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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% to 60.56% (mean 39.03%), which is not congruent with Qiagen's claim that 80% of 70 bp to 4 kb fragments are retained using this product (i.e., 20% loss). Our study also found no clear relationship between neither DNA strand length and retention, nor 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; 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., 2014; 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).

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.

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 (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), 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).

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 an 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 fragments of 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 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 in library purification and/or enrichment for high throughput sequencing (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; 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., 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:

number of copies = [amount (ng) * 6.022×10^{23}] / [length (bp) * 1×10^{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 $1x10^7$, $1x10^5$, $1x10^4$, $5x10^3$, $2x10^3$, $1x10^3$, $5x10^2$, $2.5x10^2$, $1x10^2$, $5x10^1$, $1x10^1$ copies/µL. For example, the $1x10^5$ dilution contained $1x10^5$ copies of each of the sixteen amplicons per µL (totaling $1.6 x10^6$ 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 $1x10^5$ to $1x10^1$ 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 $1x10^7$, $1x10^5$, and $1x10^4$ 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 $1x10^7$ extracted standards was conducted in duplicate against

standard curves, generated from two reactions each of $1x10^7$, $1x10^5$, $1x10^4$, $5x10^3$, $2x10^3$, $1x10^3$, $5x10^2$, $2.5x10^2$, and $1x10^2$. Two additional $1x10^7$ standards were quantified as unknowns in order to monitor the concentration of the pre-extracted standard. Quantification of the molecules that remained in the $1x10^5$ and $1x10^4$ extracted standards was similarly conducted, but against standard curves generated from $1x10^5$, $1x10^4$, $5x10^3$, $2x10^3$, $1x10^3$, $5x10^2$, $2.5x10^2$, and $5x10^1$ reactions. Two additional $1x10^5$ and $1x10^4$ 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 $1x10^4$ standards ranged from 34.68% (SD 5.2%) to 60.56% (SD 1.84%), the $1x10^5$ standards ranged from 34.83% (SD 5.25%) to 54.28% (SD 4.72%), and the $1x10^7$ standards ranged from 21.75% (SD 2.7%) to 41.17 (SD 1.86%) (Appendix A, 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. 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 this problem, 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 are retained (i.e., only a 20% loss) by the MinElute PCR Purification Kit. This might be a product of some 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]. During revision of our manuscript we tested whether mixtures of $1x10^7$, $1x10^5$, and $1x10^4$ 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 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 ($1x10^{10}$). 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 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). This makes it obvious to us that reality is more complex than any model being proposed.

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	11, 11	288	96.77	0.9989	96.07	0.9982

	GTTTGTGTGTGCATCTGTAAGCATGTATC						
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

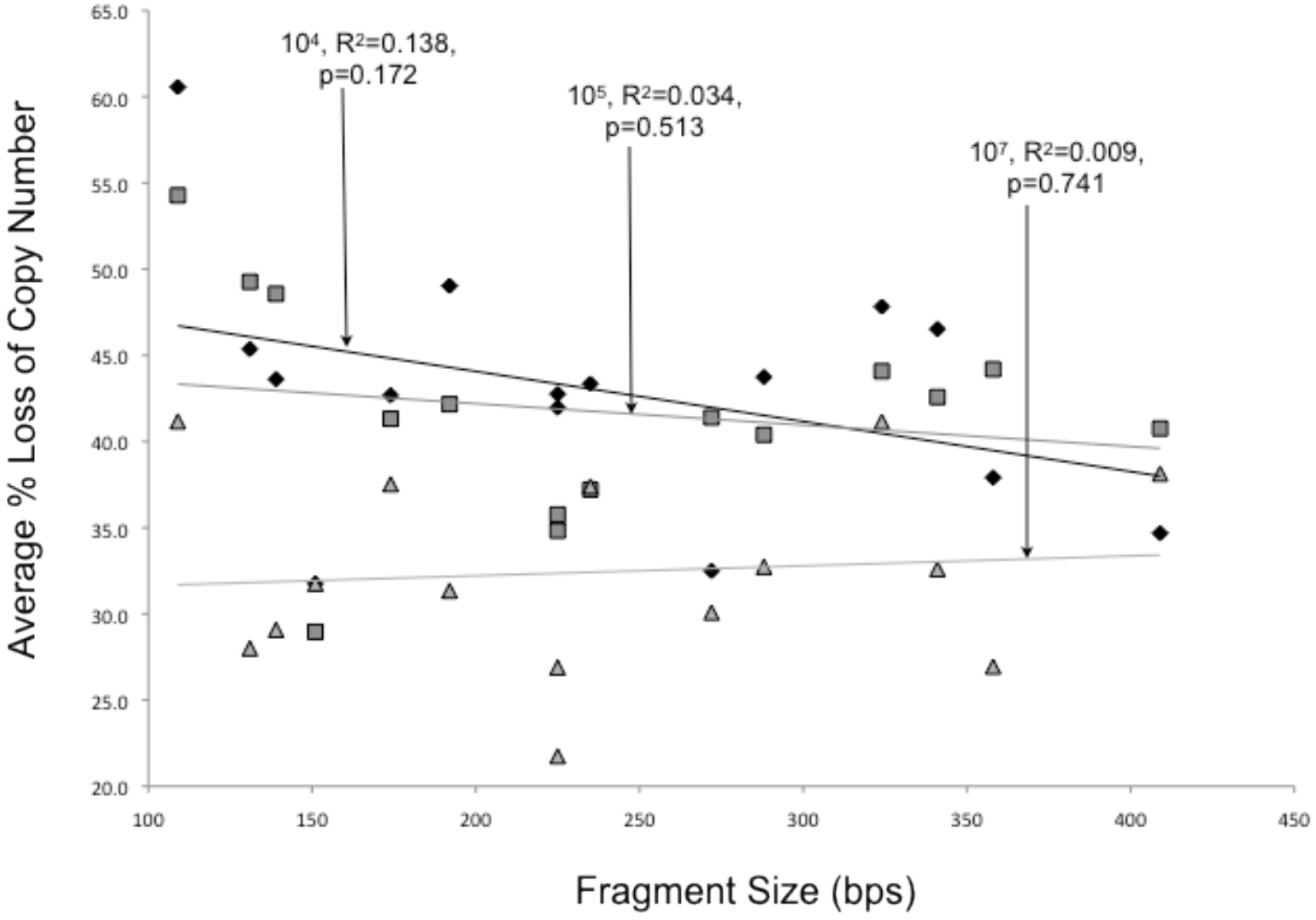
TABLE 2. Summary of percent DNA loss across all fifteen markers evaluated from three standards. See Appendix A for details.

		Percentage loss of $1X10^4$ standard						Percentage loss of $1X10^5$ standard						Percentage loss of $1X10^7$ 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**				**Average loss:**		**41.72**				**Average loss:**		**32.44**	

*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. 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. Quantitative PCR results. These data are summarized in Table 2

(Continued)

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. Quantitative PCR results. These data are summarized in Table 2

(Continued)

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		