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Evaluation of Modified Yfiler™ Amplification Strategy for Compromised Samples

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Aim To characterize the data produced using a modified amplification protocol for the AmpFℓSTR® Yfiler™ PCR Amplification Kit (Applied Biosystems) and explore the potential of Y-chromosomal short tandem repeat (Y-STR) recovery from severely degraded skeletal remains encountered at the Armed Forces DNA Identification Laboratory.

Methods Experiments were performed using two sets of Yfiler™ amplification parameters. One set of parameters reflected the manufacturer's recommendations. The second set of parameters included twice the recommended Taq concentration and 6 additional cycles. Recovery of authentic alleles and the incidence of drop-in alleles were assessed for 3 data sets: 8 different quantities of pristine DNA, 8 artificially-degraded samples, and 31 non-probative case samples.

Results Samples tested with both protocols from all 3 data sets yielded twice as many authentic alleles under the modified parameters than under the standard parameters (62% vs 31%), with only a nominal associated increase in the occurrence of non-authentic alleles (1.36% of all alleles detected). When applied to a range of representative casework samples, the modified protocol leveraged 9 or more reproducible alleles from over half of the specimens tested.

Conclusion Reproducible and informative Y-STR profiles can be recovered from a broad range of degraded and inhibited skeletal remains extracts when a commercially available kit is employed under modified amplification parameters.

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The identification of degraded skeletal remains at the Armed Forces DNA Identification Laboratory (AFDIL) is primarily achieved by mitochondrial DNA (mtDNA) typing. The cases typically undertaken by AFDIL involve severely compromised skeletal remains, and thus mtDNA offers the greatest chance of success due to its high copy number and relative abundance as compared with nuclear DNA. mtDNA data are also particularly useful in these types of historical forensic investigations because the molecule is maternally inherited without recombination. As a result, all maternal relatives possess identical mtDNA haplotypes (in the absence of mutation) and thus even distant relatives can provide reference material for comparison. This is extremely beneficial in cases of missing persons when references from immediate family members are unavailable for standard short tandem repeat (STR) testing.

Despite these obvious benefits, the forensic value of mtDNA can be limited in particular cases by the very characteristics that make it such a powerful marker in general. For strict identification purposes, the marker's practical utility is dictated by the availability of either direct or maternal reference samples. In some cases, these types of references are simply unavailable, preventing mtDNA associations to particular families, and thus identifications, from being made. In addition, because of the molecule's mode of inheritance, a number of common mtDNA (hypervariable region I and II) haplotypes exist in any given population (1-3). Individuals sharing a common type cannot be distinguished without additional genetic information; and although specific single nucleotide polymorphisms (SNP) in the coding region of the mitochondrial genome can often be used to resolve individuals, discrimination is not always achieved. Under these circumstances, mtDNA data may be of limited use and markers in the nuclear genome offer the only alternative source of genetic information.

Historically, the poor quality and limited quantity of nuclear DNA present in severely degraded skeletal remains has restricted the use of autosomal and Y-chromosomal STR data in cases involving severely aged and/or compromised remains. However, we have recently highlighted the potential of recovering nuclear DNA from degraded skeletal elements by using commercially available STR kits under modified reaction parameters and amplification conditions (4,5). The protocol employed was based on methods previously described for both forensic DNA applications, where only low levels of nuclear DNA may be recovered from trace evidentiary material (6-11), and ancient DNA applications, where nuclear DNA tends to be limited in quantity, highly fragmented, and also severely affected by cross-linkage, deamination, dimers, and other insults resulting from extensive environmental exposure and mortuary treatment (12-21). Generally speaking, these methods employ additional polymerase chain reaction (PCR) cycles in an effort to increase sensitivity to the relatively few template molecules present in the reaction. For AFDIL's specific application, the cycle number and Taq concentration were increased. Additional Taq was included so that input extract volume and thus allele sampling and recovery could be maximized in any given amplification. AFDIL primarily deals with bone specimens that allow for multiple samplings and extractions and thus permit the liberal use of extract. However, large volumes of these skeletal extracts tend to produce polymerase inhibition resulting from co-extracted humic and fulvic acids, as well as co-extracted calcium and collagen (22). Thus, additional polymerase was required to offset this inhibition. The results from this work showed great promise (4,5), particularly when the modified amplification was coupled with an improved DNA extraction (23).

In this study, we report data that further demonstrate the potential of recovering reproducible and informative Y-STR data from severely compromised skeletal remains.For AFDIL's specific purposes, one of the greatest potential benefits of Y-testing is the fact that the pool of family references can be expanded to include even distant paternal relatives. This is of great importance in these decades-old cases for which family references may not be available for standard autosomal or mitochondrial DNA comparisons. In addition, Y-STRs would find immediate utility when used for the sorting and re-association of skeletal remains. In this context, information from Y-markers would be valuable in further resolving commingled skeletal elements originating from multiple individuals. Currently, this type of sorting and re-association is based exclusively on mtDNA information. Bones sharing mtDNA haplotypes are grouped, and those that share unique or rare types are generally assumed to represent a single person. However, when common mtDNA haplotypes are encountered among skeletal assemblages clearly representing multiple individuals, bone:bone re-association cannot be made on the basis of mtDNA data alone. It is in these cases that Y-STR data – even partial Y-profiles – are likely to provide valuable additional information to help segregate individuals. Here, we present experiments conducted in an effort to further characterize the data produced with the AmpFℓSTR® Yfiler™ PCR Amplification Kit (Applied Biosystems, Foster City, CA, USA), using the modified amplification

protocol and further explore the potential of Y-STR recovery from a broad range of AFDIL casework material.

MATERIALS AND METHODS

PCR amplification and electrophoresis

Standard and modified amplifications were conducted using the AmpF*l*STR® Yfiler™ PCR Amplification Kit. This kit includes 17 loci located on the non-recombining portion of the Y-chromosome and targets amplicons ranging in size from 90 to 330 base pairs (bp). According to sensitivity tests described by Mulero et al (24), the optimal template range of the kit is 0.5 to 1.0 ng, though full profiles can be obtained with DNA quantities as low as 0.125 ng using the protocol recommended by the manufacturer (25). For standard reactions, volumes and amplification conditions as defined in the AmpF'STR® Yfiler™ PCR Amplification Kit User's Manual were followed (25). The modified reactions maintained the 25-µL total reaction volume of the standard protocol but the recommended Taq concentration was doubled and thus a maximum volume of 9.2 µL of template DNA could be added. The modified thermal cycling conditions consisted of 6 additional cycles for a total of 36 instead of the standard 30 cycles suggested by the manufacturer. All post-amplification procedures were performed according to the manufacturer's recommendations. Samples were separated on a 3130*xl* Genetic Analyzer (Applied Biosystems).

Laboratory procedures were performed according to ancient DNA guidelines intended to minimize contamination (26,27). In order to monitor contamination, reagent blanks and negative controls were carried through all steps of the process. In addition, all amplification products were compared with a staff Y-STR database.

Pristine low copy DNA

The sensitivity of the modified typing strategy was evaluated using dilutions of Raji (200 ng/µL), the human cell line used as the standard in the Quantifiler™ Human DNA Quantification Kit (Applied Biosystems). Serial stock dilutions were prepared down to 1 pg (125, 62, 31, 16, 8, 4, 2, 1 pg) and 5 replicates of each dilution were amplified with both the modified and standard protocols. Average allele recovery across all amplifications was calculated for both protocols. In addition, to assess how reproducibly alleles could be recovered at each DNA input quantity, all combinations of 3 amplifications were evaluated. Alleles observed at least twice in each set of 3 amplifications were included in a "consensus" profile, and then the average "consensus" allele recovery from the various replicate combinations was tabulated.

The amplifications of diluted, pristine DNA were also used to evaluate stutter products generated with the modified protocol and establish minimum peak height thresholds for reportable alleles.

Artificially degraded samples

Eight artificially degraded saliva samples were obtained by AFDIL as part of an investigative study organized by the European DNA Profiling Group. Swabs soaked with saliva from 2 different male contributors were degraded over 1, 2, 12, and 72 weeks by means similar to those described by Dixon et al (28). The samples were extracted using a standard phenol chloroform extraction (29) and then concentrations were determined by Quantifiler. Due to limited extract quantities, an input volume of 5 µL was utilized for both the standard and modified Yfiler™ amplification reactions of the degraded samples. In addition, only 2 amplifications could be performed for both protocols and 1 extract had no remaining volume to conduct any standard reactions.

Non-probative case samples

To evaluate the utility of the modified Yfiler™ protocol on typical AFDIL case samples, 31 skeletal elements corresponding to 15 individuals were selected for testing. The samples ranged in age from 40-50 years and varied greatly in terms quality. Only samples for which suitable Y reference material was available for comparison were selected, and thus the degraded skeletal elements used for this portion of the study were effectively controls. A total of 17 family reference samples (for 2 individuals, 2 references were available for each) were used to confirm the Yfiler™ results obtained from the skeletal specimens under modified amplification parameters.

Skeletal remains were extracted according to the protocol of Loreille et al (23), but with a final volume of 200 µL. All skeletal extracts were quantified with Quantifiler. However, because the Quantifiler™ assay tends to overestimate the quantity of amplifiable DNA templates in degraded extracts as a result of its small amplicon, the values were not used to determine optimal extract input. Instead, the Quantifiler™ data were evaluated following acquisition of

Yfiler™ data to determine whether or not the quantitation results provided any gauge of Yfiler™ amplification success. In addition, the Quantifiler™ internal positive control cycle threshold (IPC C_T) was used to gather some information on PCR inhibitors on the extracts. All inhibited extracts (sample IPC C_T 1 cycle greater than the standard IPC C_T) were subsequently diluted for final quantitation and the values reported represent only those assays in which the IPC was not inhibited, unless otherwise noted.

Optimal extract input volume per amplification was determined on a sample-by-sample basis, and assumed no additional purification or concentration of extracts. Preliminary amplification reactions were performed using extract input volumes of 1, 5, and 9.2 µL. Based on the results from the preliminary reactions, 3 modified amplifications were then conducted using the maximum possible input volume that did not also produce polymerase inhibition. In order to directly compare the modified protocol with the standard protocol on typical casework material, 6 of the 31 skeletal samples were also amplified with the standard Yfiler™ protocol. For these amplifications, the same range of input volumes used for the modified protocol was also used for the standard protocol.

Data analysis

Data were analyzed using GeneMapper® ID version 3.2 (Applied Biosystems) and alleles were assigned using the allelic ladder provided by the manufacturer. Based on the sensitivity data, a minimum peak height threshold of 75 relative fluorescent units was established for assigning alleles and peaks in a stutter position (plus and minus 1 repeat unit) were removed if the peak height ratio was less than 0.3. This stutter value is higher than values suggested by the manufacturer and was required to capture elevated stutter artifacts that are known to result from aggressive amplification protocols and low DNA input quantities (8,10,16). The 30% filter encompassed over 90% of all stutter peaks observed in the data generated for this study (data not shown).

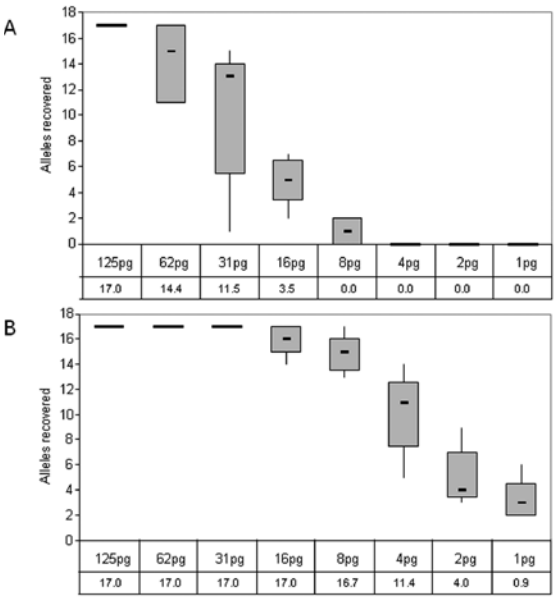
For the artificially degraded samples and non-probative case samples, all amplifications were conducted in either duplicate or triplicate. Only duplicated alleles observed were included in any finalized profiles in order to confirm data authenticity (8,10,16). In addition to allele recovery, amplifications from all 3 data sets were inspected for the occurrence of spurious, or so-called "drop-in," alleles. For our purposes and in the interest of being conservative, spurious alleles included stutter peaks if the peak height ratio was greater than the modified stutter threshold (defined as 30%). All other false alleles that could not be explained by stutter artifacts were attributed to sporadic allele contamination.

RESULTS

Pristine low copy DNA

Results from the amplification replicates of serially-diluted positive control DNA are shown in Figure 1. The results reflect a considerable increase in allele recovery with the modified amplification parameters at all DNA input quantities tested. With the modified protocol, complete Yfiler™ profiles (17 loci) were obtained for all amplification replicates with DNA input quantities of 31 pg or more. In addition, profiles consisting of 9 or more loci were regularly recovered from DNA quantities as low as 4 pg. In contrast, under standard Yfiler™ amplification conditions, complete 17-locus profiles were not observed consistently at any input quantity less than 125 pg and allele recovery per am-

Figure 1.



Allele recovery observed for (**A**) standard and (**B**) modified typing of various input quantities of Raji cell line DNA. A total of 17 alleles were expected. The number of alleles recovered per amplification (top portion of each panel) and the average number of consensus alleles obtained for all combinations of 3 amplifications (lower portion of each panel) are shown.

plification dropped to an average of 5 at DNA input quantities less than 31 pg. Overall, allele recovery was more than doubled with the increase in cycle number and Taq concentration. When all possible combinations of 3 amplifications (10 total) were evaluated for allele duplication, the modified protocol resulted in complete, 17-allele consensus profiles from DNA input quantities down to 8 pg. In comparison, the standard protocol produced no consensus alleles from 8 pg or less, and only at an input of 125 pg was a full profile of duplicated alleles obtained.

The greater sensitivity of the modified approach was not only reflected in a greater recovery of authentic alleles, but also in increased detection of contaminant DNA and greater susceptibility to amplification artifacts. While no spurious alleles were observed in the 35 standard amplifications, 4 non-authentic alleles were observed in the 35 modified reactions (0.11 per amplification). The alleles were seen at 4 loci (DYS19, DYS390, DYS456, YGATAH4) in 4 different amplifications of 8 pg or less and are shown in Table 1. Three of these alleles occurred in stutter positions, which would generally offer a clear explanation of their origin. However, one of these alleles was observed in the absence of the authentic allele and another allele exceeded the adjacent (authentic) allele's peak height. These features seem to reflect sporadic contamination as much as stutter artifacts; however, Whitaker et al (10) note that it is theoretically possible for a stutter peak to amplify in the absence of the authentic allele. Regardless of their source, the alleles were not reproducible among amplifications. Furthermore, no drop-in alleles were observed in any of the 7 negative controls amplified using the modified protocol.

Artificially degraded samples

Allele recovery differences between the modified amplification approach and the standard amplification approach on artificially degraded samples are shown in Figure 2. Based on the Quantifiler-determined DNA input quantities, results from both protocols were inconsistent with the results observed among the sensitivity data, but is also not surprising given the size of the Quantifiler™ amplicon and the degraded state of these samples. In particular, large DNA inputs (>200 pg) did not necessarily yield complete Yfiler™ profiles with either protocol. In addition, and contrary to our observations from authentic casework mate-

TABLE 1. **Unauthentic alleles observed in the modified amplifications of pristine, artificially degraded and non-probative case samples**

Sample set	Sample	Replicate	Loci	Expected	Observed	PHR (%)*
Pristine	Raji (8pg)	4	DYS390	21	21,23	53
	Raji (4pg)	2	DYS19	15	14	–
	Raji (4pg)	5	GATA	12	11,12	73
	Raji (2pg)	2	DYS456	13	13,14	121
Degraded	Male 2 (1wk)	2	DYS389II	31	29,30,31	91,170
Case	Sample 1a	1	DYS392	13	14	–
	Sample 1a	2	DYS439	11	10,11	67
	Sample 3a	3	DYS635	21	24	–
	Sample 3b	1	DYS393	13	13,14	71
	Sample 3b	2	DYS385	18,18	18,19	86
	Sample 4a	2	DYS439	12	11	–
	Sample 4c	3	DYS437	15	17	–
	Sample 5a	2	DYS456	16	15,16	43
			DYS385	11,14	12,14	15
	Sample 5d	1	DYS458	17	16,17	48
	Sample 8a	1	DYS385	11,14	10,14	90
	Sample 10a	2	DYS390	22	25	–
	Sample 10a	3	DYS389II	29	31	–
	Sample 11a	3	GATA	12	11	–
	Sample 12c	1	DYS389II	30	28,30	122
	Sample 12d	1	DYS390	25	24,25	32
	Sample 13a	3	DYS437	15	15,16	75
	Sample 14a	2	DYS458	18	17,18	96

*PHR – peak height ratio.

rial, we found that the larger loci were not necessarily the ones consistently dropping out when incomplete profiles were obtained under the modified parameters. We suspect that this is due to the extremely sensitive conditions of the modified assay. Even when the larger templates are in much lower copy number than the smaller templates, the assay can still recover the large alleles.

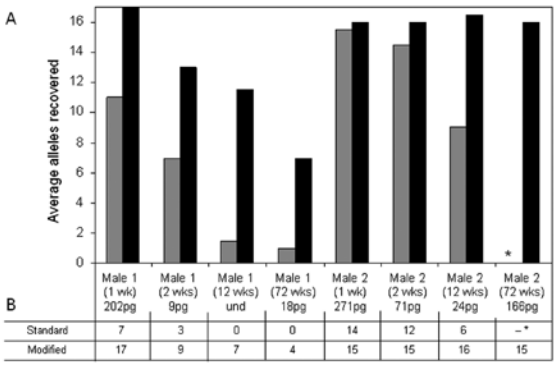
Figure 2A summarizes the average number of alleles recovered using both protocols and reflects the increase in allele recovery with the modified protocol. For Male 1, the modified approach yielded over twice as many alleles across all degradation stages. For example, only 7 and 3 alleles could be replicated under standard conditions while the modified parameters produced 17 and 9 reproducible alleles, respectively. Similarly, the standard protocol exhibited clearly diminished recoveries for Male 2, while the modified protocol produced nearly full profiles regardless of degradation interval. The difference between protocols was also evident in the consensus profiles shown in Figure 2B.

Of the 16 modified amplifications performed with the artificially degraded samples, 2 drop-in alleles resulting from elevated stutter were observed (Table 1). The 2 alleles occurred in a single reaction, and both occurred at locus DYS-389II. The artifacts represented 91% and 170% of the true allele and were found, respectively, at -8 and -4 bps from the authentic peak (Figure 3B). As with the drop-in alleles observed in the sensitivity data, neither allele was reproduced in replicate amplifications and no other drop-ins originating from sporadic contamination were observed. Allele drop-in was not observed in any standard amplifications or in the modified amplifications of negative controls and extraction blanks.

Non-probative case samples

In order to assess the practical implications of the increased data recovery observed among sensitivity and artificially degraded sample data, extracts from 31 skeletal elements were amplified under modified Yfiler™ conditions. Six of the 31 samples, representing a range of qual-

Figure 2.



Allele recovery observed for standard and modified typing of 8 artificially degraded samples. A total of 17 alleles was expected for each sample. The average number of alleles recovered in two replicates (**A**) and the number of alleles duplicated in both amplifications (**B**) are shown. The template input based on Quantifiler™ results is listed below the sample identifier. The concentration for the Male 1 (12 weeks of degradation) extract could not be determined by Quantifiler™ and therefore the DNA input is unknown. DNA inputs of less than 100 pg may be unreliable, as they reflect extract concentrations either in or below the lower dynamic range of the Quantifiler™ assay. Gray bars – standard typing; closed bars – modified typing, asterisk – due to limited extract volume, no standard amplifications were conducted for the Male 2 sample degraded over 72 weeks.

TABLE 2. Six non-probative case samples typed using both the standard and modified protocols with input volumes of 1, 5, and 9.2 µL*

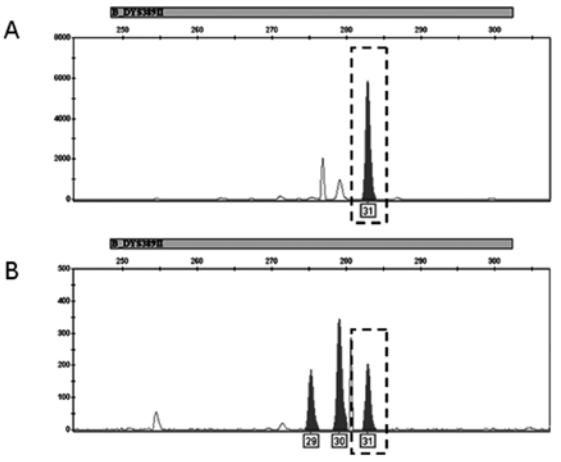
Sample	Concentration (ng/µL)†	Inhibition‡	Standard protocol 1 µL	5 µL	9.2 µL	Best standard profile recovery (%)§	Modified protocol 1 µL	5 µL	9.2 µL	Best modified profile recovery (%)§
1a	0.0184	mild	0	1	0	6	7	10	11	65
2c	0.1020	severe	8	10	7	59	16	17	17	100
3b	0.0096	mild	1	0	1	6	0	3	9	53
4b	0.0939	none	0	4	8	47	7	14	15	88
14a	0.0710	severe	1	0	0	6	15	7	0	88
15a	3.0000	none	10	12	17	100	17	17	17	100

*The expected Yfiler™ haplotypes for all 6 extracts contain 17 alleles.
†The concentrations shown are based on uninhibited Quantifiler™ reactions generated from dilutions (1:2 or 1:10) of those extracts that showed inhibition in real-time polymerase chain reactions of undiluted extract.
‡Sample IPC C_T values less than 1 cycle different from the control internal positive control cycle threshold (IPC C_T) cycles were assumed to be free of inhibitors. Samples with an IPC C_T 1-3 cycles greater than the controls were classified as mildly inhibited, samples with an IPC C_T 3 cycles or more were classified as moderately inhibited and samples with an undetermined IPC C_T were classified as severely inhibited.
§The best profile recovery (%) for each protocol represents the greatest number of alleles observed with any of the three input volumes divided by the expected number of alleles (17).

ity, were also typed with the standard protocol in order to directly gauge the benefit of the alternate PCR conditions (Table 2). Of the 6 samples amplified under both standard and modified conditions, only 1 sample produced a complete Yfiler™ profile under standard parameters. This was the only sample that exhibited no inhibition, as well as a high quant value (3.0 ng/μL), in the real-time assay. Nevertheless, recovery of the 17-locus profile required the maximum input volume of 9.2 μL under standard amplification conditions. Under modified conditions, a complete profile was recovered for this sample at all extract input volumes. Profiles generated from the other 5 samples exhibited large differences in allelic recovery when the two protocols were compared. Samples that were either inhibited (as evidenced by elevated or undetermined IPC C_T values) or low in DNA quantity demonstrated a substantial increase (41%) in allele recovery. However, the greatest difference between protocols was observed in samples exhibiting both inhibition and low DNA quantity. For these samples, the maximum number of alleles obtained with the modified protocol was as much as 82% greater than with standard amplification. Overall, the combination of additional cycles and increased Taq resulted in a marked improvement in haplotype recovery for compromised case samples tested with both protocols.

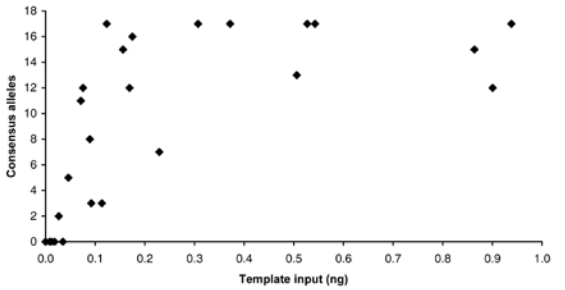
A few general comments can be made regarding amplification success with respect to the Quantifiler™ determined DNA concentrations in the non-probative case samples. The results are shown in Figure 4 and suggest that complete, or nearly complete profiles can often be obtained when more than 250 pg of Quantifiler™ measured DNA are amplified. While this result is not unexpected with 250 pg of pristine DNA (particularly amplified under "low copy number" conditions), it does suggest that when large quantities of DNA are detected by Quantifiler™ in significantly degraded samples/extracts, there is a decent chance for recovering a nearly complete STR profile despite issues relating to the Quantifiler™ amplicon size. In other words, a large quantity of highly fragmented DNA suggests that in many cases, but certainly not all, there will be enough larger templates to produce a nearly complete profile with the modified Yfiler™ protocol. The plot also suggests that when DNA input is below 100 pg, the case samples do not mirror the sensitivity data in terms of consensus alleles recovered (30). The case samples tested in this study produced far less data at comparable DNA input quantities, yielding only between 0 and 12 loci with less than 100 pg. We should mention here that DNA input values of 100 pg or less are primarily based on real-time determined concentrations of 10 pg/μL or less – which is below the reliable range of 23 pg/μL for the Quantifiler™ assay. However, the values were actually quite reproducible between real-time amplifications of dilutions and replicates of the various extracts. Nevertheless, the results should be viewed with some caution given the uncertainty in the quant values.

Figure 3.



Electropherograms for the (**A**) first and (**B**) second modified amplifications for artificially degraded sample Male 2 (1-week degradation) at Yfiler™ locus DYS389II. The authentic allele is indicated by the dashed box in each replicate.

Figure 4.



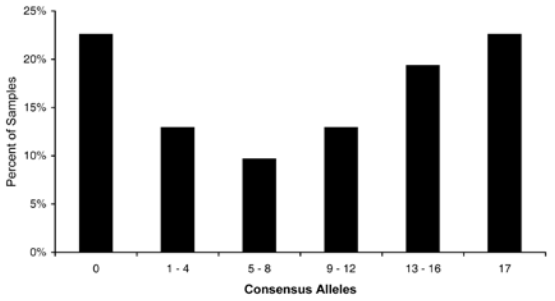
The number of consensus alleles recovered based on the amount of DNA input for 26 non-probative case samples. Input DNA values are based on the optimal input volume and the concentration established by uninhibited Quantifiler™ amplifications. Two outliers with greater than 3.0 ng of input DNA are not included in the plot. Three other samples were excluded because they showed inhibition, but could not be diluted and re-quanted due to limited extract volume. The two data points located on the y-axis represent samples with concentrations that could not be determined by Quantifiler. They produced no reproducible alleles.

TABLE 3. Optimal extract input volumes for Yfiler™ compared to the degree of inhibition observed with Quantifiler*

Degree of inhibition	Δ IPC C_T†	Input volume		
		1 µL	5 µL	9.2 µL
No to mild	<3 cycles	3	0	15
Moderate	≥3 cycles	0	1	3
Severe	undetermined	1	0	4

*Four of the 31 non-probative case samples were limited by extract volume, which did not permit the optimal input volume to be determined. They are not included in this table.
†IPC C_T – internal positive control cycle threshold.

Figure 5.



Number of alleles obtained in the finalized, consensus profile of 31 non-probative case samples.

Generally speaking, the scatterplot suggests that real-time quantification of the aged, degraded skeletal elements typically encountered at AFDIL serve as the best indicator of Yfiler™ success when the detected DNA quantities are large. In those cases, not surprisingly, some success with the modified protocol may be expected. When DNA quantities are low, however, Yfiler™ results are more difficult to predict but not necessarily poor.

The relationship between the degree of inhibition and the optimal reaction input volume utilized for modified amplifications of the non-probative case samples is shown in Table 3. Optimal input volume determinations were made on the basis of preliminary tests conducted using a range of extract volumes (1, 5, and 9.2 µL) that assessed maximum input and the effect of inhibitors. In only one case was it necessary to minimize extract volume in order to avoid inhibition. The remaining 3 samples requiring only 1 µL were not inhibited, but exhibited such high DNA concentrations that extract had to be minimized to prevent saturation. For the remaining 81% of the samples, the maximum input (9.2 µL) did not produce inhibition, and yielded the most data. Overall, the modified Y-STR typing strategy produced consensus profiles representing over half of the total possible alleles for 17 of the 31 case samples (Figure 5). Of these, full Yfiler™ profiles were generated for 7 samples. Seven of the remaining 14 samples resulted in consensus profiles of 8 alleles or less, whereas the final 7 samples resulted in no reproducible/reportable alleles.

Spurious alleles were observed in 18 of the over 100 modified amplifications (0.16 per amplification) of the non-probative case samples. The details of the drop-in alleles are shown in Table 1. Aside from one instance, no more than a single false allele was present in any given amplification. In addition, the majority of spurious peaks fell in stutter positions and can likely be attributed to elevated, or in some cases preferential, amplification of stutter artifacts. Interestingly, however, in one-third of these cases, the corresponding authentic allele was absent. Although we cannot definitively establish the source of these peaks (stutter vs contamination), neither they, nor the remaining drop-ins originating from sporadic contamination, were reproducible.

When finalized profiles reflecting duplicated, "consensus" alleles were compared with family reference data, all consensus alleles were concordant with their corresponding family reference profile(s), with one exception reflecting a mutation event at DYS438. In this particular case, 2 paternal references were available for comparison to the skeletal remains – a nephew and a cousin of the missing male. While haplotypes from the case sample and the paternal male cousin were consistent at all loci, the haplotype from the paternal nephew differed by a single tetranucleotide repeat unit at DYS438. All other loci were concordant. Both haplotypes (regardless of the allele at the discordant locus) were unique in the US Y-STR Database (31), and thus the most plausible explanation for the inconsistency is mutation. Given an average mutation rate estimate of 0.6×10^{-3} (32,33) for DYS438, a mutation at this locus can be expected in approximately 1 of every 555 uncle-nephew pairs.

DISCUSSION

We characterized a modified amplification protocol for the typing of Y-chromosomal STRs from casework material typically encountered at AFDIL and extracted via the protocol of Loreille et al (23). The approach is intended for use with aged, degraded skeletal remains, which differ in a number of respects from other forensic evidentiary samples, harboring very little DNA. Specifically, the remains typically encountered by AFDIL differ with respect

to the quantity of sample available for testing, the presence of polymerase inhibitors that are co-extracted with the DNA, and the types of DNA damage and/or degradation encountered. The quality of extracts from these types of specimens varies dramatically on a sample-by-sample basis and also depends fundamentally on the extraction protocol used (as well as any subsequent extract purification or concentration). The protocol described here targets a broad range of extract quality and specifically addresses inhibition. As a result, it slightly differs from other protocols that have been developed for samples and/or extracts that are largely uninhibited, but limited in quantity (8,10,11).

Taken as a whole and considering all alleles observed, data from the 3 sample sets illustrate that average allele recovery is significantly greater ($P < 0.001$) using the modified protocol. While only 31% of all possible alleles were recovered under standard conditions, 62% of all possible alleles were recovered with the modified Yfiler™ protocol applied to all 3 sample types. Although our data did exhibit stochastic artifacts that are known to result from the amplification of low-levels of DNA, particularly under aggressive amplification conditions (8,10,16,17), the artifacts did not complicate data interpretation. In the 3 sample types tested in this evaluation (pristine low copy DNA, artificially degraded samples, and casework samples), the primary issues encountered were elevated stutter and allelic drop-out; both of which are the result of allele sampling from the extract and preferential amplification during early cycles of the PCR. Only rarely did we observe non-authentic alleles deriving from random, low-level contaminants. In fact, of the 24 total drop-in alleles observed, only 6 of them could not be explained by stutter. All of these anomalies were evident on an amplification-by-amplification basis only, and were clearly diminished when the replicates were considered (Figure 3). Elevated stutter peaks and DYS385 heterozygote imbalance were clarified by replication. Stochastic drop-in alleles reflecting both elevated stutter and sporadic contamination occurred only rarely on a per amplification basis (0.15 overall) and were never reproduced in multiple amplifications. As a result, when finalized profiles including only replicated alleles were considered from all 3 sample sets evaluated in this study, there was no increase in reproducible artifact alleles with the modified parameters. It is the case, however, that non-specific amplification of bacterial or fungal contaminants present in degraded skeletal elements may generate reproducible non-authentic alleles, particularly under sensitive amplification conditions such as those described here. Although we expected to find signs of this based on previous experience with other assays, we saw no obvious evidence in any of the casework samples tested. Furthermore, when we directly assessed the risk of non-specific amplification under the modified amplification conditions, there was no distinguishable difference between the results obtained using the modified protocol and results obtained using the standard protocol from control DNAs of bacteria, fungi, various non-human vertebrates, and a human female (data not shown).

It is also worth noting that although the sensitivity and artificially degraded samples were important for evaluating assay sensitivity and the general characteristics of profiles produced with this protocol, they were only loose proxies for authentic casework samples in terms of mirroring the assay's utility in a practical framework. These control sample sets basically model only 2 – low template quantity and DNA fragmentation – of the myriad problems affecting the skeletal elements typically encountered at AFDIL. Authentic casework remains vary so dramatically in terms of their age, quality (inhibition), bacterial and fungal contamination, and DNA damage/fragmentation state, that the only way to adequately assess the utility of an assay is to evaluate it on representative samples. When we did that, and considered data from the casework samples alone, the modified amplification approach produced 9 or more reproducible alleles in over 50% of the specimens tested and full 17-locus profiles from nearly one-fourth of all samples tested.

Overall, we recovered forensically informative Y-STR profiles from skeletal elements ranging from 40 to 50 years postmortem, that were degraded and generally subject to extreme environmental conditions and/or harsh mortuary treatments. This effectiveness can be attributed largely to the modified parameters, but also partially to the nature of the multiplex itself. The commercially available Yfiler™ kit (and, indeed, many other described Y-STR assays, ref. 34,35) is well-suited for these types of remains because the multiplex targets relatively small amplicons. The assay, therefore, provides the well-documented benefits of other described "mini-amplicon" approaches for degraded samples (36-39), although the smaller amplicons alone, in the absence of additional cycles and extra Taq, were not enough to leverage comparable profiles from the 6 casework samples tested under both conditions. We note that another distinct advantage of targeting Y-STRs is the fact that data interpretation issues resulting from allelic drop-out, allelic drop-in, and heterozygote peak imbalance in profiles generated with aggressive protocols on poor quality specimens are mitigated by the haploid nature of the marker. AFDIL deals almost exclusively with single-source samples, and

thus Y-profile interpretation tends to be relatively straightforward. Furthermore, some of the more difficult data interpretation issues encountered with autosomal "low copy number" profiles, including assignment of homozygosity and the interpretation of alleles found in stutter positions at otherwise "homozygous" loci, are restricted to just a few Y-markers (a single marker in the Yfiler™ kit).

In summary, we have demonstrated that reproducible and informative Y-STR profiles can be recovered from a broad range of sample types when a commercially available kit is employed under modified amplification parameters. Artifacts resulting from the sensitive conditions are clearly mitigated by conducting amplification replicates, but are also fairly easy to identify as a result of the haploid nature of the Y-markers assayed and the single source origin of the samples tested.

At AFDIL, Y-STR data will be useful for skeletal element sorting and re-association, as well as for missing persons identification. Although the latter effort will need to be undertaken with a heightened awareness of both Y-STR mutations and non-paternity; and although the application of any Y-chromosome based methods for identification and/or re-association will be restricted by both the quality of the evidence profile generated and the availability of appropriate reference material, the mere potential of acquiring Y-STR data promises to provide significantly more investigative options in the large-scale missing persons efforts at AFDIL.

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Official Disclaimer

The opinions and assertions contained herein are solely those of the authors and are not to be construed as official or as views of the U.S. Department of Defense and the U.S. Department of the Army.

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