Evaluation of Demineralization Parameters for DNA Extraction Methods from Skeletal Remains

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Introduction

Skeletal samples are often the only remaining source of DNA for human identification in investigations involving missing persons, unidentified remains, or cold cases. The protective hydroxyapatite matrix encasing the cells in these samples helps to preserve the DNA but also requires an additional demineralization step to access the DNA present in these cells.[1] Partial demineralization methods require limited incubation time but result in unused skeletal material whereas total demineralization methods often require overnight incubations but result in complete dissolution of skeletal samples.[1,2]

Similarly to the variation in incubation time, the temperature can also impact the demineralization process, subsequent DNA extraction, and downstream processing. Traditionally, 56°C is used, however the ancient DNA community utilizes lower incubation temperatures (e.g. 37°C) to limit any degradation to an already challenging sample type. However, there is speculation that the digestive process may be hindered at these lower temperatures due to incomplete activation of the Proteinase K enzyme.[3,4] To combat this issue, one adaptation of our investigated extraction methods involved an additional spike of Proteinase K followed by an increase in incubation temperature to allow for its activation, similar to those performed by Xavier et al.[5]

Overall, several adaptations of DNA extraction methods were investigated to examine the effect of incubation time and temperature on the quality and quantity of DNA recovery.

Materials and Methods

- Sample Selections: 6 challenging skeletal samples – 2 burned, 2 buried, 2 surface decomposed
- Extraction Methods: performed in duplicate (**Figure 1**)
  - Total Demineralization – Adaptation of Loreille et al.[1]
  - Partial Demineralization – PrepFiler™ BTA (ThermoFisher Scientific) Quantification:
- Investigator® Quantiplex® Pro (QIAGEN)
- Traditional STR Typing: Globalfiler™ (ThermoFisher Scientific); AT–50 RFU, ST–250 RFU

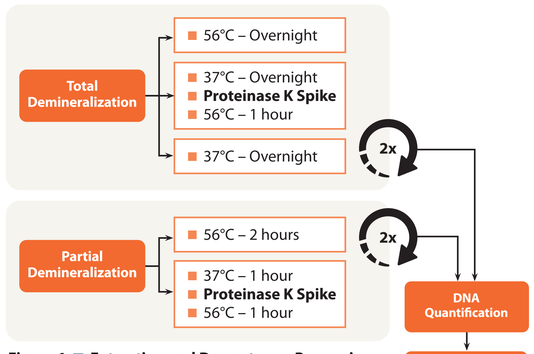

Figure 1 ■ Extraction and Downstream Processing Schematic – Total and partial demineralization methods were examined in duplicate (i.e. 2x) using various incubation temperatures and Proteinase K volumes.

Results and Discussion

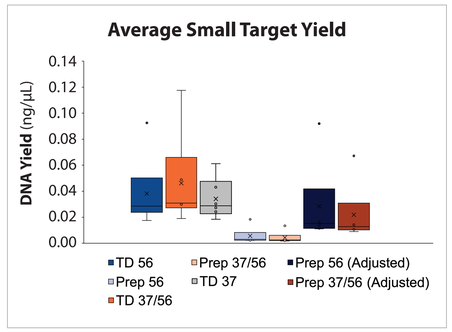

Figure 2 ■ Average Small Target Yield – Comparison of average small target DNA yield for total and partial demineralization methods with various incubation temperatures. NOTE: The adjusted extracts indicate a theoretical yield of 5x the original PrepFiler™ BTA extract yields to account for the difference in sample input of the total and partial demineralization methods.

Degradation Index

Sample ID	TD 56°C	TD 37°C/56°C	TD 37°C	Prep 56°C	Prep 37°C/56°C
A	11.03	–	14.73	18.60	10.84
B	7.35	–	6.94	6.58	7.37
C	73.42	–	106.41	27.19	27.57
D	–	–	–	–	–
E	11.82	–	14.13	91.60	25.35
F	17.38	–	13.39	12.18	11.41

Table 1 ■ Degradation Index – Comparison of average degradation index for all methods. Sample D was highly degraded therefore no large target was recovered. The total demineralization 37°C/56°C method failed to produce any large DNA fragments in all samples.

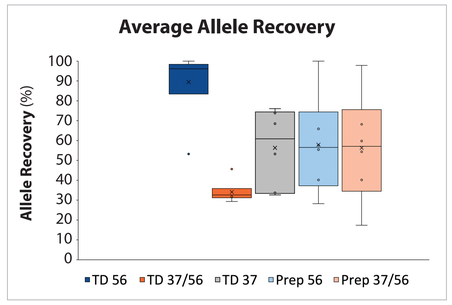

Figure 3 ■ Average Allele Recovery – Comparison of average STR allele recovery for total and partial demineralization methods with various incubation temperatures.

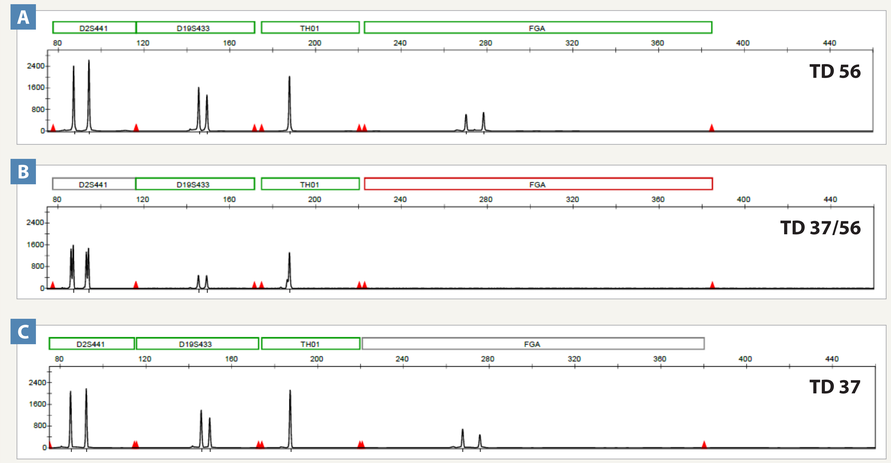

Figure 4 ■ Total Demineralization Impact on Profile Quality – Yellow dye channel of A Total demineralization at 56°C only, B Total demineralization at 37°C followed by an additional spike of Proteinase K and an increase to 56°C, and C Total demineralization at 37°C only.

- No significant difference in average small target DNA yields were observed when examining the total demineralization methods, regardless of the incubation temperature (**Fig. 2**).
  - Total demineralization methods had statistically significantly higher small target DNA yields compared to the partial demineralization methods (p-value: 56°C - 0.0128, 37°C/ 56°C - 0.0236). However, the partial demineralization methods utilized only a fifth of the bone powder needed for total demineralization. When adjusting for this difference, the theoretical DNA yields were more comparable (**Fig. 2**).
- The total demineralization method that began at 37°C and was raised to 56°C following an additional Proteinase K spike had no large fragment recovery and an inability to determine the degradation index (**Table 1**). The Investigator® Quantiplex® Pro handbook[6] states that high levels of inhibition may lead to degradation being flagged. This issue did not appear when samples were later processed using a total demineralization method at only 37°C without an additional Proteinase K spike.
- Total demineralization at 56°C had a 56.37% increase in STR calls compared to the total demineralization method that began at 37°C and was raised to 56°C following an additional Proteinase K spike (**Fig.3**).
  - When the total demineralization method was repeated using only 37°C and no additional spike of Proteinase K, the STR recovery improved with an average of 57.61% compared to the 34.29% recovery with the additional Proteinase K spike (**Fig 3**). Overall, total demineralization at 56°C still had the highest percentage recovery of 89.66% and was more consistent in its recovery compared to the 37°C method.
  - Incubation temperature did not appear to impact allele recovery for the partial demineralization method, rather it appeared sample dependent (**Fig. 3**).
- Total demineralization at 37°C with an additional spike of Proteinase K and an increase in incubation temperature resulted in poor peak morphology indicating signs of inhibition (**Fig. 4B**). This inhibition is likely due to the added Proteinase K as it does not appear in the total demineralization methods at 56°C (**Fig. 4A**) or 37°C (**Fig. 4C**).

Conclusions

- Total demineralization at 56°C was identified as the best DNA extraction method for skeletal samples as it had the highest STR allele recovery, resulted in clean profiles, and performed the most consistently.
- The additional spike of Proteinase K that was intended to aid in sample digestion resulted in PCR inhibition and limited STR allele recovery.
- Partial demineralization methods appeared sample dependent and can be a good alternative method when there is limited sample material or the results are time sensitive.

Acknowledgements

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References

1. O.M. Loreille, T.M. Diegoli, J.A. Irwin, M.D. Coble, T.J. Parsons, High efficiency DNA extraction from bone by total demineralization, *Forensic Sci. Int. Genet.* 1 (2007) 191–195. https://doi.org/10.1016/j.fsigen.2007.02.006
2. J. Stray, A. Holt, M. Brevnov, L.M. Calandro, M.R. Furtado, J.G. Shewale, Extraction of high-quality DNA from biological materials and calcified tissues, *Forensic Sci. Int. Genet.* Suppl. Ser. 2 (2009) 159–160. https://doi.org/10.1016/j.fsigss.2009.08.086
3. N. Rohland, M. Hofreiter, Comparison and optimization of ancient DNA extraction, *Biotechniques.* 42 (2007) 343–352
4. M.P. Evison, D.M. Smillie, A.T. Chamberlain, Extraction of single-copy nuclear DNA from forensic specimens with a variety of postmortem histories, *Journal of Forensic Science.* 42 (1997) 1032–1038
5. Xavier C, Eduardoff M, Bertoglio B et al (2021) Evaluation of DNA Extraction Methods Developed for Forensic and Ancient DNA Applications Using Bone Samples of Different Age. *Genes* 12: https://doi.org/10.3390/genes12020146
6. Investigator Quantiplex Pro Handbook. QIAGEN, V. HB-2335-002 (2018)

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