A Pooling Method for Use on Low Copy Number and Historic Samples

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Abstract

Degraded skeletal remains generally contain limited quantities of nuclear DNA (nucDNA) and thus DNA-based identification efforts often target the mitochondrial DNA (mtDNA) control region due to the relative abundance of intact mtDNA as compared to nucDNA. In many cases, however, the discriminatory power of mtDNA is inadequate to permit identification and therefore, STR analysis becomes essential. Unfortunately, commercial Short-Tandem-Repeat (STR) kits such as the AmpFlSTR® Applied Biosystems® Y filer™ PCR Amplification kit (Life technologies), AmpFlSTR® MiniFiler™ PCR Amplification Kit (Life technologies) or PowerPlex16 HS PCR Amplification kit (Promega) require input DNA quantities greater than what is typically extracted from highly degraded bone samples and as a result, amplification is generally unsuccessful when using the manufacturer's instructions.

Presented here is a protocol where multiple 0.2-0.25g bone powder samples were extracted, pooled and concentrated in a way to limit humic acid competition for the silica membrane. The extracts were quantified and (LCN) Y Filer amplified before and after pooling. Results show that pooling 3 or 4 extracts produces the most significant increase in alleles meeting minimum reporting guidelines.

Introduction

The project described in this paper sought to improve the extraction of historic samples by introducing a pooling method into the process that would concentrate the extracted DNA while at the same time limiting the amount of inhibitors carried along in the extraction. This research was developed based on the idea that it would be beneficial to any groups that worked commonly with low copy number DNA samples.(3)

The K208 cases being anthropologically analyzed by Joint POW/MIA Accounting Command (JPAC) were part of the driving force behind this project. The K208 cases are two hundred and eight "caskets" that contained the remains of US service members from the Korean War were given to the US by the Democratic People's Republic of Korea between the years of 1993 and 1994. (1) Each casket appeared to contain the remains of a single individual, but anthropological and mtDNA testing identified that multiple individuals were represented in each casket. Due to the mixing of remains and poor documentation the K208 cases have become a giant puzzle that utilize multiple sources of evidence in order to identify each service member represented. DNA has helped in making identifications in the K208 cases but it has its limitations. Most often when working with ancient to historic remains it is more common to retrieve a mitochondrial DNA (mtDNA)profile instead of a nuclear profile.(1) While mtDNA was able to separate remains based on region and sequence it has limitations due to being a lineage marker. In cases lacking strong DNA evidence AFDIL will test the samples using Y-STR kits or auSTR kits to create a combined likelihood statistic to support the identification.

Methods & Materials

Instrumentation and Chemistries

Extraction:

- Demin 2 Extraction via AFDIL Standard Operating Procedure (SOP)

Process: Many of the samples were prepared and extracted in the lab prior to the research beginning. A few of the samples were extracted during the research using Demin 2.

Purification:

- MinElute Qiagen®

Process: Every sample had to go through at least one round of MinElute purification prior to analysis.

Quantification:

- Quantifiler Duo Applied Biosystems™
- Applied Biosystems™ 7500 Real-Time PCR Instrument

Process: Quantification was performed by laboratory techs that worked at AFDIL.

Amplification:

- Low Copy Number Y-Filer Applied Biosystems™
- MiniFiler Applied Biosystems™
- Applied Biosystems™ 9700 Thermocycler

Process: Amplification was done in triplicate to ensure the data was accurate.

Capillary Electrophoresis:

- Applied Biosystems™ 3130XL and 3500XL

Data Analysis:

- Genemapper® ID-X Applied Biosystems™

Results

Number of Extracts Pooled	Cases with Equal or Better Results	Total Number of Cases	Success Rate
Two	7	14	50%
Three	3	4	75%
Four	8	9	88.9%
Total	18	27	66.6%

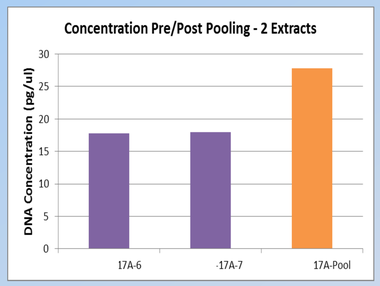
Table 1: Results were separated based on the number of extracts pooled. As mentioned earlier the samples were divided based on the number of extracts. By splitting up the extracts, two questions were answered. First was the pooling successful and second how many extracts are ideal for the pooling method.

Number of Extracts Pooled	Number of Drop in Alleles	Total Number of Alleles	Percent of Alleles deemed Drop in
Two	15	726	2.1%
Three	12	366	3.3%
Four	16	451	3.5%
Total	43	1543	2.7%

Table 2: The number of drop in alleles never exceeded 3.5% for the separate pooled extracts and only 2.7% for the combined total. When it came to the artifacts occurring within amplifications it was more common for the drop in alleles to occur in the cases where three extracts were pooled.

Number of Extracts Pooled	Amplifications with Drop in Alleles	Total Number of Amplifications	Percent Affected by Artifacts
Two	13	130	10%
Three	9	48	18.8%
Four	12	108	11.1%
Total	34	286	11.9%

Table 3: shows the amount of amplifications that were affected by drop in alleles. The total percentage was only 11.9% of the 286 amplifications performed.



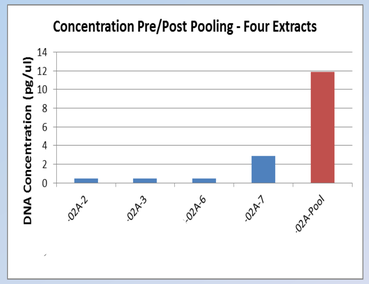


Figure 1 & 2: are graphical representations of successful cases using two and four extracts within the pool.

Discussion

Overall the project succeeded in proving that pooling of samples is a viable option for improving STR results of historic and ancient samples. The success of the project, as seen in Table 1, shows the samples had an 88.9 % success rate. This simple result supported both that pooling four extracts led to the best result and the method could improve STR results. The pooling of the four extracts led to an almost complete YSTR profile when the extracts (left untreated) managed to result in a maximum of 6 alleles. The 09 and 89 cases were unique because they were samples that had been refrigerated for over a year after being used for a previous validation. The 09 and 89 samples had been previously amplified with the AmpFlSTR® MiniFiler™ PCR Amplification Kit in which they had resulted in a maximum of five alleles. When 09 and 89 were used with the pooling method it was astonishing to see almost complete profiles come back in the results for YSTR as well as MiniFiler.

The two extract pooling was not as successful as the four extract pooling. In Table 4 it shows that at times it was not always the concentration that would have been theoretically reached with the combination of the two extracts. For example if you take 03A-2 and 03A-7, 6.7 pg/µl and 9.8 pg/µl respectively, should have a combined concentration of 16.5 pg/µl but instead have a pooled concentration of 7.7 pg/µl. The final pool concentration was seen to have lower amounts than expected multiple times and the reason was considered to be due to the MinElute™ purification step. Further testing of DNA retention could be beneficial at ensuring a maximum amount of the DNA is recovered during the pooling process.

Conclusion

In conclusion this process was developed to mitigate the inhibition brought on from samples buried in the soil. As mentioned earlier a major inhibitor found in historic burial samples is humic acid which competes with DNA for the silica filter of the MinElute™. By using multiple small samples (instead of one large sample) and pooling them in order to limit the amount of inhibitors associated with each portion. With the results found throughout the project show that the pooling method can improve results with a high success rate and even on long stagnant samples.

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