Megatherium americanum **MAPB4R 3965 bone sample preparation**

Three subsamples between 187-285 mg were taken from the rib cross section sample of MAPB4R 3965. Subsamples were manually pulverized and washed with 300 µl of 0.5 M EDTA (shaking at 1000 RPM; 20 min; 25°C) to remove dust and surface contaminants. Samples were then subject to alternating rounds of demineralization (0.75 ml of 0.5 M EDTA; shaking at 1000 RPM; ~24 hours; 25°C) and digestion (0.75 ml of ProK Digestion Buffer (0.01M Tris-Cl (pH 9), 0.20 % Sarcosyl, 0.25 mg/ml Proteinase K, 0.01 M $CaCl_2$); rotation; ~5 hours; 45°C). Samples were centrifuged at max speed for 5 minutes following each round of demineralization or digestion, collected, and pooled between rounds. A total of three alternating rounds each of demineralization and digestion were completed as above, followed by one set of long demineralization (0.75 ml of 0.5 M EDTA; shaking at 1000 RPM; ~72 hours; 25°C) and digestion (0.75 ml of ProK Digestion Buffer; rotation; ~24 hours; 45°C). Supernatants were collected as above and pooled.

Samples were extracted using the "Method B" extraction procedure outlined in Glocke & Meyer[1], except eluted off the column in 50 µl of EBT. Due to extensive visible inhibitor carry over (characterized as a darkening of the extract solution), samples were additionally purified with the MinElute PCR Purification Kit with the following modifications: 6:1 volume of Buffer PB to extract volume; PB-extract mixture was subject to a cold spin for approximately 20 minutes (4°C; 22 kG) before being bound to the column; two washes with 750 µl of Buffer PE; elution in 50 µl of EBT.

Library preparation followed protocols outlined in Meyer & Kircher[2] with modifications from Kircher et al.[3]. Libraries were constructed using 20 µl of purified extract as input in 40 µl reactions. A heat deactivation (20 min; 80°C) was used in place of a final MinElute purification following adapter fill-in. Final concentrations and cycling conditions for each step in library preparation can be found in TABLES 1-8.

Indexing was carried out in 40 µl reactions with unique P5 and P7 indexing primers[3] using 12.5 µl of heat-deactivated library as input (TABLES 9,10). Indexing reactions were purified over MinElute PCR Purification columns with the following modifications: 6:1 volume of Buffer PB to reaction; two washes with 750 µl of Buffer PE; elution in 13 µl of EBT.

Mitochondrial in-solution capture was carried out using the optimized Xenarthran Enrichment protocol outlined in Karpinski et al.[4]. Enrichment reactions were carried out using 5 µl of purified indexed library and 100 ng of a previously designed xenarthran bait set[5].

Enriched libraries were size-selecteed for fragment sizes between 150 bp to 600 bp, and sequenced on an Illumina HiSeq 1500 using a 2x90 bp paired-end double-index protocol at the McMaster University Francombe Metagenomics Facility.

Table 1: Final concentration for all components in the Oligo Hybridization Buffer.

Reagent	Final Concentration
NaCl	500 mM
Tris-Cl, pH 8.0	10 mM
EDTA, pH 8.0	1 mM

Table 2: Final concentration for all components in the Adapter Mix. Adapter Mix was prepared separately for the P5 and P7 adapter, and combined following a 10 second incubation (95°C), and a ramp from 95°C to 12°C (rate of 0.1°C/sec).

Reagent	Final Concentration
IS1_adapter_P5.F or IS2_adapter_P7.F	200 µM
IS3_adapter_P5+P7.R	200 µM
Oligo Hybridization Buffer	1x

Table 3: Final concentration for all components in the End Repair master mix. Water (not listed) was used to bring up the final volume to 40 µl.

Reagent	Final Concentration
NE Buffer 2.1	1x
DTT	1 mM
dNTPs	100 µM
ATP	1 mM

T4 polynucleotide kinase	0.5 U/µl
T4 DNA polymerase	0.1 U/µl

Table 4: Cycling conditions for the End Repair step of library preparation.

Temperature	Time
25°C	15 min
12°C	15 min
4°C	hold

Table 5: Final concentration for all components in the Adapter Ligation mixture. Water (not listed) was used to bring up the final volume to 40 µl.

Reagent	Final Concentration
T4 DNA Ligase Buffer	1x
PEG-4000	5%
Adapter Mix	0.25 µM
T4 DNA Ligase	0.125 U/µl

Table 6: Cycling conditions for the Adapter Ligation step of library preparation.

Temperature	Time
16°C	15 hours
4°C	hold

Table 7: Final concentration for all components in the Adapter Fill-in master mix. Water (not listed) was used to bring up the final volume to 40 µl.

Reagent	Final Concentration
ThermoPol Reaction Buffer	1x
dNTP Mix	250 µM
BST Polymerase (large fragment)	0.4 U/µl

Table 8: Cycling conditions for the Adapter Fill-in step of library preparation.

Temperature	Time
37°C	30 min
80°C	20 min
4°C	hold

Table 9: Final concentration for all components used during indexing PCR of prepared libraries. Water (not listed) was used to bring up the final volume to 40 µl.

Reagent	Final Concentration
KAPA SYBR®FAST qPCR Master Mix (2X)	1x
P5 Indexing Primer	750 nM
P7 Indexing Primer	750 nM

Table 10: PCR cycling conditions used during indexing. Steps in bold and marked with two astericks (*** X ***) were repeated a total of 10 times.

Temperature	Time
95°C	5 min

*** 95°C ***	*** 30 sec ***
*** 60°C ***	*** 45 sec ***
60°C	3 min

References

1. Glocke, I. & Meyer, M. Extending the spectrum of DNA sequences retrieved from ancient bones and teeth. *Genome Res.* **27,** 1–8 (2017).
2. Meyer, M. & Kircher, M. Illumina sequencing library preparation for highly multiplexed target capture and sequencing. *Cold Spring Harb. Protoc.* **2010,** pdb.prot5448 (2010).
3. Kircher, M., Sawyer, S. & Meyer, M. Double indexing overcomes inaccuracies in multiplex sequencing on the Illumina platform. *Nucleic Acids Res.* **40,** 1–8 (2012).
4. Karpinski, E., Mead, J. I. & Poinar, H. N. Molecular identification of paleofeces from Bechan Cave, southeastern Utah, USA. *Quat. Int.* **443,** 140–146 (2016).
5. Delsuc, F. *et al.* The phylogenetic affinities of the extinct glyptodonts. *Curr. Biol.* **26,** R155–R156 (2016).