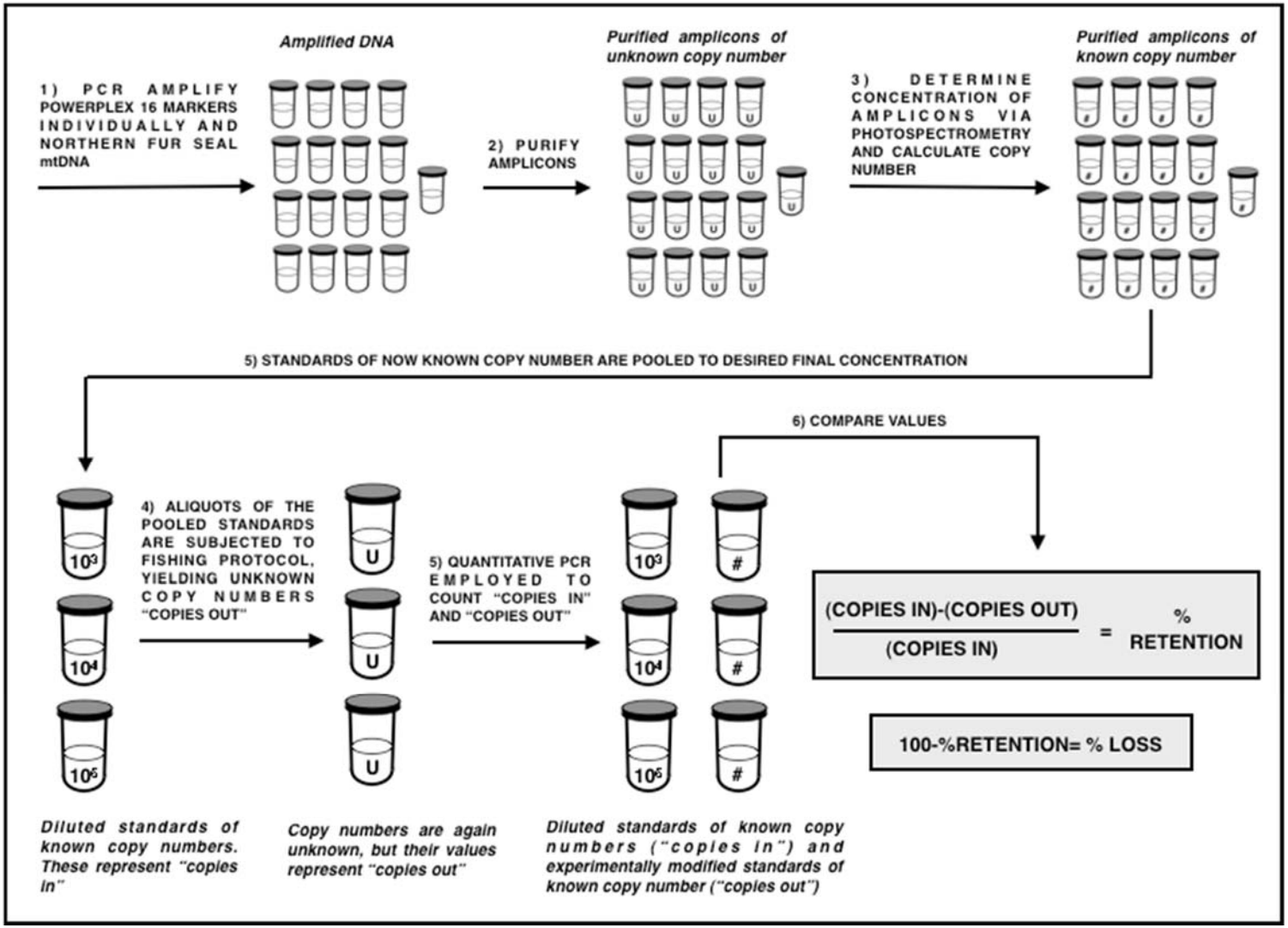
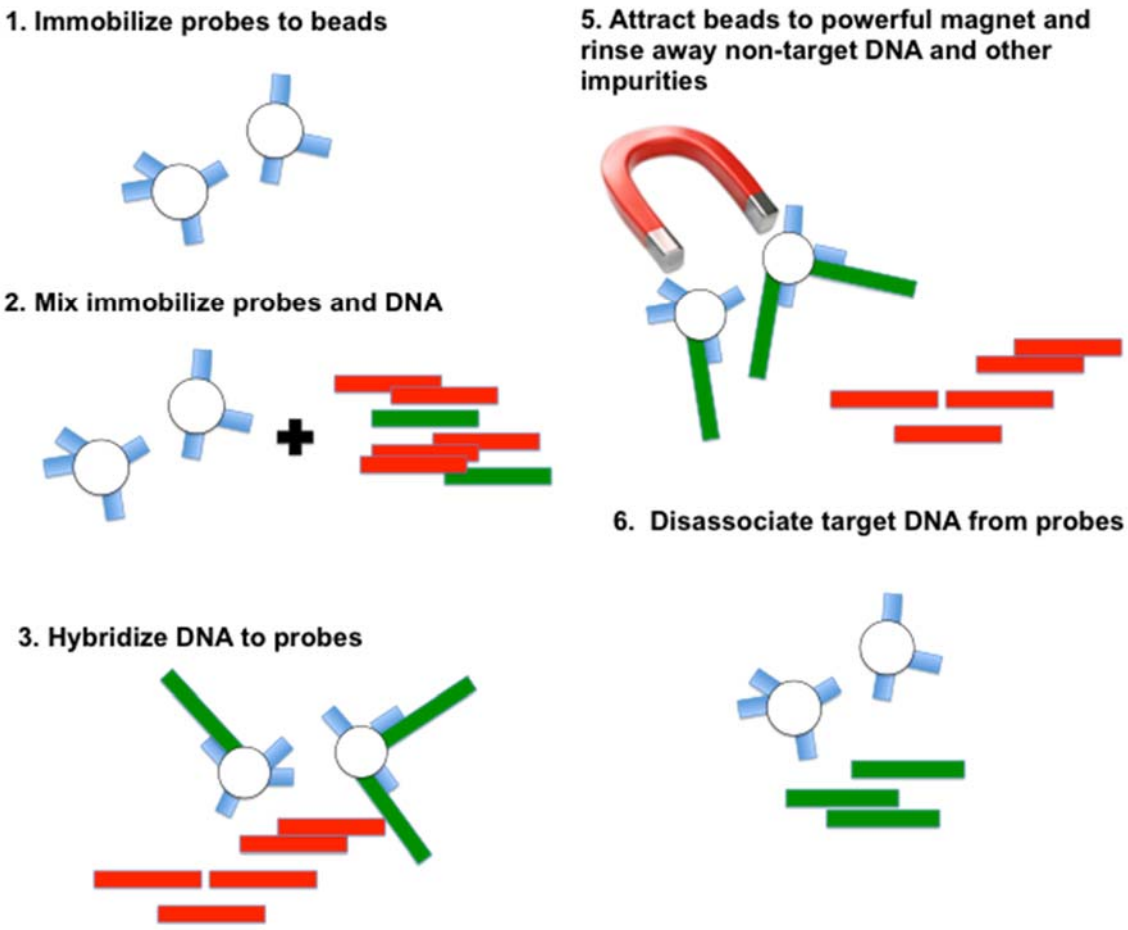


Figure 3. Depiction of the Direct Fishing Protocol. Target molecules are green (in this study, representing the 16 fragments targeted by PowerPlex 16® System**)**, non-target molecules are red (in this study, representing NFS mtDNA), biotinylated probes are blue, and the streptavidin coated magnetic beads are white.



Chapter 3: Evaluating the Efficiency of Primer Extension Capture as a Method to Enrich DNA Extractions

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ABSTRACT

In this study we sought to document the efficiency of primer extension capture (PEC) as a method to enrich DNA extractions by two measures, first in its ability to retain targeted DNA molecules and, secondly, in its ability to remove non-target DNA molecules from a common pool containing both. Efficiencies were estimated following the approach of comparing number of "copies in" to "copies out" by quantitative polymerase chain reaction (qPCR). Conducted as previously described, PEC retention of DNA targets ranging 109-288 base pairs (bps) in length was only 15.88-2.14% (i.e., loss of 84.12-97.86%). Experimental modifications of PEC aimed to potentially increase this efficiency were generally of no avail. However, the benefit of PEC, as originally designed, is in its ability to remove most non-target DNA molecules (99.99%). We discovered that many (56.69%) of the target molecules are "lost" prior to their immobilization on the streptavidin-coated beads, that is following extension and purification of the extended products. Our results will allow researchers to carefully weigh the general inefficiency of PEC in retaining target molecules over its high efficiency in removing non-target molecules. Moreover, these efficiencies are directly comparable to previous ones estimate for "fishing" for DNA, any alternative method for DNA enrichment.

INTRODUCTION

High throughput DNA sequencing [also commonly referred to as next generation sequencing (NGS) or massive parallel sequencing (MPS)] holds promise for the continued study of low copy number (LCN) and degraded DNA samples. While forensic DNA practitioners recognize its utility (e.g., Chang et al., 2009; Butler, 2010; Berglund et al., 2011; Parson et al., 2013; Melton, 2014; Yang et al., 2014), it has already been established as a reliable approach to the study ancient DNA (aDNA). Reports of the first two ancient genomes, a ~4,000 year old Paleo-Eskimo genome (Rasmussen et al., 2010) and that of the Neanderthal (Green et al., 2010), has led to the rise of a field now dubbed "Paleogenomics" (Shapiro and Hofreiter, 2014). Recently Raghavan et al. (2014) was able to conduct population level genetic analysis of genomic data, serving as an example for the utility of paleogenomics.

However, with short tandem repeats (STRs) as the current gold standard for forensic identification, it has been unclear how soon high throughput sequencing (HTS) would become widely used by the forensic DNA community, despite its advantages (Holland et al., 2009; Butler, 2010; Yang et al., 2014). One obvious drawback of "standard" HTS is that it is analogous to screening DNA prior to the invention of PCR, in that, it is not directed at molecules of interest, but rather represents a "shot gun" approach. Thus, standard HTS is not well suited for screening specific markers of interest (i.e., with much coverage at any particular site) or mapping repetitive units (Metzker, 2010), such as those represented by the STRs in the Combined DNA Index System (CODIS) panel. However, forensic researchers have recently demonstrated that, following STR multiplex amplification and indexed library preparation of those amplicons, HTS can be leveraged to produce reliable STR profiles of adequate depth (Zeng et al., 2015). Here coverage (or depth) refers to the number of molecules screened. For example, with HTS, if a site has two or more times coverage, the site has been observed on two or more independent molecules derived from the biological material from which they have been purified. This is vastly different than screening markers with PCR, which theoretically begins from a pool of molecules intact enough to contain both priming sites. In this case, the copy number available [e.g., as assessed by quantitative PCR (qPCR)] from a sample dictates the "coverage", which is observed downstream as an average in the amplicons sequenced using the Sanger method or typed by other means (e.g., RFLP, length variance).

As another means to leverage the power of HTS, aDNA researchers have developed a variety of "DNA capture" methods that allow for pre-selection of regions of interest, which can be later subjected to HTS (e.g., Briggs et al., 2009; Maricic et al., 2010; Carpenter et al., 2013). DNA capture is also commonly referred to as "sample enrichment", "pull down", or "fishing". Most DNA capture methods, while variable, follow the same general principles. First, the DNA sample is denatured and target molecules are hybridized to synthetic biotinylated DNA molecules (called "probes" or "bait"). Immobilization of this hybridized complex on streptavidin coated magnetic beads, effectively "captures" the targets. A strong magnet is used to attract the beads to the side or bottom of the tube. Buffers are used to wash away impurities and non-target DNA, and then the beads are released from the magnet and the target DNA disassociated from the probes. In the end, there should be enrichment of target over non-target DNA molecules, and it should also be free of impurities (e.g., PCR inhibitors).

While most DNA capture methods have been developed to enrich from aDNA libraries (i.e., already amplified DNA) that typically contain comparatively little endogenous DNA (Carpenter et al., 2013), it is possible that similar methods could be used to capture target molecules from a sample's total DNA eluate, that is, to prescreen and enrich for DNA markers of interest prior to PCR amplification or even library builds. Taking this perspective it is essential to further consider that when targeting CODIS markers (or any set of markers) by traditional PCR, that these reactions contain far more non-target genomic DNA of the individual than target DNA [not to mention the possible complicating factor of non-target exogenous DNA associated with the sample (Noonan et al., 2005; Poinar et al., 2006)]. For example, the sum of the average amplicon lengths targeted in Promega Male 9948 DNA by the Promega PowerPlex 16® System is 3573 base pairs (bps) (Promega, 2008; Kemp et al., 2014) (Chapter 1). Given that the human genome is ~3.2 billion bps in length, only ~$1.12x10^{-4}$% of it need be present in a DNA extract to generate a complete profile. In other words, the vast majority of the genome is of no consequence to generating a full CODIS profile. In general, amplification efficiency of targeted DNA is sub-optimal because non-target DNA can interfere between polymerase and targeted DNA molecules and/or compete as binding sites for primers (Wilson, 1997; Nielsen et al., 2008). If "capture extracted" DNA is efficient, it could possibly lead to more reliable DNA profiling.

Anderung et al (2008) described one such method of "fishing" for DNA with biotinylated probes from a pool of DNA extracted from ancient cattle bones and teeth. Nandineni and Vedanayagam (2009) also used a fishing approach to capture CODIS STRs. In both studies, the researchers demonstrated the feasibility of the methods, but did not document its efficiency against a standard (e.g., Barta et al., 2014; Kemp et al., 2014) (Chapter 1). Using such an approach, Winters et al. (In Prep) (Chapter 2) recently observed that the method is generally inefficient at both retaining target molecules and removing non-target molecules. Through a series of experiments, the best average retention of DNA targets ranging 109-288 bps in length was 31.41-12.08%, which was similar to the average retention of non-target molecules (22.07-24.65%, two tailed t-test p=0.623). While the method may be useful in the study of DNA compromised by polymerase chain reaction (PCR) inhibitors (Wang and McCord, 2011), one must carefully weigh this possible advantage against the degree of expected DNA loss and the non-selectivity of the method for targeted over non-targeted DNA.

Primer extension capture (PEC) is an alternative capture method developed by Briggs et al (2009) (Figure 1). The PEC method expands on fishing for DNA with the addition of one cycle of annealing and polymerase extension of the biotinylated probes on the target molecules. Following extension, the biotinylated probes become complementary to the length of the target molecule. Thus, the target DNA should be more tightly bound to the probes, in comparison to fishing for DNA with short probes. After primer extension the products are immobilized onto streptavidin coated magnetic beads and separated from the non-target DNA and impurities present in the supernatant. Briggs et al. (2009) used this method to capture and sequence pieces of the complete Neanderthal mitochondrial genome.

In this study we sought to document, by two measures, the efficiency of PEC as an extraction method, first in its ability to retain targeted DNA molecules and secondly in its ability to remove non-target DNA molecules. Efficiencies were estimated following the approach of comparing number of "copies in" to "copies out" by qPCR (Barta et al., 2014; Kemp et al., 2014) (CHAPTER 1). Since the efficiency of fishing for DNA as a

generalized pre-PCR extraction method has already been documented in this manner, it permits direct evaluation of PEC against that method (Winters et al., In Prep) (Chapter 2).

MATERIALS AND METHODS

Creation of the Pooled Standards

Pooled standards containing the 16 fragments of genomic DNA amplified by the Promega PowerPlex 16® System (i.e., representing targeted DNA for PEC) and a 181 bp fragment of northern fur seal mitochondrial DNA (i.e., representing non-targeted DNA, and abbreviated "NSF mtDNA" throughout the remainder of the study) were created following Winters et al (In Prep) (Chapter 2) to final concentrations of 1×10^5, 1×10^4, and 1×10^3 copies/µL. For example, the $1x10^3$ pooled standard contained 1000 copies of each of the sixteen PowerPlex 16® System amplicons and 1000 copies of NFS mtDNA per microliter.

Primer Extension Capture [Largely Following Briggs et al. (2009)] (Figure 1)

Primer Extension

One hundred microliter PCRs contained: 1X Gold Buffer, 2.5 mM $MgCl_2$, 250 µM of each dNTP, 200 µg/µL BSA, 0.05 pmol of each of the 32 Promega PowerPlex 16® System biotinylated primers (Winters et al., In Prep) (Chapter 2), 5 U Amplitaq® Gold, and 30 µL of pooled standard DNA. One round of PCR was conducted (i.e., to extend the biotinylated primers). Following twelve minutes of activation at 95°C, reactions were held at 60°C for one minute, followed by a two minute hold at 72°C.

Primer extension products were purified with the Qiagen MinElute® PCR Purification Kit, following the manufacturer's instructions except that the pH indicator was not added. Final elution was performed in 30 µL of Buffer EB.

Bead Preparation

Two hundred and fifty microliters of Dynabeads® M-270 Streptavidin-coated beads were prepared by placing them on the magnet (Dynamag™) for 2 minutes and removing the supernatant. The beads were then washed in 500 µL of 2X Binding and Wash (B&W) buffer [10mM Tris-HCl pH 8.0, 1mM EDTA, 1M NaCl, 0.2% Tween-20 (v/v) — it is notable that the recipe of Briggs et al. (2009) for B&W buffer differs from that described by Invitrogen (2012)]. The mixture was vortexed and placed back on the magnet for 2 minutes and the supernatant removed. This wash was repeated and, subsequently, the beads were resuspended in 250 µL of 2X B&W buffer.

Immobilization/Capture

A 25 µL aliquot of the prepared beads was added to the purified primer extension products. This mixture was allowed to rotate at room temperature for 30 minutes. The immobilized mixture was transferred to a new 1.5 mL tube to which 500 µL of 1X B&W buffer was added. This was placed on the magnet for 2 minutes and the supernatant removed. Washing with 1X B&W buffer in this manner was repeated an additional four

times. Following the fifth wash, 500 µL of "hot wash" buffer [1X Amplitaq Gold Buffer, 2.5 mM $MgCl_2$, 0.1% Tween-20 (v/v)] were mixed with the beads and this volume transferred to a new 1.5 mL tube. The tube was rotated at 65°C for two minutes, placed on the magnet for two minutes, and the supernatant removed. Beads were resuspended in 30 µL of Buffer EB and the mixture heated for 2 minutes and 30 seconds at 95°C. The tube was immediately placed back on the magnet and the supernatant was collected and placed in a new 1.5 mL tube after one minute. The eluate volume was brought to 50 µL with dH_2O.

Modified Primer Extension Capture #1 (Platinum® Taq, Modified Wash Following Capture)

In this modified version of the Briggs et al. (2009) PEC, we substituted Platinum® Taq in the primer extension step and used a modified final wash step prior to elution.

Primer Extension

One hundred microliter PCRs contained: 1X PCR Buffer, 1.5 mM $MgCl_2$, 0.32 µM of each dNTP, 0.05 pmol of each of the 32 Promega PowerPlex 16® System biotinylated primers (Winters et al., In Prep) (Chapter 2), 1.9 U Platinum® Taq, and 60 µL of pooled standard DNA. Following a three minute hold at 94°C, reactions were held at 94°C for 15 seconds, 60°C for 1 minute, and 72°C for 2 minutes. After returning to room temperature, volumes were transferred to 1.5 mL tubes. Primer extension products were purified as described above for the primer extension capture protocol.

Bead Preparation

Beads were prepared as described for the primer extension capture protocol.

Immobilization/Capture

Capture and final elution were conducted as described for the primer extension capture protocol, except that following the fifth wash, the beads were washed with 500 µL of 1X Invitrogen PCR buffer.

Modified Primer Extension Capture #2 (Platinum® Taq, Primer Extension Products Not Purified, Modified Beads and Capture)

In this modified version of the Briggs et al. (2009) PEC, we substituted Platinum® Taq in the primer extension step. The primer extension products were not purified prior to capture and we modified the final wash and elution steps.

Primer Extension

Composition of PCRs followed that described for modified PEC #1. Following a three minute hold at 94°C, reactions were held at 94°C for 15 seconds, 60°C for 15 seconds, and 72°C for 15 seconds. After returning to room temperature, volumes were transferred to 1.5 mL tubes.

Bead Preparation

Eighty microliters of Dynabeads® M-270 Streptavidin-coated beads were prepared by adding 200 µL of 1X Binding and Wash (B&W) buffer [following Invitrogen (2012): 5 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 1M NaCl], vortexing the tube, placing it on the magnet for 2 minutes, and removing the supernatant. This was repeated two additional times for a total of three washes with 1X B&W buffer. Finally, beads were eluted in 1000 µL of 2X B&W buffer.

Immobilization/Capture

A 100 µL aliquot of the prepared beads was added to the 100 µL of primer extension products. This results in a 1X final concentration of B&W buffer, the ideal condition (i.e., 1 M NaCl) for immobilization (Invitrogen, 2012). This mixture was rocked gently at room temperature for 30 minutes before being placed back on the magnet for 2 minutes, which was followed by three washes with 200 µL of 1X B&W. The beads were eluted in 60 µL with dH_2O. This volume was heated to 95°C for five minutes, immediately placed on the magnet, and, after one minute, the supernatant was collected into a new 1.5 mL tube.

Modified Primer Extension Capture #3 (Platinum® Taq, Primer Extension Products Not Purified, 2nd Modified Wash and Elution)

In this modified version of the Briggs et al. (2009) PEC, we substituted Platinum® *Taq* for the primer extension. The primer extension products were not purified prior to immobilization and we modified the final wash and elution steps over that described for the modified PEC #2.

Primer Extension

Composition of PCRs and conditions were as described for the modified PEC #1.

Bead Preparation

Beads were as described for the modified PEC #2.

Immobilization/Capture

Immobilization was modified over that described for the modified PEC #2. Following the three washes with 200 µL of 1X B&W, the beads were resuspended in 500 µL of hot wash buffer and the volume transferred to a new 1.5 mL tube. The tube was rotated at 65°C for two minutes, placed on the magnet for two minutes, and the supernatant removed. Beads were resuspended in 60 µL of dH_2O and the mixture heated for five minutes at 95°C. The tube was immediately placed back on the magnet and the 60 µL of supernatant was collected and placed in a new 1.5 mL tube after one minute.

Analysis of Primer Extension Efficiency Between Amplitaq® Gold and Platinum® Taq

Thirty microliters of pooled standard were subjected to primer extension with Amplitaq® Gold or Platinum® *Taq* and purified with the Qiagen MinElute® PCR Purification Kit as described above for primer extension capture. Final elutions were performed with 30 µL Buffer EB, then eluate volumes were brought to 50 µL with dH_2O.

Quantitative PCR (qPCR) and Calculating Percent Retention and Loss

The amount of Amelogenin, D13s317, D16s539, and NFS DNA remaining in the standard after experimental treatment (i.e., "copies out") was determined by qPCR following Winters et al (Winters et al., In Prep) (Chapter 2). Quantification of each marker was conducted in duplicate against standard curves generated from two reactions each of standard pools at 1×10^5, 1×10^4, 5×10^3, 2×10^3, 1×10^3, 5×10^2, 2.5×10^2, 1×10^2 and 50 copies/µL. Two additional 1×10^5, 1×10^4, and 1×10^3 copies/µL standards (untreated) each were quantified as unknowns to monitor the concentration of pre-experimental standards. These provide estimates of "copies in".

Subtraction of the number of "copies out" (measured as the average of duplicate qPCR amplifications from the molecules retained following purification) from "copies in" (measured as the average of duplicate qPCR amplifications of the standards treated as unknowns) divided by "copies in" multiplied by 100 provides the percent retention:

[(copies in - copies out)/ copies in] × 100

One hundred minus retention provides a measure of percent loss. Retention and loss of Amelogenin, D13s317, D16s539, and NFS mtDNA was determined in this manner three times, from which the average loss and the uncorrected sample standard deviation (i.e., the standard deviation of the sample) were calculated.

Statistical Analyses

Two-tailed t-tests were used to statistically evaluate differences in mean percentage retention at the 0.05 level of probability. Statistical analyses were conducted in StatPlus (AnalystSoft, Inc.).

RESULTS

Quantitative PCR efficiencies for Amelogenin, D13s317, and D16s539 ranged from 92.64-98.08%, with R^2 values ranging 0.9988-0.9994 (Tables 1-6), comparable to previous observations (Kemp et al., 2014; Winters et al., In Prep) (Chapters 1 & 2). The NSF mtDNA qPCR efficiencies ranged from 96.62-93.06% with R^2 values from 0.9965-0.9991, also comparable to previous observations Winters et al (In Prep) (Chapter 2).

Across all three standards (1×10^5, 1×10^4, and 1×10^3 copies/µL), average DNA retention of targeted Amelogenin, D13s317, and D16s539 DNA following PEC was 7.66% (i.e., loss of 92.34%) (Table 1). Almost all of the non-targeted NFS mtDNA was removed during the process, with an average loss of 99.99%, which is statistically more loss than that of the targeted molecules ($p=0.037$). Subjected to modified PEC #1, 1.72% of the targets were retained (i.e., loss of 98.28%) and 99.98% of non-target molecules removed (Table 2). Average loss of target and non-target molecules was not statistically different ($p=0.075$) following modified PEC #1. Subjected to modified PEC #2, 10.90% of the targets were retained (i.e., loss of 89.10%) and 90.65% of non-target molecules removed (Table 3). Average loss of target and non-target molecules was not statistically different ($p=0.698$) following modified PEC #2. Modified PEC #3 was associated with the retention of 7.47% of the targets (i.e., loss of 92.53%) and a loss of

99.59% of non-target molecules (Table 4). Average loss of target and non-target molecules was not statistically different (p=0.023).

The mean retention of targeted molecules by PEC (7.66%) was larger than that of modified PEC # 1 (1.72%, p=0.005), but not statistically different from that retained by modified PEC #2 (10.90%, p=0.265) or from modified PEC #3 (7.47%, p=0.936). The mean loss of non-target molecules by PEC (99.99%) was not different from that lost following modified PEC # 1 (99.98%, p=0.932), but statistically greater than the average loss associated with modified PEC #2 (90.65%, $p=2.5 \times 10^{-6}$) and modified PEC #3 (99.59%, p=0.003).

On average, more than half of the target DNA in the standards (56.59%) subjected solely to primer extension with Amplitaq® Gold, followed by purification with the Qiagen MinElute® PCR Purification Kit, were lost (Table 5). This was statically indistinguishable from the loss of the non-target DNA (65.78%, p=0.237). Less than half of the target DNA in the standards was lost (45.26%, but not statistically different from Amplitaq® Gold results, p=0.054) when subject to primer extension with Platinum® *Taq*, followed by purification with the Qiagen MinElute® PCR Purification Kit. Loss of non-target DNA (50.18%) was not statistically different that loss of target DNA (p=0.628).

DISCUSSION

In this study we sought to document the efficiency of PEC (Figure 1) as an extraction enrichment method by two measures, first in its ability to retain targeted DNA molecules and, secondly, in its ability to remove non-target DNA molecules. Following the protocol of Briggs et al (2009) results in the loss of 92.34% of the three targeted molecules (Amelogenin, D13s317, and D16s539). Since these three markers were chosen as representatives of the fragments targeted for amplification by the Promega PowerPlex 16® System, presumably the same percentage of the other thirteen targets was lost. While PEC might be considered largely inefficient, the method removes proportionally more non-target DNA molecules (i.e., 99.99% of NFS mtDNA molecules were removed, p=0.037). This is precisely why PEC can be of value, in focusing the power of HTS on molecules of interest over non-target regions of the genome and also act to exclude DNA exogenous to the individual under study. Regardless of absolute efficiency, PEC is probably more efficient at removing non-target DNA because, even if the probes were able to anneal to non-target molecules, mismatches at or near the 3' end of the primers would result in poor extension of the molecule by the polymerase (Palumbi, 1996). Thus, they would be subsequently more difficult to immobilize.

Across our experimental modifications to the PEC protocol, none were equivalent to the original protocol. Modified PEC #1 retained less target molecules (1.72%, p=0.005) but was equivalent in its removal of non-target molecules (99.98%, p=0.932). Both modified PEC #2 and #3 were equivalent in their retention of CODIS molecules (10.90%, p=0.265 and 7.47%, p=0.936, respectively), but associated with less efficient removal of the NSF mtDNA molecules (90.65%, $p=2.5 \times 10^{-6}$ and 99.59, p=0.003, respectively). Thus, based on the results of our study, if PEC is to be used as a DNA capture method, it is recommendable to follow the protocol as originally described (Briggs et al., 2009). However, it is notable that employment of Amplitaq® Gold may not be ideal with all samples and/or sample types, as previous investigation has shown that this polymerase is particularly prone to inhibition (Monroe et al., 2013).

Further modification to the basic PEC method should be directed at improving the retention of target molecules, as was possible with modifications to the fishing method (Winters et al., In Prep) (Chapter 2). However, while the optimized fishing protocol retained on average more target molecules (21.70%, $p=4 \times 10^{-5}$) than possible with PEC, removal of non-target molecules reach only 76.99% (thus, it inadvertently retained far more non-target DNA, 23.01%, $p=9.8 \times 10^{-6}$). This illustrates a strength in our assessment of efficiency using common standards; the outcomes are directly comparable, resulting in both relative and absolute assessments of efficiency.

Our unique approach to quantifying efficiency by comparing "copies in" to "copies out" estimated from quantitative PCR (Barta et al., 2014; Kemp et al., 2014) (Chapter 1) permitted us to begin identifying wherein DNA loss occurs during the PEC protocol. Specifically, we observed an average loss of 56.59% of the target DNA following extension by Amplitaq® Gold and purification with the Qiagen MinElute® PCR Purification Kit (Table 5). Previously, losses of Amelogenin, D13s317, and D16s539 at 52%, 40.85%, and 38.95% (average loss across pooled standards starting at $1x10^7$, $1x10^5$, and $1x10^4$ copies/µL) have been observed following purification with the Qiagen MinElute® PCR Purification Kit (Kemp et al., 2014) (Chapter 1). From this, it might be concluded that the remaining fraction of lost molecules (~4-17%) is attributable to the twelve minute activation at 95°C required by the enzyme. More appropriately, these lost molecules might be better termed "unamplifiable", probably having lost one or both priming sites as a result of degradation of the single strands during heat activation. Yet, these numbers are not additive, given the theoretical expectation that primer extension should double the number of target molecules. Working through an example will aid in illuminating this point.

The average copy number of D16s539 retained from the 1×10^5 standard was 67124.71 per microliter. If 38.95% loss is attributable to purification by the Qiagen MinElute® PCR Purification Kit, there should have been 172335.58 copies present prior to purification, representing only a 42.9% rise over the pre-amplification count of 120591 copies. Moreover, since NFS mtDNA copies should not have increased following one round of extension (i.e., because northern fur seal probes were not added), it is hard to reconcile why loss of these molecules (58.89%) was not greater than that observed for target molecules ($p=0.237$). Perhaps all of these numbers are not simply additive because of the loss of amplifiable molecules due to the prolonged heat activation step necessary for the polymerase that leaves the DNA exposed while in a state of single strandedness. Therefore, it is reasonable to conclude that primer extension probably does not double the copy number, which uniquely contributes to the overall low efficiency of PEC in retaining molecules of interest compared to fishing for DNA. Since losses associated with extension by Platinum® *Taq* were not statistically different from that employing Amplitaq® Gold, this substitution provides no relief from the heat activation issue.

It is worth emphasizing that the outcome of the experiments described here were not compared with standard methods of DNA extraction and genotyping, which would have pointed to the strength and weakness of PEC over standard methods. Nevertheless, our study is the first to evaluate the efficiency of PEC for DNA against 100% recovery of molecules sized exactly for amplification with the Promega PowerPlex 16® System. A possible future direction would be to experimentally degrade native DNA (e.g., Ambers et al., 2014) and evaluate the efficiency of PEC for capture of target molecules from total genomic DNA that is degraded and chemically modified.

While PEC is an established method for enriching target DNA from libraries prior to HTS (Briggs et al., 2009), our experiments raise concern over its use as method for enriching DNA extractions prior to PCR amplification. Before adopting this method, researchers should carefully weigh its general inefficiency in retaining target molecules over its high efficiency in removing non-target molecules.

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Table 1. Retention of Amelogenin, D13s317, D16s539, and northern fur seal mitochondrial DNA (NSF mtDNA) molecules using primer extension capture.

					Capture 1		Capture 2		Capture 3		Average			
		R^2	Copies In	SD	Copies Out	SD	Copies Out	SD	Copies Out	SD	Copies Out	SD	% Retention	% Loss
Amelogenin	97.53	0.9993	111394.00	2364.17	2964.54	58.87	2273.65	n/a	2170.82	239.57	2469.67	431.65	2.22	97.78
			11340.70	481.00	235.92	8.95	217.99	52.94	272.59	9.39	242.17	27.83	2.14	97.86
			1072.08	47.40	21.99	2.31	27.99	0.19	23.23	3.22	24.40	3.17	2.28	97.72
D13s317	93.24	0.9993	82378.40		11532.43	1370.28	11071.29	50.81	9593.43	1298.55	10732.39	1012.95	13.03	86.97
			8294.56	943.00	1008.36	179.15	1237.77		1181.44	111.11	1142.52	119.56	13.77	86.23
			841.48	52.50	130.71	10.80	140.45	6.13	129.65	5.75	133.60	5.95	15.88	84.12
E														
D16s539	96.26	0.9991	115868.00	4075.82	7427.77	248.48	6422.53	435.67	6310.06	212.29	6720.12	615.42	5.80	94.20
			11735.90	358.00	658.02	6.03	768.50	115.41	865.75	135.18	764.09	103.94	6.51	93.49
			1099.80	2.63	68.92	0.67	84.98	11.50	87.32	0.59	80.40	10.02	7.31	92.69
												Average Targeted DNA	7.66	92.34
NFS mtDNA	90.78	0.9980	70310.00	n/a	1.15	0.08	1.25	1.14	1.21	0.80	1.20	0.05	0.00	100.00
			8222.18	1490.78	0.32	0.19	0.71	0.41	0.14	0.17	0.39	0.29	0.00	100.00
			905.29	73.50	0.09	n/a	undetermined	n/a	0.22	0.02	0.15	0.09	0.02	99.98
												Average Non-Targeted DNA	0.01	99.99

Table 2. Retention of Amelogenin, D13s317, D16s539, and northern fur seal mitochondrial DNA (NSF mtDNA) molecules using the modified primer extension capture #1 (Platinum® *Taq*, modified wash following capture)

					Capture 1		Capture 2		Capture 3		Average			
	E	R^2	Copies In	SD	Copies Out	SD	Copies Out	SD	Copies Out	SD	Copies Out	SD	% Retention	% Loss
Amelogenin	96.54	0.9994	115291.00	704.00	304.77	38.10	481.53	15.00	415.77	12.10	400.69	89.34	0.35	99.65
			9145.61	296.00	29.90	1.73	29.33	3.44	23.75	1.22	27.66	3.39	0.30	99.70
			832.37	43.20	2.65	0.91	2.83	0.72	2.18	0.19	2.55	0.33	0.31	99.69
D13s317	92.78	0.9993	96424.40	1069.58	2808.71	63.90	4456.11	98.90	3513.25	166.00	3592.69	826.57	3.73	96.27
			7643.45	430.00	283.24	12.40	316.82	11.00	208.75	48.10	269.60	55.31	3.53	96.47
			753.47	43.70	35.40	0.67	21.11	1.67	19.50	0.45	25.33	8.75	3.36	96.64
D16s539	94.48	0.9990	114253.00	8780.81	1393.46	69.10	1788.37	17.90	1632.64	25.80	1604.82	198.92	1.40	98.60
			8674.43	56.60	121.15	5.09	129.06	0.78	116.72	24.10	122.31	6.25	1.41	98.59
			891.48	74.50	12.40	0.20	8.31	0.29	8.75	1.01	9.82	2.25	1.10	98.90
Average Targeted DNA													1.72	98.28
NFS mtDNA	86.62	0.9989	86073.40	2627.17	6.19	n/a	9.59	0.90	10.38	2.50	8.72	2.23	0.01	99.99
			7142.19	417.00	0.42	0.29	0.39	0.08	0.34	0.12	0.38	0.04	0.01	99.99
			736.50	15.00	0.40	n/a	0.39	n/a	0.27	0.01	0.35	0.07	0.05	99.95
Average Non-Targeted DNA													0.02	99.98

Table 3. Retention of Amelogenin, D13s317, D16s539, and northern fur seal mitochondrial DNA (NSF mtDNA) molecules using the *modified primer extension capture #2 (Platinum® Taq, primer extension products not purified, modified beads and capture)*

					Capture 1		Capture 2		Capture 3		Average			
	E	R^2	Copies In	SD	Copies Out	SD	Copies Out	SD	Copies Out	SD	Copies Out	SD	% Retention	% Loss
Amelogenin	96.69	0.9994	114610.00	47.40	3143.43	111.00	1767.23	26.00	2780.16	2.58	2563.61	713.20	2.24	97.76
			11290.50	448.00	292.97	12.30	281.91	9.15	349.40	20.80	308.09	36.20	2.73	97.27
			983.09	80.10	26.44	1.80	32.44	3.85	30.76	2.74	29.88	3.09	3.04	96.96
D13s317	93.23	0.9991	77567.50		15833.90	812.00	12419.80	258.00	13216.50	3693.68	13823.40	1786.13	17.82	82.18
			8589.38	1040.81	1303.18	312.00	1083.57	395.00	1506.55	274.00	1297.77	211.54	15.11	84.89
			887.22	53.80	160.48	11.50	144.54	24.70	176.87	0.73	160.63	16.17	18.11	81.89
D16s539	96.25	0.9992	128385.00	2112.88	15539.90	235.00	12201.20	186.00	15051.70	585.00	14264.27	1803.27	11.11	88.89
			11548.70	546.00	1439.92	13.00	1483.18	114.00	1691.36	35.50	1538.15	134.43	13.32	86.68
			1092.93	26.10	147.37	1.54	160.30	0.25	172.88	16.00	160.18	12.76	14.66	85.34
Average Targeted DNA													10.90	89.10
NFS mtDNA	93.06	0.9991	79960.20	n/a	9473.66	13.80	6732.82	68.40	7354.30	2173.29	7853.59	1437.02	9.82	90.18
			9377.67	647.00	894.68	n/a	753.99	n/a	911.09	n/a	853.25	86.36	9.10	90.90
			882.16	18.20	80.65	4.23	74.74	n/a	86.03	11.30	80.47	5.65	9.12	90.88
Average Non-Targeted DNA													9.35	90.65

Table 4. Retention of Amelogenin, D13s317, D16s539, and northern fur seal mitochondrial DNA (NSF mtDNA) molecules using the modified primer extension capture #3 (Platinum® Taq, primer extension products not purified, 2nd modified wash and elution)

					Capture 1		Capture 2		Capture 3		Average			
	E	R^2	Copies In	SD	Copies Out	SD	Copies Out	SD	Copies Out	SD	Copies Out	SD	% Retention	% Loss
Amelogenin	96.54	0.9991	99253.60	10778.90	2277.71	18.80	2093.12	183.00	2633.96	362.00	2334.93	274.92	2.35	97.65
			10814.70	207.00	183.91	n/a	89.95	15.50	217.03	38.30	163.63	65.92	1.51	98.49
			1023.70	30.80	17.86	1.53	14.31	0.92	10.34	1.44	14.17	3.76	1.38	98.62
D13s317	93.64	0.9992	91774.60	11837.60	11485.40	736.00	10439.10	445.00	11870.20	1034.46	11264.90	740.59	12.27	87.73
			9683.25	69.00	1045.31	45.70	1076.99	67.10	1235.51	33.70	1119.27	101.91	11.56	88.44
			874.93	60.60	108.98	2.27	94.28	0.59	77.01	0.67	93.42	16.00	10.68	89.32
D16s539	92.64	0.9994	114193.00	15981.50	10383.30	362.00	9563.37	140.00	11299.90	118.00	10415.52	868.71	9.12	90.88
			10293.50	352.00	864.72	114.00	805.66	30.90	1201.67	14.70	957.35	213.64	9.30	90.70
			1028.35	69.20	115.59	1.59	73.05	0.74	90.46	4.96	93.03	21.39	9.05	90.95
Average Targeted DNA													7.47	92.53
NFS mtDNA	90.96	0.9965	97258.50	1994.34	602.79	184.90	253.37	63.62	243.30	155.87	366.49	204.71	0.38	99.62
			9489.92	319.00	46.22	9.47	26.10	12.44	17.37	1.56	29.90	14.79	0.32	99.68
			898.32	8.80	5.74	1.13	4.00	1.67	4.38	1.44	4.70	0.91	0.52	99.48
Average Non-Targeted DNA													0.41	99.59

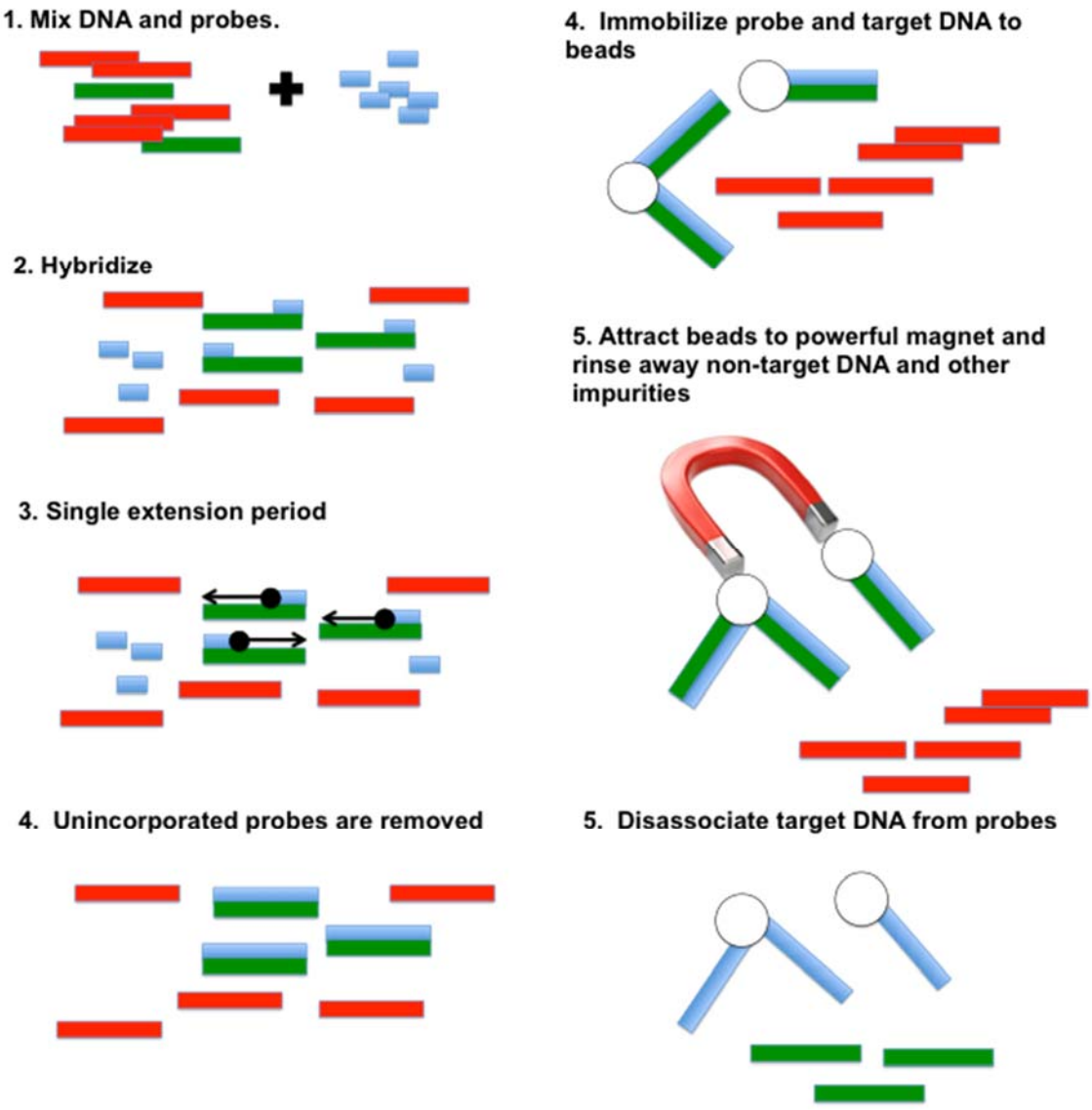
Table 5. Loss of Amelogenin, D13s317, D16s539, and northern fur seal mitochondrial DNA (NSF mtDNA) molecules following primer extension with Amplitaq Gold ® and Minelute® purification

					Round 1		Round 2		Average		%
	E	R^2	Copies In	SD	Copies Out	SD	Copies Out	SD	Copies Out	SD	Loss
Amelogenin	96.01	0.9994	114718.00	5144.78	35232.75	2100.93	40658.83	n/a	37945.79	3836.82	66.92
			11004.50	333.00	3182.93	305.35	2735.33	n/a	2959.13	316.50	73.11
			1035.97	3.88	286.37	48.62	346.41	n/a	316.39	42.45	69.46
D13s317	93.36	0.9988	63422.90	n/a	43762.33	8355.65	33143.33	n/a	38452.83	7508.77	39.37
			9423.94	n/a	4972.89	1519.04	4094.92	42.69	4533.90	620.82	51.89
			952.52	14.20	370.24	39.34	345.01	n/a	357.62	17.83	62.46
D16s539	93.94	0.9989	120591.00	936.00	64514.83	578.65	69734.58	344.24	67124.71	3690.92	44.34
			12238.80	454.00	5692.23	49.57	5995.17	387.54	5843.70	214.21	52.25
			1215.81	47.20	634.35	10.89	592.43	99.59	613.39	29.64	49.55
Average Targeted DNA											56.59
NFS mtDNA	91.20	0.9986	81128.30	n/a	31073.92	5699.87	35056.42	9125.80	33065.17	2816.05	59.24
			9426.45	35.50	3069.90	245.70	3344.06	81.09	3206.98	193.86	65.98
			1012.44	35.50	286.49	18.51	278.16	n/a	282.33	5.89	72.11
Average Non-Targeted DNA											65.78

Table 6. Loss of Amelogenin, D13s317, D16s539, and northern fur seal mitochondrial DNA (NSF mtDNA) molecules following primer extension with Platinum Taq® and Minelute® purification

					Round 1		Round 2		Round 3		Average		
	E	R^2	Copies In	SD	Copies Out	SD	Copies Out	SD	Copies Out	SD	Copies Out	SD	% Loss
Amelogenin	98.08	0.9991	126717.00	543.00	52663.50	1867.68	58274.00	681.00	70503.90	2168.71	60480.47	9122.57	52.27
			11855.30	543.00	4622.48	232.00	5381.83	197.00	8257.73	44.10	6087.35	1917.57	48.65
			1091.40	6.38	394.34	0.33	358.22	5.74	470.23	54.50	407.60	57.16	62.65
D13s317	92.73	0.9990	72830.60	n/a	49816.20	n/a	52510.20	n/a	58979.30	5866.78	53768.57	4709.38	26.17
			9175.94	n/a	5028.88	231.00	5321.58	489.00	6533.42	1097.68	5627.96	797.69	38.67
			879.23	25.80	386.82	18.40	382.00	13.00	533.03		433.95	85.84	50.64
D16s539	95.84	0.9992	129488.00	2467.53	69658.00	2581.48	74550.20	3908.34	88620.80	3040.57	77609.67	9844.65	40.06
			12265.70	136.00	6008.81	75.50	7830.31	427.00	10506.20	171.00	8115.11	2262.18	33.84
			1130.15	41.60	532.45	23.50	490.53	13.30	523.66	21.10	515.55	22.11	54.38
											Average Targeted DNA		45.26
NFS mtDNA	92.36	0.9980	76093.70	n/a	50820.40	n/a	56341.80	n/a	63146.60	5153.41	56769.60	6174.23	25.40
			10229.30	n/a	4714.22	114.00	4775.21	88.20	5258.20	n/a	4915.88	298.02	51.94
			1000.43	21.40	267.03	4.46	227.25	n/a	309.78	n/a	268.02	41.27	73.21
												Average Non-Targeted DNA	50.18

Figure 1. Depiction of Primer Extension Capture [largely following Briggs et al. (2009)] Target molecules are green (in this study, representing the 16 fragments targeted by PowerPlex 16® System), non-target molecules are red (in this study, representing NFS mtDNA), biotinylated probes are blue, and the streptavidin coated magnetic beads are white.



Chapter 4: Evaluating the Efficiency of "Mega-Probe" Capture as a Method to Enrich DNA Extractions

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ABSTRACT

In this study we sought to document the efficiency of a previously described DNA capture method, one that we call “mega-probe” capture, for enriching DNA extracts of target molecules over non-target ones. Efficiencies were estimated following the approach of comparing number of “copies in” to “copies out” by quantitative polymerase chain reaction (qPCR). Mega-probe capture increased target molecules from standards by 702.46%, an impossible outcome. From our observations of negative controls, 6217.43, 14555.55, and 36621.00 copies/µL of D16s539, D13s317, and amelogenin bait molecules, respectively, became counted as captured copies when, in fact, there were no copies to capture. Due to unexpected experimental outcomes, we were not able to estimate the efficiency of this method in its removal of non-target molecules. Nevertheless, our principal concern about mega-probe capture is the possibility that the mega-probe bait becomes counted as captured target molecules. Thus, any attempt to measure its efficiency in retaining targeted molecules will be biased. Additional experiments conducted on DNA retention following hybridization steps of the mega-probe protocol reveal tremendous loss of molecules attributable to both purification by the Qiagen MinElute® PCR Purification Kit and heat exposure. These experiments highlight the critical need for those working with LCN and degraded DNA samples to be mindful of the loss of already limited DNA strands available for analysis due to the experimental treatments used to study them.

INTRODUCTION

Attempts to more fully leverage the power of high throughput sequencing (HTS) of DNA [also commonly referred to as next generation sequencing (NGS)] have produced various DNA enrichment methods (Briggs et al., 2009; Carpenter et al., 2013). While the protocols vary in their details, the end goal of these methods is to enrich target DNA molecules (i.e., genomic regions of interest of the organism under investigation) over non-target ones (i.e., genomic regions not of interest from the organism under investigation, as well as any exogenous DNA present in the extract) from a pool containing both.

Most DNA capture methods follow the same general principles. The DNA sample is denatured and target molecules are hybridized to synthetic biotinylated DNA molecules (called "probes" or "bait"). The hybridized complex is immobilized on streptavidin coated magnetic beads, and a strong magnet is used to attract the beads to the side or bottom of the tube. Buffers are used to wash away impurities (e.g., PCR inhibitors) and non-target DNA, and then the beads are released from the magnet and the target DNA disassociated from the probes. The enriched DNA can now be used for downstream applications. While many of the capture methods were designed to enrich from DNA libraries (i.e., already amplified DNA) prior to HTS (Briggs et al., 2009; Carpenter et al., 2013), they might also be useful in enriching DNA extracts prior to amplification. In fact, the earliest, and arguably the simplest method of capture, known as "fishing" for DNA was presented as an alternative extraction method to a far more common silica-based method (Anderung et al., 2008). However, prior to adopting any capture method, it would be useful to have some estimates of its efficiency.

Winters et al (In Prep-a) (Chapter 2) used a quantitative PCR (qPCR) approach to measure the efficiency of "fishing" for DNA targets ranging 109-288 base pairs (bps) in length from standards containing known numbers of copies of these target molecules. This permitted the efficiency to be measured against 100%, rather than relative against some set of alternative methods (Barta et al., 2014; Kemp et al., 2014) (Chapter 1). They found that average retention of target molecules reached only 31.41-12.08%, which was similar to the average retention of non-target molecules (22.07-24.65%, two tailed t-test p=0.623). In other words, fishing for DNA simply lowered the concentration of DNA in the extract. Thus, the method would appear to be a poor choice, despite its potential usefulness for enriching DNA in the presence of inhibitors (Anderung et al., 2008; Nandineni and Vedanayagam, 2009; Wang and McCord, 2011). Yet, it is important to highlight that the absolute efficiencies of many routinely used DNA extraction methods are unknown (Barta et al., 2014; Kemp et al., 2014) (Chapter 1).

Primer extension capture (PEC) is an alternative capture method that builds on the idea of "fishing" for DNA with the addition of one cycle of annealing of the biotinylated probes and extension with polymerase on targeted molecules (Briggs et al., 2009). Theoretically, this should result in target DNA that is bound more tightly to the probes over that achieved with fishing with short probes alone. Using a directly comparable qPCR approach, Winters et al (In Prep-b) (Chapter 3) determined that PEC retains only 15.88-2.14% of DNA targets ranging 109-288 base pairs (bps) but simultaneously removes 99.99% of non-target molecules. These results permit researchers to carefully weigh the general inefficiency of PEC in retaining target molecules against its high efficiency in removing non-target molecules.

The capture method of Maricic et al (2010) builds long DNA fragments into biotinylated probes that are subsequently used to capture other DNA molecules, those complementary to the long probes. We refer to this approach as "mega-probe" capture (Figure 1) because it employs biotinylated probes much longer than those used in the other two methods described above. Maricic et al (2010) made copies of the full mitochondrial genome with two long range polymerase chain reactions (PCRs). The amplicons were then sonicated to fragments ~150-850 bps in length. The various sized fragments were then purified, biotinylated, and immobilized on streptavidin beads. They used their mtDNA mega-probes to capture and sequence 46 complete human mtDNA genomes and the method has also been used to capture and sequence the complete mitochondrial genome from an extinct Denisovian hominin (Reich et al., 2010).

Mega-probe capture theoretically holds promise for enrichment of DNA recovered from low copy number (LCN) and degraded DNA samples, however it would be instructive to estimate its efficiency so as to be directly comparable to efficiency estimates of fishing (Winters et al., In Prep-a)(Chapter 2) and PEC (Winters et al., In Prep-b) (Chapter 3). Thus, in this study we sought to document, by two measures, the efficiency of mega-probe capture as a method for enriching DNA extractions, first in its ability to retain targeted DNA molecules and secondly in its ability to remove non-target DNA molecules. Efficiencies were estimated following the approach of comparing number of "copies in" to "copies out" by qPCR (Barta et al., 2014; Kemp et al., 2014) (Chapter 1).

MATERIALS AND METHODS

Creation of the Pooled Standards

Pooled standards containing the 16 fragments of genomic DNA amplified by the Promega PowerPlex 16® System (i.e., representing targeted DNA for mega-probe capture) and a 181 bp fragment of northern fur seal mitochondrial DNA (representing non-targeted DNA, and abbreviated "NSF mtDNA" throughout the remainder of the study) were created following Winters et (In Prep-a) (Chapter 2) to final concentrations of 1×10^5, 1×10^4, and 1×10^3 copies/µL. For example, the 1×10^3 pooled standard contained 1000 copies of each of the sixteen PowerPlex 16® System amplicons and 1000 copies of NFS mtDNA per microliter.

Mega-probe Capture (Largely Following Maricic et al. (2010) (Figure 1)

Preparation of the Mega-probe Bait

To make double stranded Bio-T/B adaptors, Bio-T (5'-Biotin-TCAAGGACATCCG-3') and Bio-B (5'-CGGATGTCCTTG-3') were resuspended in a solution of 10 mM Tris and 10 mM NaCl to 100 µM. Mixed together in equal portions (with final concentration of each primer at 50 µM), the solution was heated to 98°C for two minutes, then allowed to cool to room temperature on the bench top.

A pooled standard containing 16 fragments of genomic DNA amplified by the Promega PowerPlex 16® System at a concentration of $1x10^7$ copies/µL was made blunt ended with the Quick Blunting™ Kit (New England Biolabs®). The 100 µL reaction incubated for 30 minutes at room temperature included: 1X Blunting Buffer, 0.1 mM dNTP mix, 4 µL Blunting Enzyme Mix, and 76 µL of the $1x10^7$ standard DNA. This reaction was purified

using the Qiagen MinElute® PCR Purification Kit, following the manufacturer's instructions except that the pH indicator was not added and final elution was performed in 15 µL of Buffer EBT (EB Buffer, 0.05% Tween-20).

The double stranded Bio-T/B adaptors were ligated to the blunt-ended pooled DNA standard with the Quick Ligation™ Kit (New England Biolabs®). The 40 µL reaction incubated at room temperature for 15 minutes included: 1X Quick Ligation Buffer, 1 µL of the Bio-T/B adaptors (50 µM), 4 µL of Quick T4 DNA Ligase, and the 15 µL of purified, blunt-ended pooled DNA standard. This reaction was purified using the Qiagen MinElute® PCR Purification Kit, following the manufacturer's instructions except that the pH indicator was not added and final elution was performed in 15 µL of Buffer EBT

Adding Mega-probe Bait to the Beads

The 15 µL of prepared mega-probe bait was mixed with 15 µL of 2X BWT Buffer (2M NaCl, 10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0, 0.1% Tween-20). This mixture was heated to 98°C for one minute and immediately placed on ice until further use.

An aliquot of 50 µL of Dynabeads® M-270 Streptavidin-coated beads was prepared by placing them on the magnet (Dynamag™) for 2 minutes and removing the supernatant. An aliquot of 200 µL of 1X BWT Buffer was added to the beads. The mixture was vortexed and placed back on the magnet for 2 minutes and the supernatant removed. This was repeated twice more with volumes of 200 µL of 1X BWT Buffer added to the beads. Finally the beads were resuspended in 200 µL of TET Buffer [10 mM Tris-HCl (pH 8.0), 1mM EDTA (pH 8.0), 0.05% Tween-20].

The 30 µL of chilled, prepared mega-probe bait was added to the bead suspension. The tube was rotated for 20 minutes at room temperature, placed on the magnet for two minutes, and the supernatant removed. An aliquot of 200 µL of 1X BWT, preheated to 50°C, was added, and then the tube was vortexed and placed back onto the magnet for two minutes. This was repeated once again. Finally the beads with immobilized mega-probes were resuspended in 50 µL of TET Buffer.

Mega-probe capture of DNA Standards

Three standards each at 1×10^5, 1×10^4, and 1×10^3 copies/µL, as well as one negative control (dH_2O) were subjected to mega-probe capture. A 26 µL aliquot of the pooled DNA standard was combined with 26 µL of Agilent hybridization buffer. This mixture was heated to 95°C for three minutes, then held at 37°C for 30 minutes. The tube was briefly centrifuged to spin down the liquid.

The tube containing bead-immobilized mega-probes was placed on the magnet for two minutes and the supernatant removed. The immobilized mega-probes were resuspended in the 52 µL of pooled DNA standard in Agilent hybridization buffer. The tube lid was secured with parafilm prior to incubation at 65°C for two nights.

The tube was subsequently placed on the magnet for two minutes and the supernatant removed. An aliquot of 200 µL of 1X BWT Buffer was mixed with the captured DNA on the magnetic beads, the tube was placed back on the magnet for two minutes, and the supernatant removed. This was repeated twice more with 200 µL aliquots of 1X BWT Buffer. Preheated to 60°C, 200 µL of 1X HWT Buffer (2.5 mM $MgCl_2$, 50 mM KCl, 15mM

Tris-HCl ph 8.0, 0.1% Tween-20) was added. The mixture was incubated at 60°C for two minutes, the tube placed back on the magnet for two minutes, and the supernatant removed. This was repeated again with the addition of 200 µL of 1X HWT Buffer preheated to 60°C. The captured DNA on the magnetic beads was washed once more in this fashion with 200 µL of 1X BWT. Finally the beads were resuspended in 100 µL of TET Buffer and this mixture was transferred to a new 1.5 mL tube.

Dissociation of Captured DNA from Mega-probes

The tube was placed on the magnet for two minutes and the supernatant discarded. An aliquot of 50 µL of 125 mM NaOH was added, the tube vortexed for ten seconds, and then placed back on the magnet for two minutes. The supernatant was transferred to a tube containing a neutralizing solution (250 µL of PB Buffer, 14 µL of 0.5 M HCl). An additional 50 µL aliquot of 125 mM NaOH was added to the beads, the tube vortexed for ten seconds, and then placed back on the magnet for two minutes. The supernatant was transferred to the same tube containing the neutralizing solution and an additional 250 µL of PB Buffer and 14 µL of 0.5 M HCl were added. This mixture was purified using the Qiagen MinElute® PCR Purification Kit, following the manufacturer's instructions except that the pH indicator was not added and final elution was performed in 15 µL of TET Buffer. The eluate volume was brought to 50 µL with dH_2O.

Hybridization Steps Only with Qiagen MinElute® PCR Purification Kit Purification

In order to assess the influence on resulting copy number of the hybridization steps of the mega-probe capture protocol, two 26 µL aliquots of each 1×10^5, 1×10^4, and 1×10^3 copies/µL pooled DNA standard were mixed with 26 µL of Agilent hybridization buffer heated to 95°C for three minutes, followed by a hold at 37°C for 30 minutes and then at 65°C for two nights. Two 26 µL aliquots each of the 1×10^5 and 1×10^4 copies/µL standard were mixed with 26 µL of Agilent hybridization buffer and subjected to the following heat treatments: 1) 95°C for three minutes, 2) 37°C for 30 minutes, or 3) 65°C for two nights. All of these experimentally heat treated standards were purified by Qiagen MinElute® PCR Purification Kit, following the manufacturer's instructions except that the pH indicator was not added and final elution was performed in 26 µL of dH_2O. Additional dH_2O was added to bring the final volume to 50 µL.

Quantitative PCR (qPCR) and Calculating Percent Retention and Loss

The amount of Amelogenin, D13s317, D16s539, and NFS DNA remaining in the pooled DNA standard following experimental treatment (i.e., "copies out") was determined by qPCR following Winters et al (In Prep-a) (Chapter 2). Quantification of each marker was conducted in duplicate against standard curves generated from two reactions each of standard pools at 1×10^5, 1×10^4, 5×10^3, 2×10^3, 1×10^3, 5×10^2, 2.5×10^2, 1×10^2 and 50 copies/µL. Two additional 1×10^5, 1×10^4, and 1×10^3 copies/µL standards (untreated) each were quantified as unknowns to monitor the concentration of pre-experimental standards. These provide estimates of "copies in".

Subtraction of the number of "copies out" (measured as the average of duplicate qPCR amplifications from the molecules retained following purification) from "copies in" (measured as the average of duplicate qPCR amplifications of the standards treated as unknowns) divided by "copies in" multiplied by 100 provides the percent retention:

[(copies in - copies out)/ copies in] × 100

One hundred minus retention provides a measure of percent loss. Retention and loss of Amelogenin, D13s317, D16s539, and NFS mtDNA was determined in this manner three times, from which the average loss and the uncorrected sample standard deviation (i.e., the standard deviation of the sample) were calculated.

Statistical Analyses

Two-tailed t-tests were used to statistically evaluate differences in mean percentage retention at the 0.05 level of probability. Statistical analyses were conducted in StatPlus (AnalystSoft Inc.).

RESULTS

Quantitative PCR efficiencies for amelogenin, D13s317, and D16s539 ranged from 91.20-97.10%, with R^2 values ranging 0.9984-0.9994 (Tables 1-5), comparable to previous observations (Kemp et al., 2014; Winters et al., In Prep-b; Winters et al., In Prep-a) (Chapters 1-3). The NSF mtDNA qPCR efficiencies ranged from 87.48-92.04% with R^2 values from 0.9987-0.9993, also comparable to previous observations (Winters et al., In Prep-b; Winters et al., In Prep-a) (Chapters 2 & 3).

Across all three standards (1×10^5, 1×10^4, and 1×10^3 copies/μL), average DNA retention of targeted amelogenin, D13s317, and D16s539 DNA following mega-probe capture was 702.46%, with tremendously variable retention percentages across markers and the standards (Table 1). This was likewise true of the non-targeted NFS mtDNA, with an average retention of 307.04%.

An average of 83.04% of the DNA was lost from the purified standards (1×10^5, 1×10^4, and 1×10^3 copies/μL) that were heated to 95°C for three minutes, followed by a hold at 37°C for 30 minutes and then at 65°C for two nights. An average of 51.31% of the DNA was lost from the purified pooled DNA standards (1×10^5 and 1×10^4 copies/μL) that were heated to 95°C for three minutes. An average of 63.94% and 68.25% of the DNA was lost from the same purified standards following subjection to 37°C for 30 minutes and 65°C for two nights, respectively.

DISCUSSION

In this study we sought to document the efficiency of mega-probe capture (Figure 1) as a method to enrich DNA extracts by two measures, first in its ability to retain targeted DNA molecules and secondly in its ability to remove non-target DNA molecules. However, unlike our previous efforts to measure the efficiency of fishing and PEC by these same measures (Winters et al., In Prep-b; Winters et al., In Prep-a) (Chapters 2 & 3) the results are not as straightforward to interpret. Instead of being less than 100% efficient at retaining target DNA molecules, our observations reveal an average 702.46% increase in these molecules. This was an unexpected outcome, one that is impossible and, thus, demands some explanation. One clue is that our results demonstrate that the absolute number of copies retained across each targeted DNA fragment is relatively consistent (Table 1). For example, across the amelogenin standards that span three

orders of magnitude in copy number (1×10^3 to 1×10^5 copies/µL), the number of retained target molecules differ only by ~11000 molecules (29610.26-50603.96). It is conceivable that a portion of the bait molecules became disassociated from the streptavidin-coated beads, losing their status as bait and, thus, gaining the ability to be counted as part of the pooled DNA standard (i.e., inadvertently adding to the number of copies out). This idea is supported by our observation of 6217.43, 14555.55, and 36621.00 copies/µL of D16s539, D13s317, and amelogenin, respectively, in the mega-probe negative control [bait molecules and dH_2O (Table 1)].

More difficult to explain is our observation of 5169.17 copies/µL of NSF mtDNA in the mega-probe negative control, given that it contained neither NSF mtDNA nor NSF mtDNA mega-probes to capture them. Looking again at the dissociation curve for these reactions, nothing abnormal was observed. One likely explanation is that the incorrect 1×10^7 standard was used to create the mega-probe bait, one that contained 1×10^7 copies/µL of NSF mtDNA. If this is the case, we would not be able to draw conclusions about the efficiency of mega-probe capture in removing non-target molecules. However, observing NSF mtDNA in a negative control is consistent with the notion of bait molecules being transformed into countable molecules. While follow up experiments are warranted to account for this, overall the results reported here, combined with mega-probe capture being a relatively cumbersome and time intensive method, we did not do so.

In our previous studies aimed to evaluate the efficiency of fishing for DNA (Winters et al., In Prep-a)(Chapter 2) and PEC (Winters et al., In Prep-b)(Chapter 3), we were able to identify some of the steps within the respective protocols that contribute to their inefficiencies. Specifically, we demonstrated that 61.35-69.49% of the molecules are "lost" during the *essential* hybridization step of the fishing protocol and that 56.69% of the molecules are "lost" following extension and purification of the extended products, steps that are *essential* steps to the PEC protocol. Since this loss of molecules follows heat treatment of the DNA, it is likely that the molecules are losing one or both priming regions, leading to them being non-amplifiable and, thus, not quantified during qPCR (Winters et al., In Prep-b; Winters et al., In Prep-a).

During the current study we observed losses of DNA molecules following subjection specifically to the hybridization steps employed in the mega-probe protocol. An average of 83.04% of the DNA was lost following subjection to 95°C for three minutes, followed by a hold at 37°C for 30 minutes and then at 65°C for two nights. Subjection to each of these hybridization steps separately resulted in 51.31%, 63.94%, and 68.25% loss of the DNA molecules, respectively. There appears to be a positive relationship between increasing DNA loss and time exposure to heat. While the accumulation of DNA damage with heat exposure is an expected outcome (Lindahl, 1993), the magnitude of loss was quite surprising to us. It is important to keep in mind that some of the DNA is lost following purification with the Qiagen MinElute® PCR Purification Kit (Kemp et al., 2014) (Chapter 1) and, thus, a correction factor may be applied to gain a better perspective on DNA loss due to the heat treatment alone. As an example, we observed an average of 80.87% loss of amelogenin DNA from the 1×10^5 standard, resulting in and outcome of 21044.19 copies (Table 2). Since we can expect to lose 52% of these molecules during purification (Kemp et al., 2014) (Chapter 1), prior to purification we could estimate the copy number to have been ~40469.60. Thus, heat treatment of 95°C for three minutes, followed by a hold at 37°C for 30 minutes and then at 65°C for two

nights is responsible for ~63% of the loss. It is critically important for those working with LCN and degraded DNA samples to be mindful of the loss of already limited DNA strands available for analysis as a result of the experimental treatments used to study them.

While mega-probe is an established method for enriching target DNA from libraries prior to HTS (Maricic et al., 2010; Reich et al., 2010), our experiments raise concern over its use as a DNA extraction method, despite some of our own methodological problems in evaluating its efficiency. Of principal concern is the possibility that the mega-probe bait becomes quantified as captured target molecules, and may in turn lead to the misrepresentation of the endogenous molecules in downstream applications.

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Table 1. Retention of Amelogenin, D13s317, D16s539, and northern fur seal mitochondrial DNA (NSF mtDNA) molecules following megaprobe capture.

					Capture 1		Capture 2		Capture 3		Average				dH_2O Negative	
		R^2	Copies In	SD	Copies Out	SD	Copies Out	SD	Copies Out	SD	Copies Out	SD	% Retention	% Loss	Copies Out	SD
Amelogenin	95.97	0.9994	109074.00	1153.37	35275.19	422.63	20444.13	18.90	33111.44	591.93	29610.26	8011.48	27.15	72.85	36621.00	1174.74
			10889.20	961.00	68499.62	129.73	15717.47	79.73	67594.81	2407.43	50603.96	30215.98	464.72	-364.72		
			952.09	5.27	37968.27	1453.65	27014.13	213.08	25460.38	772.00	30147.60	6817.31	3166.47	-3066.47		
D13s317	93.04	0.9992	88395.50	1752.54	14577.77	545.29	6364.17	1621.97	13784.85	n/a	11575.60	4530.60	13.10	86.90	14555.55	1027.21
			8022.76	245.00	25185.58	n/a	6201.13	n/a	26388.27	584.18	19258.33	11323.84	240.05	-140.05		
			637.19	18.00	12428.33	1217.91	10185.25	197.66	9059.19	n/a	10557.59	1715.15	1656.91	-1556.91		
D16s539	91.20	0.9993	112950.00	1625.65	8847.77	244.79	4049.19	4.98	8407.57	351.80	7101.51	2652.53	6.29	93.71	6217.43	11.83
			11560.80	1396.11	16256.90	382.19	3240.25	201.63	15003.00	54.12	11500.05	7180.62	99.47	0.53		
			991.58	51.40	7209.66	173.25	6724.84	71.70	5342.47	76.90	6425.66	968.88	648.02	-548.02		
												Average	702.46	-602.46		
NFS mtDNA	87.48	0.9993	84906.70	n/a	7722.26	255.93	4183.27	n/a	8235.33	n/a	6713.62	2206.31	7.91	92.09	5169.17	670.34
			9264.85	299.00	13962.54	n/a	2818.17	n/a	11968.96	726.63	9583.22	5942.90	103.44	-3.44		
			727.21	74.90	7018.73	n/a	5996.15	3.15	4651.54	n/a	5888.81	1187.24	809.79	-709.79		
												Average	307.04	-207.04		

Table 2. Loss of Amelogenin, D13s317, D16s539, and northern fur seal mitochondrial DNA (NSF mtDNA) molecules purified by Qiagen MinElute® PCR Purification Kit following subjection to 95°C for three minutes, followed by a hold at 37°C for 30 minutes and then at 65°C for two nights.

					Standard 1		Standard 2		Average		
		R^2	Copies In*	SD	Copies Out	SD	Copies Out	SD	Copies Out	SD	% Loss
Amelogenin	94.97	0.9991	109491.00	2601.30	13026.08	682.74	29062.31	744.09	21044.19	11339.33	80.78
			10445.10	1410.22	1624.72	22.54	1394.12	16.76	1509.42	163.06	85.55
			1165.84	30.50	396.61	54.45	87.54	4.12	242.07	218.55	79.24
D13s317	94.39	0.9990	93568.80	13842.40	15503.57	249.65	26938.08	n/a	21220.82	8085.42	77.32
			9239.72	609.00	1498.68	n/a	1492.54	n/a	1495.61	4.34	83.81
			861.66	26.30	343.61	n/a	90.30	n/a	216.96	179.11	74.82
E											
D16s539	92.01	0.9991	128737.00	3534.84	12630.38	532.07	21645.19	3273.09	17137.79	6374.43	86.69
			12879.10	485.00	1221.24	21.18	1080.24	47.40	1150.74	99.70	91.07
			1302.15	131.00	293.94	8.85	77.91	6.23	185.92	152.76	85.72
NFS mtDNA	88.09	0.9987	79412.90	15408.50	11002.62	195.65	19855.87	330.84	15429.24	6260.19	80.57
			9810.36	n/a	1344.97	n/a	1221.51	n/a	1283.24	87.30	86.92
			918.90	90.20	234.05	77.57	60.88	10.86	147.47	122.45	83.95
										Average	83.04

*values for $1X10^5$ and $1X10^4$ copies in are associated with efficiencies (E) and coefficients of determination (R^2) found in Tables 3 and 4

Table 3. Loss of Amelogenin, D13s317, D16s539, and northern fur seal mitochondrial DNA (NSF mtDNA) molecules purified by Qiagen MinElute® PCR Purification Kit following subjection to 95°C for three minutes.

					Standard 1		Standard 2		Average		
		R^2	Copies In	SD	Copies Out	SD	Copies Out	SD	Copies Out	SD	% Loss
Amelogenin$_E$	96.50	0.9990	109491.00	2601.30	53315.58	2397.09	54138.27	748.99	53726.92	581.73	50.93
			10445.10	1410.22	4120.40	305.39	5616.32	233.96	4868.36	1057.77	53.39
D13s317	94.80	0.9984	93568.80	13842.40	52795.67	1786.94	54998.27	1070.45	53896.97	1557.47	42.40
			9239.72	609.00	4511.63	n/a	5496.54	n/a	5004.09	696.43	45.84
D16s539	97.10	0.9990	128737.00	3534.84	58365.87	458.40	63959.71	1931.63	61162.79	3955.45	52.49
			12879.10	485.00	4558.87	337.43	5975.36	201.62	5267.11	1001.61	59.10
NFS mtDNA	92.04	0.9988	79412.90	15408.50	39985.77	818.88	41285.58	4472.99	40635.67	919.10	48.83
			9810.36	n/a	3734.65	n/a	4612.77	n/a	4173.71	620.92	57.46
										Average	51.31

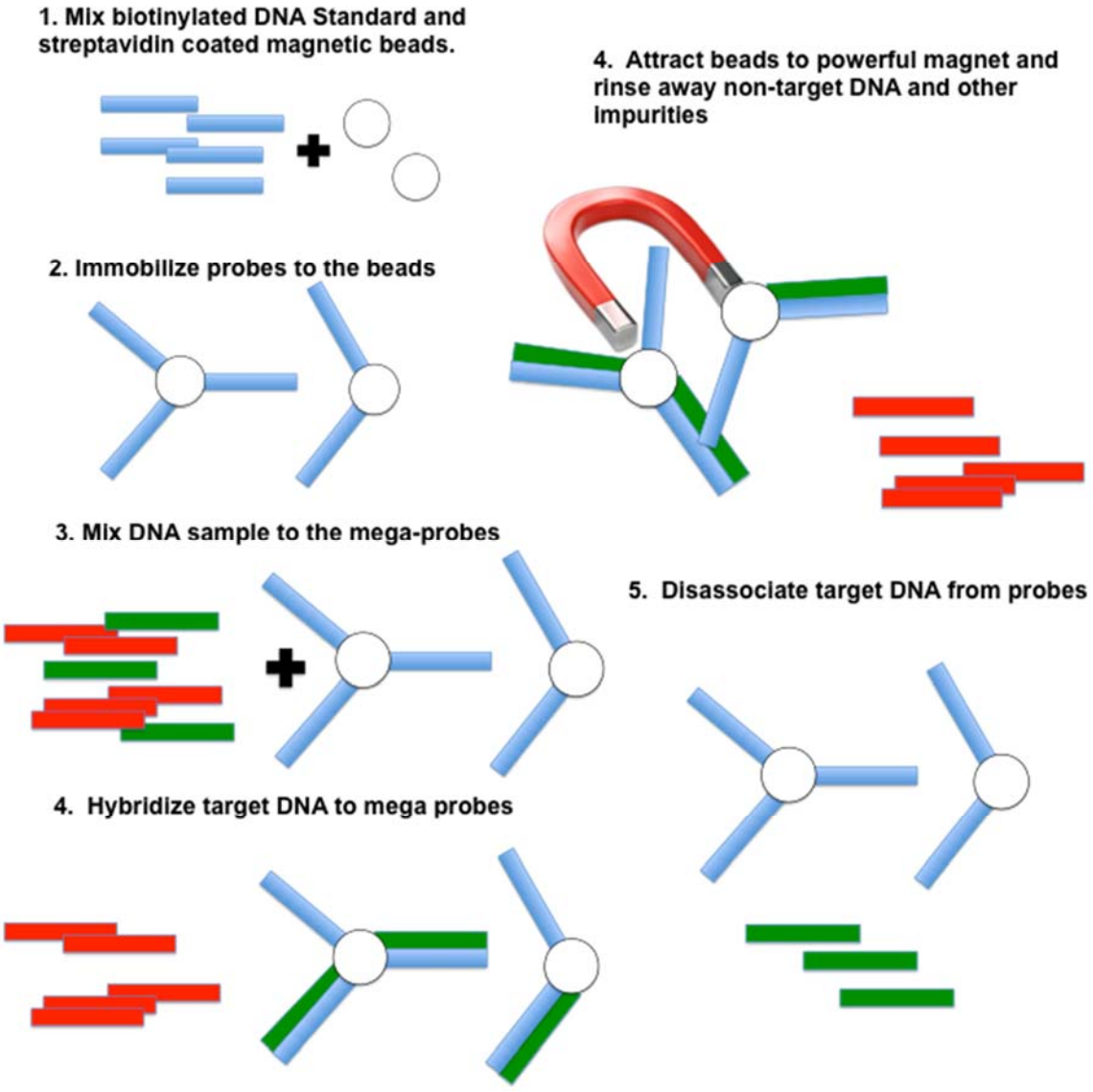
Table 4. Loss of Amelogenin, D13s317, D16s539, and northern fur seal mitochondrial DNA (NSF mtDNA) molecules purified by Qiagen MinElute PCR Purification Kit following subjection to 37°C for 30 minutes.

					Standard 1		Standard 2		Average		
		R^2	Copies In	SD	Copies Out	SD	Copies Out	SD	Copies Out	SD	% Loss
Amelogenin$_E$	96.50	0.9990	109491.00	2601.30	45645.48	2859.84	42972.12	5295.14	44308.80	1890.35	59.53
			10445.10	1410.22	2429.00	329.43	3071.35	30.51	2750.17	454.21	73.67
D13s317	94.80	0.9984	93568.80	13842.40	44799.23	n/a	41970.38	n/a	43384.81	2000.30	53.63
			9239.72	609.00	2963.44	208.00	3250.19	34.29	3106.82	202.76	66.38
D16s539	97.10	0.9990	128737.00	3534.84	54235.67	150.80	51800.19	1462.62	53017.93	1722.14	58.82
			12879.10	485.00	3346.28	319.79	4052.28	7.14	3699.28	499.22	71.28
NFS mtDNA	92.04	0.9988	79412.90	15408.50	38022.12	n/a	34478.08	n/a	36250.10	2506.01	54.35
			9810.36	n/a	2382.46	49.80	2739.90	382.30	2561.18	252.75	73.89
										Average	63.94

Table 5. Loss of Amelogenin, D13s317, D16s539, and northern fur seal mitochondrial DNA (NSF mtDNA) molecules purified by Qiagen MinElute® PCR Purification Kit following subjection to 65°C for two nights.

					Standard 1		Standard 2		Average		
		R^2	Copies In	SD	Copies Out	SD	Copies Out	SD	Copies Out	SD	% Loss
Amelogenin$_E$	94.97	0.9991	109491.00	2601.30	25622.60	341.99	40484.52	1928.91	33053.56	10508.97	69.81
			10445.10	1410.22	3285.85	78.41	3650.63	389.63	3468.24	257.94	66.80
D13s317	94.39	0.9990	93568.80	13842.40	24188.56	424.67	37541.54	n/a	30865.05	9441.98	67.01
			9239.72	609.00	2870.63	150.26	3821.10	485.51	3345.87	672.08	63.79
D16s539	92.01	0.9991	128737.00	3534.84	27149.04	83.22	45039.71	2301.22	36094.38	12650.62	71.96
			12879.10	485.00	3546.63	136.35	4735.40	105.90	4141.01	840.59	67.85
NFS mtDNA	88.09	0.9987	79412.90	15408.50	20007.69	923.05	31290.58	n/a	25649.13	7978.20	67.70
			9810.36	n/a	2484.29	14.88	3192.18	551.94	2838.24	500.56	71.07
										Average	68.25

Figure 1. Simplified Schematic of Mega-Probe Capture [largely following Maricic et al (2010)]. Target DNA molecules are green (in this study, representing the 16 fragments targeted by PowerPlex 16® System), non-target molecules are red (in this study, representing NFS mtDNA), biotinylated mega-probes are blue, and the streptavidin coated magnetic beads are white.



DISSEMINATION OF RESEARCH FINDINGS

Publications

2014 **Kemp BM**, Winters M, Monroe C, and Barta JL. "How Much DNA is Lost? Measuring DNA Loss of STR Length Fragments Targeted by the PowerPlex 16® System using the Qiagen MinElute® Purification Kit." *Human Biology* 86(4): 313-329.