

Three Reagents for in-Solution Enrichment of Ancient Human DNA at More than a Million SNPs

Nadin Rohland^{1,2,*}, Swapan Mallick^{1,2,3,*}, Matthew Mah^{1,2,3}, Robert Maier^{1,2,4}, Nick Patterson^{2,4} and David Reich^{1,2,3,4}

¹ Department of Genetics, Harvard Medical School, Boston, Massachusetts 02115, USA.

² Broad Institute of MIT and Harvard, Cambridge, MA 02142, USA.

³ Howard Hughes Medical Institute, Boston, MA 02115, USA

⁴ Department of Human Evolutionary Biology, Harvard University, Cambridge, MA 02138.

* Contributed equally: N.R. and S.M.

To whom correspondence should be addressed: N.R. (nrohland@genetics.med.harvard.edu), S.M. (shop@genetics.med.harvard.edu), D.R. (reich@genetics.med.harvard.edu)

In-solution enrichment for hundreds of thousands of single nucleotide polymorphisms (SNPs) has been the source of >70% of all genome-scale ancient human DNA data published to date. This approach has made it possible to generate data for one to two orders of magnitude lower cost than random shotgun sequencing, making it economical to study ancient samples with low proportions of human DNA, and increasing the rate of conversion of sampled remains into working data thereby facilitating ethical stewardship of human remains. So far, nearly all ancient DNA data obtained using in-solution enrichment has been generated using a set of bait sequences targeting about 1.24 million SNPs (the ‘1240k reagent’). These sequences were published in 2015, but synthesis of the reagent has been cost-effective for only a few laboratories. In 2021, two companies made available reagents that target the same core set of SNPs along with supplementary content. Here, we test the properties of the three reagents on a common set of 27 ancient DNA libraries across a range of richness of DNA content and percentages of human molecules. All three reagents are highly effective at enriching many hundreds of thousands of SNPs. For all three reagents and a wide range of conditions, one round of enrichment produces data that is as useful as two rounds when tens of millions of sequences are read out as is typical for such experiments. In our testing, the “Twist Ancient DNA” reagent produces the highest coverages, greatest uniformity on targeted positions, and almost no bias toward enriching one allele more than another relative to shotgun sequencing. Allelic bias in 1240k enrichment has made it challenging to carry out joint analysis of these data with shotgun data, creating a situation where the ancient DNA community has been publishing two important bodies of data that cannot easily be co-analyzed by population genetic methods. To address this challenge, we introduce a subset of hundreds of thousands of SNPs for which 1240k data can be effectively co-analyzed with all other major data types.

ancient DNA | human | sequencing | in-solution enrichment | SNP capture | minimizing bias

The strategy of using artificially synthesized oligonucleotides as baits to fish out complementary sequences in a DNA library has been transformative in ancient human DNA studies. Enrichment has involved diverse approaches including oligonucleotides of various lengths affixed to glass slides (1), or baits that are free in solution (“in solution enrichment”) (2). Under appropriate chemical and temperature conditions, these baits hybridize to targeted molecules so that other molecules can be washed away, allowing the bound molecules to be isolated, released, and then

49 sequenced. Enrichment has allowed researchers to achieve orders of magnitude enrichment for
50 sequences that provide high information content to address important scientific questions.

51
52 In medical genetics, the most common application of target enrichment has been capturing the
53 approximately two percent of the genome that constitutes the coding sequences of genes (the
54 “exome”). When whole genome sequencing at high coverage was still prohibitively expensive,
55 exome sequencing dropped the cost for surveillance of the coding regions for mutations causing
56 rare diseases to affordable levels (2, 3). In ancient DNA analysis, the benefits of target
57 enrichment are even greater (4). Not only is a tiny fraction of the genome in practice relevant for
58 the great majority of analyses, but typically only a small proportion of molecules in the DNA
59 library come from the individual of interest due to microbial contamination. Occasional ancient
60 DNA libraries do contain most of their molecules from the individual whose bone or tooth was
61 sampled, but it is more typical for most of the analyzed molecules not to be of human origin. For
62 example, of more than 3,000 ancient individuals for which our research group published
63 genome-wide data by the end of calendar year 2021, about half had less than 10% percent human
64 DNA. Whole genome sequencing of such samples is prohibitively expensive for all but the most
65 important samples given the typical budgets accessible to ancient DNA laboratories.

66
67 As an example of the challenge, consider a researcher who is interested in targeting a set of about
68 600,000 SNP positions genotyped in diverse modern human populations. Only perhaps one in a
69 hundred ancient DNA sequences mapping to the human genome will overlap these positions,
70 given the typical lengths of ancient molecules. If a DNA library is only one percent human, the
71 proportion of sequences that will be informative for analysis will only be about one in ten
72 thousand. Thus, if ~400 million DNA sequences are read from a library which is a typical
73 number used to produce a ~30-fold whole human genome from modern DNA, only ~40,000
74 SNPs will be retrieved that overlap those genotyped in diverse modern populations. An
75 individual with this much information will be only weakly informative for many analyses. In
76 contrast, 25 million sequences from the same ancient DNA library after in-solution enrichment
77 can provide coverage on nearly all targeted SNP positions by multiple unique molecules,
78 allowing accurate inferences about population history at much lower cost.

79
80 Practical in-solution enrichment for ancient human DNA libraries was pioneered between 2010-
81 2013 in studies that enriched for mitochondrial DNA (5, 6), nearly all of the unique sequences of
82 chromosome 21 (5) and all or part of the exome (1, 7). In 2015, several papers were published
83 that enriched for sequences overlapping sets of SNPs. The reagent that has been most extensively
84 used and that we evaluate here is the ‘1240k reagent’: it targeted slightly fewer than 1.24 million
85 SNPs chosen to be particularly valuable for studying variation among modern human
86 populations (8-10). It has proven highly effective, and has been used to generate more than 70%
87 of all genome-wide ancient human dataset: over five thousand individuals published in more
88 than seventy papers (compiled at <https://reich.hms.harvard.edu/allen-ancient-dna-resource-aadri-downloadable-genotypes-present-day-and-ancient-dna-data>). The large body of data produced
89 using the 1240k reagent has also created an important legacy dataset whose existence needs to be
90 taken to account by researchers contemplating shifting to a new method: any future enrichment
91 data benefits by targeting the same set of sites which can then be co-analyzed with existing data.
92 However, the 1240k reagent also has limitations including variability in effectiveness of
93 enrichment of targeted SNPs, and bias toward capturing some alleles more than others at the
94

95 same sites, leading to technical artifacts when such data are co-analyzed with other data types
 96 such as random ‘shotgun’ sequencing data. Because of these technical challenges, researchers
 97 often restrict analyses to 1240k data only, or to shotgun data only, excluding key datapoints
 98 generated using the other strategy, and thus reducing the value of the world’s combined dataset.
 99

100 **Table 1: Twenty-seven ancient DNA libraries experimentally characterized in this study**

Library ID	Library type	% human in shotgun sequencing	No. of 1,150,639 autosomal targets covered after downsampling to 25 million sequences			Ref. for earlier publication of data from same library	
			1240k	Arbor	Twist	Shotgun	Capture
S20720.Y1.E1.L1	DS	0.10%	4,247	3,129	4,383	new	new
S20721.Y1.E1.L1	DS	1.18%	38,513	29,958	43,375	new	new
S21299.Y1.E1.L1	DS	2.04%	332,624	227,616	379,349	new	new
S20703.Y1.E1.L1	DS	6.57%	648,971	483,408	823,496	new	new
S1633.E1.L1	DS	86.68%	812,084	647,823	1,042,602	AGDP	(11)
S8432.E1.L9	SS	0.17%	10,719	4,353	13,013	new	new
S2818.Y1.E4.L1	SS	1.17%	19,856	13,245	24,538	new	new
S13982.Y1.E8.L1	SS	6.92%	92,627	58,034	148,083	new	(12)
S10872.E1.L4	SS	4.20%	711,014	378,014	808,591	new	(12)
S10871.E1.L6	SS	42.21%	857,393	659,199	1,048,225	new	(12)
S2949.E1.L7	DS	1.67%	7,513	2,476	8,624	new	new
S11857.E1.L1	DS	7.46%	26,697	9,726	32,107	new	new
S10871.E1.L1	DS	52.59%	857,393	659,199	1,048,225	(13)	(13)
S4532.E1.L1	DS	69.12%	803,925	652,927	1,083,523	new	new
S1734.E1.L1	DS	73.92%	808,314	676,065	1,076,264	AGDP	(14)
S4795.E1.L1	DS	79.31%	817,750	649,362	1,066,996	AGDP	(15)
S1507.E1.L1	DS	66.59%	816,665	683,200	1,077,678	AGDP	(10)
S1961.E1.L1	DS	76.18%	808,645	685,996	1,063,387	new	new
S2514.E1.L1	DS	75.82%	753,037	621,223	1,008,821	new	new
S1960.E1.L1	DS	93.22%	824,903	700,631	1,072,129	new	new
S1965.E1.L1	DS	78.34%	810,646	669,482	1,066,051	new	new
S2861.E1.L1	DS	94.90%	789,102	675,731	1,074,256	AGDP	(11)
S2520.E1.L1	DS	87.29%	763,183	646,338	1,022,068	new	new
S1583.E1.L1	DS	68.66%	789,976	645,082	1,042,853	new	new
S5950.E1.L1	DS	69.63%	793,523	678,635	1,076,585	new	(12)
S5319.E1.L1	DS	95.54%	806,669	679,549	1,074,390	new	(12)
S1496.E1.L1	DS	85.45%	809,418	683,539	1,072,954	new	(12)

101 Note: We analyzed both double-stranded (DS) and single-stranded (SS) libraries. The first 10 lines are for single- and double-
 102 stranded libraries of a range of complexities and percentages of human DNA for which we carried out a full characterization,
 103 obtaining results for both 1 and in almost every case also 2 rounds of enrichment. The final 17 lines are for double-stranded libraries
 104 that in general had very extensive shotgun sequencing data and for which we only performed the recommended number of rounds of
 105 enrichment in the original protocol (2 for 1240k, 2 for Arbor Complete, and 1 for Twist Ancient DNA). The statistics in this table are
 106 computed on a core set of 1,150,639 SNPs on chromosomes 1-22 targeted by all three reagents. The final columns indicate if data
 107 from this library is first reported in this paper (“new”) or has previously been reported in a paper or in the Allen Genome Diversity
 108 Project pre-publication data release (“AGDP”) (<https://reich.hms.harvard.edu/ancient-genome-diversity-project>).
 109

110 A particular challenge with the 1240k reagent is that many ancient DNA laboratories have not
 111 been able to effectively access the technology. Secondary distribution of the reagent was not
 112 permitted by the company that synthesized the oligonucleotides. While the bait sequences were
 113 fully published in 2015, resynthesis of the reagent was prohibitively expensive on a per-reaction
 114 basis for laboratories interested in using the reagent on a scale of fewer than hundreds of

115 samples. To make it possible for any ancient DNA researcher to carry out in-solution enrichment
116 of more than a million SNPs, in 2021 two companies, Daicel Arbor and Twist Biosciences, made
117 available in-solution enrichment reagents that target the core panel of 1.24 million SNPs as well
118 as additional SNPs meant to address perceived gaps in the coverage of the original reagent. The
119 co-authors of this study advised on the creation of these reagents, but were not paid as
120 consultants and will not receive any remuneration from sale of the reagents. Here we describe a
121 systematic comparison of all three reagents on a common set of 27 ancient DNA libraries chosen
122 to span a range of library qualities from low to high percentages of human DNA, and from low
123 to high complexities with respect to the number of unique human molecules present in the
124 libraries (Table 1). In the interests of providing an independent assessment, our manuscript has
125 not been reviewed by the companies that generated the reagents.

126

127 **Results**

128

129 **Design of the three reagents.** For completeness we begin by summarizing the original ‘1240k’
130 design, first reported in 2015 (8). The almost 1.24 million probes (1,233,013 after filtering to
131 sites that could be robustly analyzed) were published in the supplementary materials of that
132 study. Each SNP was targeted by four probes of 52 bp. To reduce bias toward capturing one
133 allele or the other, two probes abutted but did not overlap the SNP in either direction. Another
134 two probes were centered on the SNP, each with an alternative allele (again to reduce bias). The
135 probes were appended on one side by an 8 bp universal flanking sequence and the 60 bp
136 oligonucleotides were printed on Agilent 1M custom arrays. The baits were then biotinylated (5).

137

138 The SNP targets in the 1240k reagent were chosen to achieve a variety of purposes, summarized
139 in Table 2 and the original publications (8-10). They included all the designable content of the
140 Affymetrix Human Origins genotyping array (16) that has now been used to publish data on
141 >8,800 present-day people from >840 human populations around the world (more than 90% of
142 these data were published in thirteen studies (11, 16-28)). They included all the designable
143 content of the Illumina 650Y genotyping array, part of a family of similar Illumina arrays whose
144 content was iteratively optimized for genome-wide association studies and which have been
145 widely used in genome-wide studies of human history. The 1240k targets furthermore included
146 SNPs on the Affymetrix GeneChip Human Mapping 50K Xba Array; SNPs on the X
147 chromosome to enable comparative studies of male and female population history; and SNPs on
148 the Y chromosome to determine haplotypes. Finally, they included SNPs of phenotypic interest
149 from association studies, scans of selection, or particularly important loci such as the HLA
150 region of chromosome 6. In practice, 1240k reagent SNP enrichment experiments have also
151 often include spiked-in oligonucleotide baits allowing enrichment of mitochondrial DNA (5, 6).

152

153 For the Daicel Arbor “myBaits Expert Human Affinities” reagent, the oligonucleotide bait
154 design is proprietary and the authors of this study do not have access to the technical details.
155 Several modules are available (<https://arborbiosci.com/genomics/targeted-sequencing/mybaits/mybaits-expert/mybaits-expert-human-affinities/>). “Prime Plus” targets the
156 exact same set of SNPs as the 1240k reagent along with the mitochondrial genome and a
157 supplementary set of 46,218 Y chromosome SNPs. The “Complete” product adds an additional
158 852,068 transversion polymorphisms (“Ancestral Plus”) discovered as variable among archaic
159 humans and validated as polymorphic in present-day humans (<https://arborbiosci.com/wp->

160

161 [content/uploads/2021/03/Skoglund_Ancestral_850K_Panel_Design.pdf](#)). These sites were
162 chosen with the goal of facilitating analyses of African human population history, where biases
163 due to the ancestry of the individuals in whom SNPs are discovered has the potential to
164 complicate inferences (29). The fact that these SNPs are transversions is also useful when
165 enriching ancient DNA libraries not enzymatically treated to remove ancient DNA damage
166 which causes high error rates at transition SNPs. All the Arbor reagents also include baits to
167 enrich mitochondrial DNA. We characterized the “Arbor Complete” reagent, which after
168 accounting for the intersections of various SNP panels constitutes 2,131,299 SNPs.

169
170 For the Twist Biosciences “Twist Ancient DNA” reagent, a single 80 bp probe was centered on
171 each targeted SNP. To avoid bias toward one allele or another, the nucleotide at the position of
172 the SNP was chosen to be different from the two SNP alleles. The reagent was built around a
173 core of 1,200,343 1240k SNPs (all 1240k SNPs on chromosomes 1-22 and X). It replaced the
174 32,670 1240k chromosome Y SNPs with 81,925 chosen to provide improved haplogroup
175 resolution. It also added 94,586 additional phenotypically relevant targets chosen to target SNPs
176 that were significant in genome-wide association studies in large sample sizes (32), as likely to
177 have been affected by natural selection (33), as possibly implicated in rare disease (34), or as
178 useful for computing heritability of complex traits (35) (Supplementary Section 1). These SNPs
179 were only added to the reagent if they were not in high linkage disequilibrium with the core
180 1240k set (Supplementary Section 1, Online Table 1). The Twist reagent also targeted non-SNP
181 locations. It tiled 857,339 bp in 3,171 Human Accelerated Regions (HARS); 2,577 bp in 3 genes
182 relevant to α -thalassemia, β -thalassemia, and favism; and 40,000 CpG dinucleotides where
183 methylation rates are known to be correlated to human age (Supplementary Section 2). After
184 filtering to probes that designed well, the final reagent included 1,434,155 probes targeting
185 1,352,535 SNPs. A mitochondrial panel from Twist Biosciences can be added to the bait pool; in
186 practice we did not spike in sufficient concentrations of the mitochondrial DNA reagent to
187 achieve consistently high mitochondrial DNA coverage, but subsequent experiments with more
188 baits achieved results comparable to the other methods (data not shown).

189
190 **Empirical characterization of the three reagents.** We experimentally characterized reagent
191 performance in 27 libraries on which we performed 109 enrichment experiments (Table 1). All
192 our sequencing was performed on HiSeqX10 instruments, and we report data on 12.2 billion
193 merged sequences obtained for the enrichment experiments, and 43.3 billion merged sequences
194 from shotgun sequencing. Basic statistics on the sequencing results for these libraries both before
195 enrichment (shotgun sequencing), and after enrichment, are reported in Supplementary Table 1.

196
197 (i) For 10 libraries of a range of complexities and percentages of endogenous human DNA (5
198 double-stranded and 5 single-stranded), we produced 0.006-26.7 mean coverage on the
199 1240k autosomal targets (assessed from 2 rounds of 1240k capture after removing duplicated
200 molecules), and ranging in percentage of human DNA from 0.1% - 87%. We carried out 58 =
201 10 x 6 - 2 enrichment experiments on these libraries (the two most complex libraries were not
202 captured for 2 rounds for Twist Ancient DNA). We carried out enrichment using all three
203 reagents with the settings specified in the Methods, and deeply sequenced capture products
204 both after the first and second round of sequencing, with 25-395 million merged sequences
205 (median 95 million merged reads) for each experiment (Supplementary Table 1).

206

207 (ii) For 17 double-stranded libraries 15 of which were of high complexity and high percentage of
 208 human DNA, we carried out extensive shogun sequencing, in 14 cases to more than 20-fold
 209 coverage. The shotgun data for four libraries has been fully published (12, 13, 36, 37), and
 210 the shotgun data for an additional 8 libraries has been released pre-publication as part of the
 211 Allen Ancient Genome Diversity Project / John Templeton Ancient DNA Atlas
 212 (<https://reich.hms.harvard.edu/ancient-genome-diversity-project>) (Table 1). We carried out
 213 51=17x3 enrichments on these libraries with the experimental settings specified in the
 214 recommended protocols. Thus, we sequenced after two rounds of capture for 1240k and
 215 Arbor Complete, and one round of capture for Twist Ancient DNA. We sequenced the
 216 enriched products far more deeply than the ~25 million sequences typically generated for
 217 such experiments (median of 104 million merged sequences, Supplementary Table 1).
 218

219 **Variation in effectiveness of enrichment in different parts of the genome.** Table 2 highlights
 220 different targeted subsets of the genome, and shows the mean coverage in each category relative
 221 to the average achieved at the core set of 1,150,639 autosomal SNP positions (to assess coverage
 222 we use number of sequences obtained prior to removal of PCR duplicates as our goal here is to
 223 study the relative effectiveness of enrichment). In Online Table 1, we provide a SNP-by-SNP
 224 breakdown (this table also reports meta-information including why each SNP was targeted).
 225 Online Table 2 assesses the methylation targets (40,000 CpG dinucleotides). Online Table 3
 226 covers Human Accelerated Regions (3,171 regions). Online Table 4 covers resequencing targets
 227 (in 3 regions). Online Table 5 reports 10.4 million alignable nucleotides on the Y chromosome.
 228 Online Table 6 reports results for 15,569 nucleotides of mitochondrial DNA.
 229

230 **Table 2: Effectiveness of enrichment in different targeted subsets of the genome**

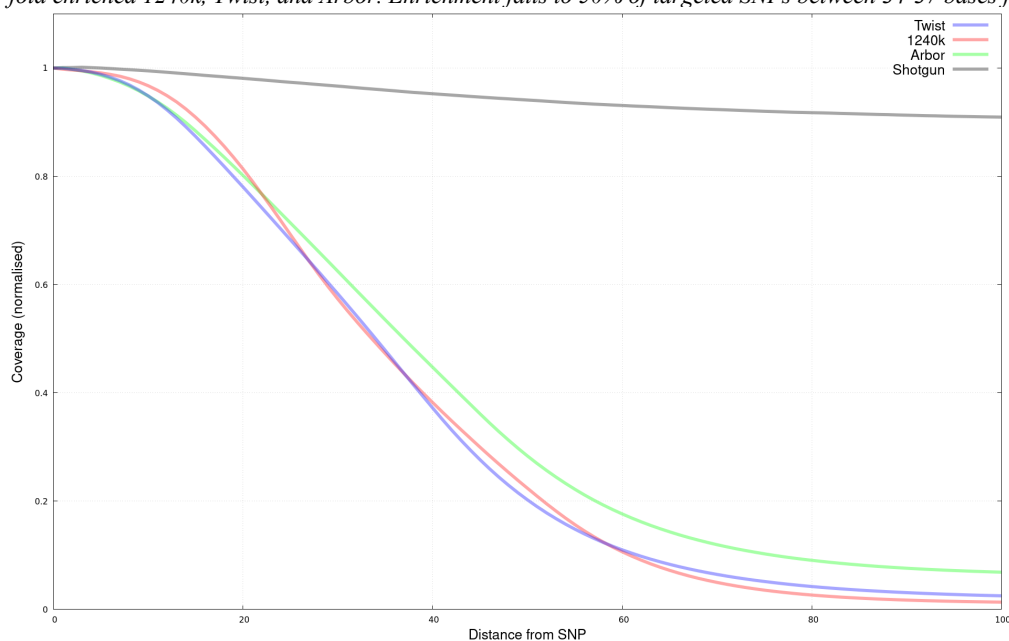
Targeted subset of the genome (some categories overlap)	# positions (either SNPs or tiled nucleotides)	1240k coverage (vs. core set)	Twist coverage (vs. core set)	Arbor coverage (vs. core set)
SNPs				
Affymetrix Human Origins	597,573	1.003	1.127	1.086
Illumina 650Y	660,611	0.951	0.882	0.927
Affymetrix 50K	58,559	0.371	0.516	0.71
1240k phenotypic supplement	45,969	0.988	0.929	0.936
1240k X content	49,704	1	1	1
1240k Y content	32,670	1	1	1
Twist phenotypic supplement	94,587	0.059	0.943	0.136
Twist Y content	81,925	0.475	1.016	0.813
Arbor ancestral supp.	852,068	0.136	0.147	0.586
Arbor Y supplement	46,218	0.184	0.952	0.774
Tiling nucleotides				
Mitochondrial DNA	16,569	6.17	2.955	28.51
Twist HAR supplement	857,339 (3171 HARs)	0.039	2.448	0.09
Twist gene sequencing supplement	2,577 (in three genes)	0.54	3.206	0.088
Twist methylation targets	80,000 (40,000 CpGs)	0.086	3.584	0.109

231 Note: Relative coverage is computed by taking the average in this part of the genome after pooling data from all 27
 232 libraries (2 rounds for 1240k, 2 rounds for Arbor, and 1 round for Twist), and dividing by either 1,150,639 SNPs on
 233 chromosomes 1-22, 49,704 SNPs on chromosome X (for SNPs there), or 32,670 SNPs for chromosome Y (for SNPs
 234 there). Coverage computations are based on sequence counts prior to removing PCR duplicated sequences.
 235

236 All three methods not only enrich for the targeted content, but also for other positions usually
 237 within dozens of nucleotides on either side of explicitly targeted content (Figure 1). To obtain a
 238 better understanding of the patterns of enrichment near targeted locations and to assess if they
 239 can be useful, we annotated all 81.2 SNPs in the 1000 Genomes project dataset (38) by the
 240 coverage relative to the 1240k autosomal SNP targets (Online Table 7). All reagents effectively
 241 enriched not just the target SNPs, but hundreds of thousands of polymorphic positions nearby;
 242 for example, we identified ~130,000-170,000 SNPs that were enriched to $\geq 50\%$ of the autosome-
 243 wide average coverage and had a minor allele frequency $\geq 5\%$ in at least one 1000 Genomes
 244 Project continental population (Table 3). Researchers wishing to choose such non-targeted SNPs
 245 for inclusion in their analyses can select them based on the metrics in Online Table 7.

246 **Figure 1: Distribution of sequence coverage as a function of distance from targets.**

247 Results are for the 15 high coverage sequencing libraries prior to removal of PCR duplicates, normalized by average coverage at
 248 targeted SNPs (position 0). Compared to nucleotides 100 base pairs from the closest target, coverage is 74-fold, 40-fold, and 15-
 249 fold enriched 1240k, Twist, and Arbor. Enrichment falls to 50% of targeted SNPs between 34-37 bases from SNP targets.



250 **Table 3: Enrichment of hundreds of thousands of near-target SNPs.**

Reagent (no. of targeted SNPs)	Maximum minor allele frequency	Coverage $\geq 10\%$ of the average at core set of 1,150,639 SNPs	Coverage $\geq 50\%$ of the average at core set of 1,150,639 SNPs
1240k (1,233,013)	$\geq 1\%$	474,617	265,743
	$\geq 5\%$	236,478	130,478
Arbor Complete (2,131,299)	$\geq 1\%$	759,543	270,247
	$\geq 5\%$	375,620	130,811
Twist Ancient DNA (1,322,529)	$\geq 1\%$	661,221	361,077
	$\geq 5\%$	330,066	172,835

Note: This analysis restricts to SNPs within 50bp of explicitly targeted nucleotides.

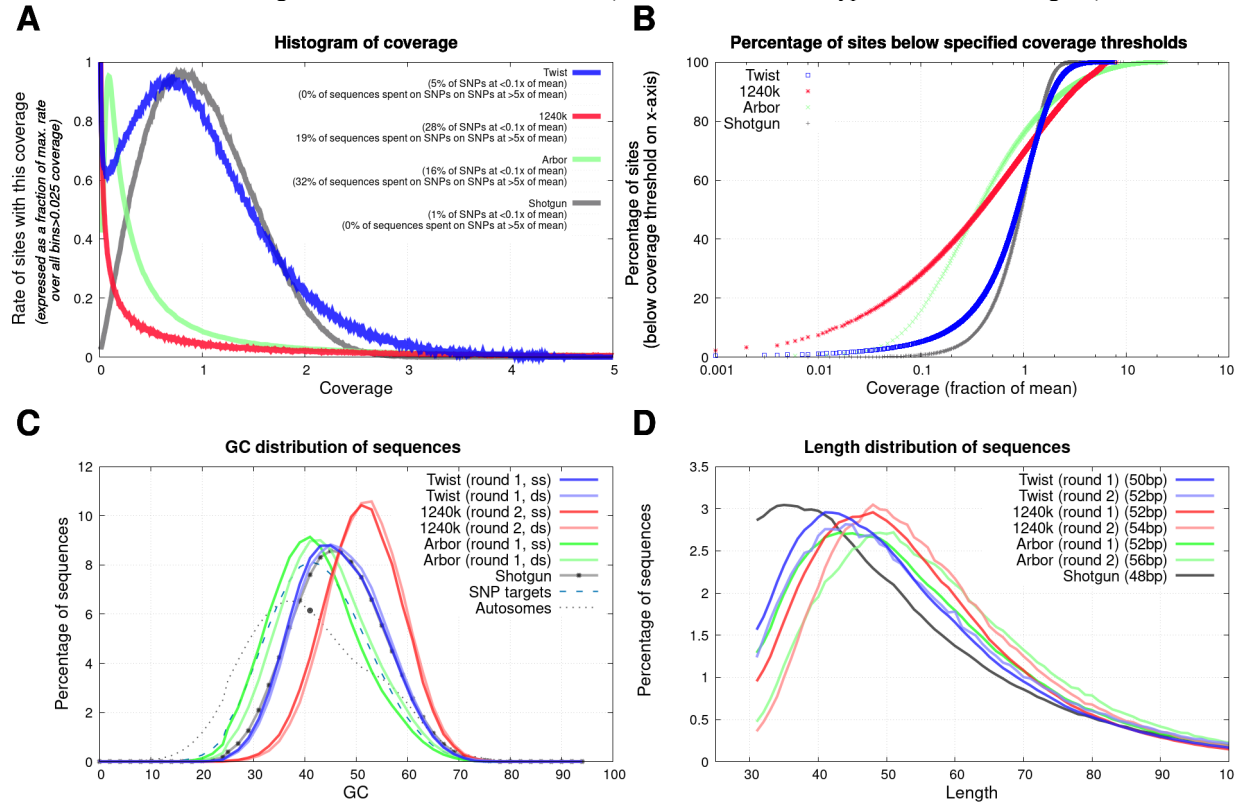
251 **Enrichment is less biased for Twist Ancient DNA than for other methods.** We built
 252 histograms of coverage on targeted SNPs pooling over the libraries for which we had deep
 253 sequencing data (Figure 2A,B). The histograms are centrally peaked for shotgun sequencing

254 (1% of SNPs with coverage <0.1-fold of the mean) and for Twist Ancient DNA (5% of SNPs),
 255 as expected for more homogeneous enrichment. In contrast, we observe skewed enrichment for
 256 1240k (28% of SNPs with coverage <0.1-fold of the mean) and Arbor Complete (16% of SNPs).
 257

258 Further evidence for a relatively homogeneous enrichment for Twist Ancient DNA comes from
 259 the proportion of guanines and cytosines in sequenced molecules, which is similar for Twist data
 260 and shotgun data, whereas Arbor Complete data shows a downward bias and 1240k an upward
 261 bias (Figure 1C). The Twist Ancient DNA also shows less of a bias toward an increase in the
 262 length of molecules than the other two enrichment methods (Figure 2D).
 263

264 **Figure 2: Biases in enrichment.**

265 We restrict to the 1,150,639 autosomal SNPs targeted by all three reagents. The top two panels analyze 15 libraries with high
 266 coverage shotgun sequencing data; the bottom two analyze 10 libraries with full results from both rounds of capture. (A)
 267 Variation in coverage across targeted SNPs is shown as a smoothed histogram where we normalize the y-axis by the maximum
 268 rate in bins with >0.025 of the average coverage. (B) The fraction of sites with coverage below different multiples of the mean.
 269 (C) The proportion of nucleotides that are either guanine or cytosine (GC) has a downward bias relative to the unenriched library
 270 for Arbor, an upward bias for 1240k, and little bias for Twist Ancient DNA. (D) All reagents preferentially enrich for longer
 271 molecules, with the least length effect for Twist Ancient DNA (medians for each data type are show in the legend).

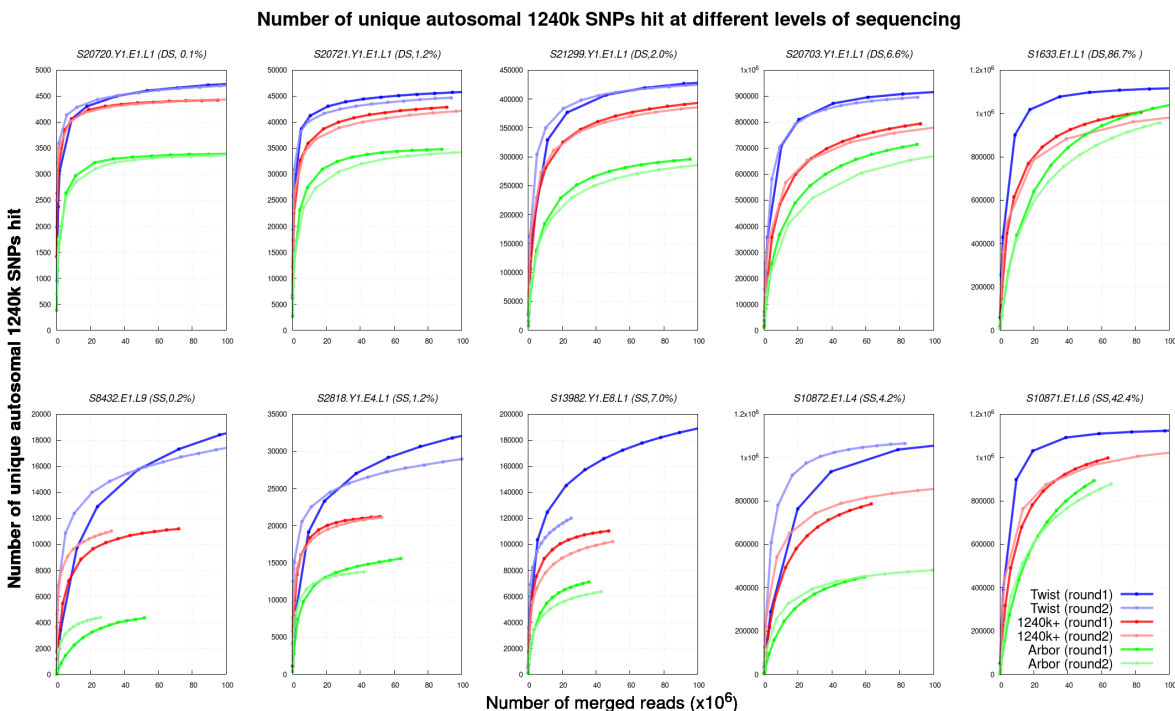


272 **All reagents are effective with Twist Ancient DNA consistently achieving highest coverage.**
 273 As expected from its greater homogeneity in enrichment, Twist Ancient DNA achieves
 274 consistently high genome-wide coverage when measured by the number of SNPs hits at least
 275 once, for an amount of sequencing (25 million read pairs) that is typical for such experiments
 276 (Table 1). Compared to 1240k data the average increase in targeted SNP count is 1.21-fold, and
 277 compared to Arbor Complete it is 1.46-fold. We observe similar patterns for a range of
 278 sequencing coverages (Figure 3 and Supplementary Figure 1).

279
280
281

Figure 3: Performance of the three reagents over a range of sequencing depths.

This analysis is based on various amounts of downsampling relative to the full sequencing data.



282

283 The increased yield for Twist Ancient DNA relative to the other protocols is particularly
284 apparent for low complexity and single-stranded libraries, the condition for which we optimized
285 this reagent over multiple rounds of testing. However, the Twist Ancient DNA reagent also
286 outperforms the 1240k reagent for low-complexity double stranded libraries for which that
287 methodology was optimized, highlighting how the Twist reagent is a definitively better reagent
288 than 1240k from a technical point of view. For the Arbor Complete experimental settings we
289 performed no optimization; instead, we used the manufacturer's recommended protocol before
290 product launch which differs from the one now available in the online manual. Better enrichment
291 performance (perhaps much better) could likely be achieved with the Arbor Complete reagent
292 through multiple rounds of optimization such as we performed for Twist Ancient DNA and
293 1240k. The correct lesson to take from these results is that the Arbor Complete reagent is
294 effective and that these results place a minimum not a maximum on its utility.

295

296 A remarkable feature of all three enrichment method is the similar genome-wide coverage
297 obtained from one round and two rounds of sequencing when a typical amount of data is
298 collected after enrichment (~25 million sequences). This is striking in light of the fact that the
299 proportion of sequences overlapping targets being much higher after two than one rounds of
300 enrichment (average of 10-fold, median of 4-fold higher for the experiments in Figure 3)
301 (Supplementary Table 1). The explanation is that the number of molecules typically sequenced
302 after enrichment (~25 million), is far larger than the number of targeted positions. Thus, even
303 with the relatively small proportions of molecules hitting targets after one round of enrichment,
304 we in practice obtain sequences that cover the great majority of the targeted positions in the
305 library. Because each enrichment round increases bias relative to the unenriched library, and
306 because one round of enrichment is less expensive and time consuming than two, we recommend

307 that standard practice for all three reagents should be to carry out just one round of enrichment
308 (thus, the second round of enrichment for nearly all 1240k experiments to date was unnecessary).

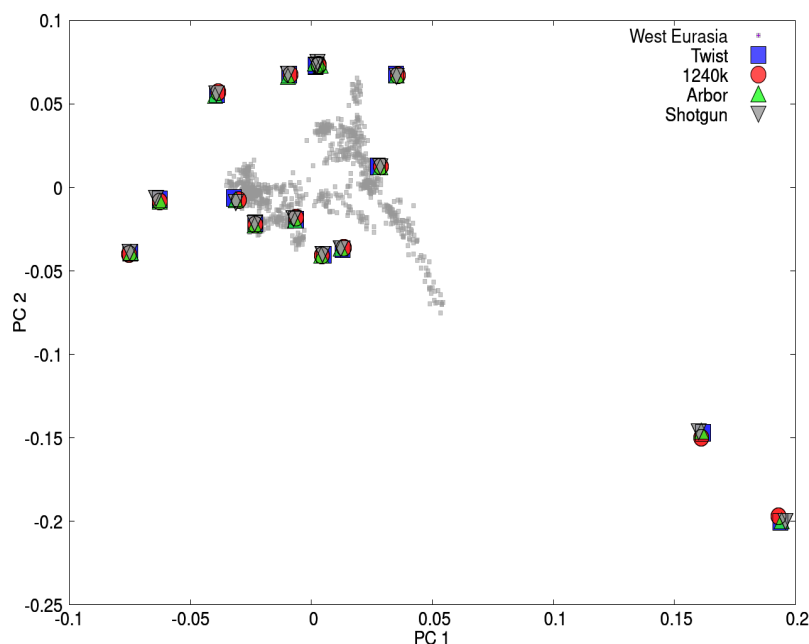
309
310 A potential concern related to our approach of comparing results only at the 1,150,639 autosomal
311 SNPs common to all three reagents is that this could be unfair to reagents that target more SNPs
312 (especially Arbor Complete and to a lesser extent Twist Ancient DNA). In practice this is not a
313 serious concern, as for a single round of enrichment which is our final recommended setting for
314 all three reagents, the great majority of sequenced molecules miss targets (Supplementary Table
315 1), and thus the rate of molecules hitting targeted positions outside the 1,150,639 evaluation SNP
316 set is small relative to the off-target content. In this setting, enrichment efficiency as assessed by
317 the ratio of sequences overlapping the core set of SNP targets (the 1,150,639) to fully untargeted
318 positions is similar to the same quantity if we do not drop sequences overlapping other targets.
319 We use the number of merged sequences on the x-axis of Figure 3 instead of a corrected number,
320 as number of merged sequences is intuitively understandable and relevant to real experiments.

321
322 **Addressing concerns about technical bias due to co-analysis of data from different sources.**

323 Biases associated with alignment and enrichment can affect population genetic analysis, causing
324 data from two ancient DNA libraries processed using the same enrichment protocol to appear to
325 have genetic affinities to each other even though the truth is that individuals from whom the
326 libraries were obtained do not have distinctive relatedness. Concerns of this type have meant that
327 in practice for population genetic analyses, researchers have often restricted their analyses to in-
328 solution enrichment data using the 1240k reagent, or shotgun data, creating a challenging
329 situation where two disjoint datasets have been built up in the community that are difficult to co-
330 analyze. Even if a technology is more accessible to the community, and even if it is more
331 efficient at capturing all targeted positions than the existing 1240k enrichment reagent, its
332 practical value could be limited if it was difficult to co-analyze with data from other methods.

333
334 **Figure 4: Principal Component Analysis shows similar ancestry regardless of data source.**

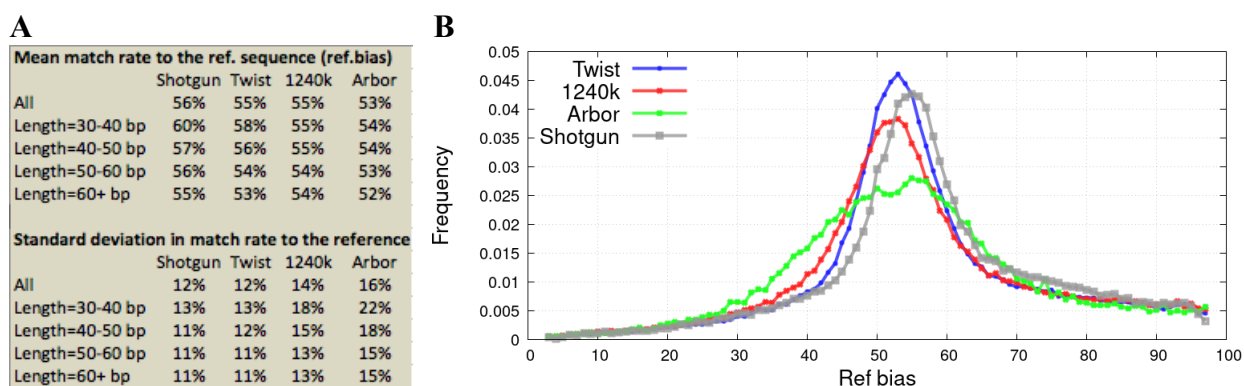
335 We performed PCA on >1000 West Eurasians, and projected data from the 15 individuals in the last rows of Table 1.



336 To explore how bias might affect our results, we began by projecting the data from the 15
 337 libraries at the bottom of Table 1 onto a Principal Component Analysis of data from diverse
 338 present-day West Eurasian people living today (Figure 4). Encouragingly, all data from the same
 339 individuals plots at the same position, consistent with the pattern observed in the first publication
 340 of Twist Ancient DNA data where Neolithic individuals from Hazleton North in southern Britain
 341 clustered tightly whether the data source was 1240k or Twist (39). That study also showed that
 342 Twist and 1240k data could be robustly co-analyzed to detect familial relatedness (39).

343
 344 Lack of evidence for bias in PCA does not mean concerns about bias should be set aside. To
 345 further probe for bias associated with the different data generation technologies, for each of the
 346 15 high coverage libraries we identified all SNP positions that were likely to be heterozygous
 347 based on observing at least one sequence matching both the reference allele and at least one
 348 matching the variant allele. For each SNP, we counted all reference and variant alleles observed
 349 at likely heterozygous positions beyond those not used in ascertainment; if there are no biases we
 350 expect 50% of these sequences to match the reference variant. We implemented an Expectation
 351 Maximization algorithm that uses these counts to estimate the distribution of reference bias for
 352 all SNPs, correcting for limited sample size which will produce more apparent variation in
 353 reference bias than is in fact the case (Supplementary Section 2).

354
 355 **Figure 5: Allelic bias due to the different enrichment strategies.**
 356 (A) Mean and standard deviation in the rate of matching to the reference genome for different data types, stratifying by sequence
 357 length, and correcting for stochastic error in the estimates using an Expectation Maximization (EM) algorithm described in
 358 Supplementary Section 2. (B) Distribution across SNPs in degree of reference bias. All analyses are based on sequences from loci
 359 ascertained as highly likely to be heterozygous, corrected for stochastic sampling variance using the EM.



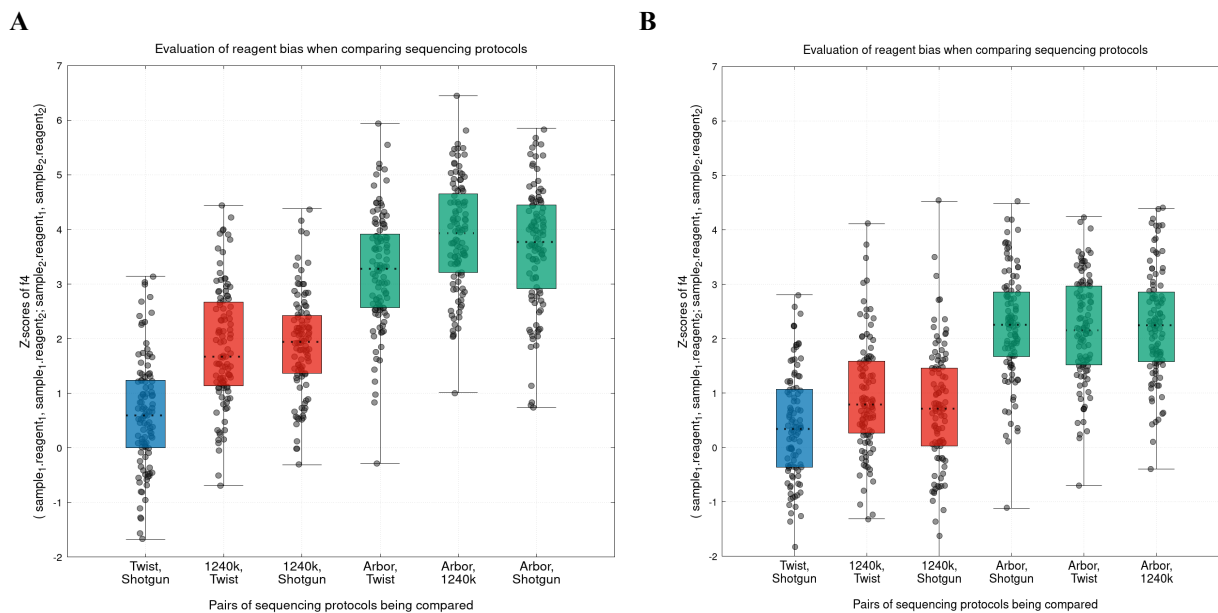
360 We observe substantial average reference bias for all methods, which as expected due to the
 361 difficulty of mapping is word for shorter reads (Figure 5). A substantial degree of average
 362 reference bias is an important problem—and methods have been developed for mapping ancient
 363 DNA sequences in a way that reduces reference bias by an order of magnitude (40, 41)—but it is
 364 not the focus of this study, especially as reference bias also affects unenriched shotgun data. The
 365 unique issue for enrichment is the wider variation in reference bias across SNPs for 1240k and
 366 especially for Arbor Complete than for either shotgun or Twist Ancient DNA, even after
 367 controlling for sequence length (Figure 5). This reflects the fact 1240k and Arbor Complete,
 368 while not more likely to capture the reference allele on average, are more likely to skew from the
 369 mean degree of reference bias. Such skews specific to a technology are expected to cause data
 370 generated from two libraries processed by the same technology to have artifactual affinity.

371

372
 373 To detect these artifactual attractions, we computed symmetry statistics of the form $f_4(\text{library 1 - reagent A, library 1 - reagent B; library 2 - reagent A, library 2 - reagent B})$. If there are no
 374 technical biases, this quantity is expected to be 0, as data from each library should be
 375 symmetrically related to that from all other libraries. In contrast, if there are technical biases, we
 376 expect positive values of the statistic reflecting greater-than-random co-occurrences of alleles
 377 from two libraries processed using the same technology. Figure 6A computes a Z-score for the
 378 deviation of these f_4 -statistics from zero based on a Block Jackknife standard error; for the one-
 379 sided test appropriate here, $Z > 1.7$ corresponds to $P < 0.05$, and $Z > 3.1$ corresponds to $P < 0.0001$
 380 (16). We observe that the Z-scores trend positive for all pairwise comparisons of the 15 libraries,
 381 as expected from the fact that any technical bias will cause a positive deviation. The statistics are
 382 most positive (mean Z of 3-4) for comparisons involving Arbor Complete captured SNPs,
 383 suggesting the strongest technical bias for this data type and consistent with the evidence that
 384 Arbor data has the largest standard deviation in reference bias across SNPs as shown in Figure
 385 5A. The statistics are also large (mean Z almost 2) for statistics comparing 1240k to Twist
 386 Ancient DNA or shotgun data, as expected from the empirical observation of problems of co-
 387 analyzability of these two data types. Bias is minimal for Twist Ancient DNA comparisons to
 388 shotgun data (mean Z-score of around 0.6 with almost all Z-scores between -2 and 2) consistent
 389 with these two data types being far more co-analyzable from a population genetic perspective.
 390
 391

392 **Figure 6: Artifactual attraction of data produced used the same methodology.**

393 (A) We compute symmetry statistics of the form $f_4(\text{library 1 - reagent A, library 1 - reagent B; library 2 - reagent A, library 2 - reagent B})$, and plot Z-scores for all $105 = 15 * 14 / 2$ pairwise comparisons of the 15 high coverage libraries as well as box-and-whisker plots showing full range, 25th and 75th percentiles, and mean. Statistics involving Arbor Complete are indicated with a green box; remaining comparisons involving 1240k with a red box; and the Twist Ancient DNA - shotgun comparison in blue.
 394
 395 (B) Same analysis but restricted to a subset of 42% of autosomal SNPs chosen to have very similar rates of matching to the reference allele for shotgun and 1240k reagent data (empirically within 4% of each other).
 396
 397
 398



399 While the minimal allelic bias associated with the data produced by the Twist Ancient DNA
 400 reagent and its easy co-analyzability with shotgun data addresses a limitation of the vast majority
 401 of capture experiments to date, it raises a new concern about co-analyzability of Twist Ancient

402 DNA data with 1240k data. We therefore set out to identify a subset of SNPs with less
403 susceptibility to such bias. To do this we mine data from 488 libraries for which we had shotgun
404 data at a median of 5-fold coverage and also good 1240k data (much of this dataset is available
405 as a pre-publication data release at [https://reich.hms.harvard.edu/ancient-genome-diversity-
406 project](https://reich.hms.harvard.edu/ancient-genome-diversity-project)). We used imputation with GLIMPSE (42) to infer diploid genotypes at each SNP
407 location (43) and counted rates of sequences matching to the reference and variant allele in all
408 individuals where the posterior probability of being heterozygous was >0.9 at a given SNP. If
409 there are no biases in enrichment, the frequency of observing the reference allele in the 1240k
410 enrichment data is expected to match that in the shotgun sequence data (both 0.5). We restricted
411 to the 42% of autosomal SNPs where difference in rate rates of matching to the reference allele
412 for shotgun data and 1240k data was empirically less than 4% in the pooled reads over 488
413 libraries (this set of SNPs is specified as a column in Online Table 1). Figure 6B shows that the
414 mean Z-scores for all f_4 -symmetry statistics comparing libraries that are shotgun sequenced,
415 libraries enriched using 1240k, and libraries enriched using the Twist Ancient DNA reagent are
416 between 0 and 1 after restricting to this set of SNPs, suggesting that this approach reduces biases.

417
418 Our goal in this analysis has been to demonstrate that a practical filter to reduce technical bias
419 between methods exists; we have not attempted to optimize the filter and believe that there is
420 substantial room to make the filter even better. The demonstration of the filter is also important
421 for a reason that has nothing to do with Twist data, as it suggests a solution to problem that has
422 been a long-standing challenge for ancient human DNA studies, namely, the difficulty of co-
423 analyzing shotgun and 1240k enrichment data in genetic studies of population history. Applying
424 a filter like has the potential to make data from diverse sources—1240k and shotgun and Twist—
425 co-analyzable even for sensitive population genetic analyses.

426

427 **Discussion**

428

429 We have systematically compared three in-solution reagents for enriching ancient DNA libraries
430 for more than a million SNPs, and found that all three are highly effective.

431

432 The 1240k reagent has a proven track record, and has been used in more than 70 publications to
433 report data from more than 5000 ancient individuals and to make robust inferences about
434 population history. While 1240k data shows more allelic bias and less target homogeneity than
435 Twist Ancient DNA data, for studies of population history, the most important requirement is to
436 regularly retrieve data from a large number of SNPs and it does this well.

437

438 The Arbor Complete reagent has several highly attractive features: it targets the same core set of
439 SNPs as the 1240k enrichment reagent so that data can be co-analyzed, it targets an additional
440 $\sim 850,000$ transversion SNPs chosen to be useful for studies of African population genetics, and it
441 can be purchased commercially. Our implementation of Arbor Complete enrichment did not
442 produce as high-quality results as the two other methods, but we also did not optimize the Arbor
443 protocols in our lab as we did for the 1240k reagent and the Twist Ancient DNA reagent, and
444 there is thus great potential for further performance improvement for this reagent.

445

446 The Twist Ancient DNA reagent was the most efficient of the three in our experiments, capturing
447 sequences overlapping almost all targeted positions with relatively high homogeneity, achieving

448 higher coverage, and having the least allelic bias making it most easily co-analyzable with
449 shotgun data at nearly all analyzed SNPs. Like Arbor Complete, the Twist Ancient DNA reagent
450 is commercially available. We have introduced a filter that makes it possible to tag SNPs most
451 affected by the bias in 1240k enrichment, and which provides confidence that Twist data will be
452 robustly co-analyzable with the great majority of ancient human DNA data generated to date.

453
454 Because of the multiple advantages associated with the Twist Ancient DNA reagent relative to
455 1240k in our testing, in June 2021 we performed our last of more than 28,500 1240k captures in
456 our laboratory. Since then, we have performed more than 4,500 captures with Twist Ancient
457 DNA reagent, and have already published our first data with this reagent (39). It is important for
458 scientific communities periodically to update their methodologies when there are enough
459 technical improvements, and we believe the advantages of new reagents are now so large that
460 this time has come for ancient human DNA.

461 **Materials and Methods**

462

463 **DNA extraction and library preparation.** We extracted DNA from tooth or bone powder with
464 a manual (44, 45) or automated protocol (46) using Dabney buffer and silica coated magnetic
465 beads. We built the extract into indexed single-stranded USER-treated libraries (47) or into
466 partial-UDG-treated barcoded double-stranded libraries (48). For cleanups after automated
467 library preparation, we used silica coated magnetic beads and PB (Qiagen), and for cleanups
468 after amplification we used SPRI.

469

470 **Target enrichment.** The three target enrichment reagents all consist of biotinylated DNA
471 probes, and while Arbor Complete and 1240k use single-stranded probes (52 bp for 1240k,
472 unknown to us for Arbor Complete), Twist Ancient DNA uses double-stranded 80 bp probes.
473 The original protocol for Twist reagents specified one round of enrichment, whereas the original
474 protocols for Arbor Complete and 1240k specified two consecutive rounds of enrichment. Arbor
475 Complete and 1240k had the mitochondrial panel included in our testing (1240k reagent: 3 bp
476 tiled probes of mitochondrial genome of 52 bp length, spiked in at 0.033%), whereas for Twist
477 Ancient DNA we only added the Twist Mitochondrial Panel to 19 of the 27 libraries (120 bp
478 long probes, spiked in at 1.67%). In our Twist testing, we added in the mitochondrial DNA
479 probes at a tenth of the concentration we had intended (our plan had been to spike in at 16.7%
480 but effectively we used 10x less because the concentration in the kit was 10x lower than
481 expected). In subsequent experiments with the intended concentration, we have obtained more
482 efficient mitochondrial retrieval for Twist than we show in Online Table 6.

483

484 For a total of 10 ancient human DNA libraries (5 single-stranded and 5 double-stranded) of
485 varying genomic complexity and endogenous content (Table 1), we enriched for one and in
486 almost every case two rounds following the conditions below for each enrichment reagent.
487 Additionally, we enriched 15 high-complexity libraries and 2 low-complexity libraries for which
488 we had generated large amounts of shotgun sequence data to further investigate the performance
489 of each reagent. For these libraries, we used only the originally recommended settings: 1 round
490 for Twist Ancient DNA, 2 rounds for 1240k, and 2 rounds for Arbor Complete.

491

492 **1240k reagent.** Since the development (5) of the in-solution enrichment technology that is the
493 basis for the 1240k reagent, we have changed temperature settings in our implementation, but not
494 buffer composition or volumes. For this study, we started with 1 μ g of library and hybridized to
495 1 μ g of single-stranded biotinylated bait in a total volume of 34 μ l for at least 16 h at 73 $^{\circ}$ C. We
496 bound the biotinylated probes to 30 μ l MyOne streptavidin C1 beads in Binding Buffer for 30
497 min, and washed the beads 5 times with 3 different wash buffers (stringent washes were
498 performed 3 times at 57 $^{\circ}$ C). We melted the library molecules from the probes, precipitated onto
499 magnetic beads, washed, eluted and amplified for 30 cycles using appropriate primer pairs
500 (depending on whether they were single- or double-stranded libraries) and Herculase II Fusion
501 polymerase. We cleaned up the product with 38% SPRI reagent and eluted round 1 in 15 μ l TE.
502 For round 2, we used 5 μ l of the round 1 product (usually 500-700 ng total) and hybridized with
503 500 ng of single-stranded baits again for about 16 h. Capture and washes were identical to round
504 1, but we eluted the cleaned PCR product in 50 μ l usually resulting in 50 - 90 ng/ μ l product.

505

506 **Arbor Complete.** We used the ‘myBaits Expert Human Affinities - Complete panel’. The kit
507 was not commercially available at the time of testing, and we therefore used reagents and buffers
508 also used for 1240k as recommended by representatives of Daicel Arbor. Experimental settings
509 are similar to the 1240k settings, with the following adjustments. Hybridization was performed at
510 70 °C and binding to 30 µl MyOne Streptavidin beads in binding buffer was recommended at 70
511 °C for 5 min. All washes were identical to 1240k, but the 3 stringent washes were performed at
512 55 °C and amplification cycles were reduced to 20 in round 1. The entire product was used in
513 round 2 (except for the 10 libraries we tested 1 and 2 rounds of capture, 1/7th was kept for round
514 1 indexing PCR and sequencing) and the final amplification was only performed for 12 cycles.
515 The now commercially available kit is slightly different and the recommended settings can be
516 found online ([https://arborbiosci.com/wp-](https://arborbiosci.com/wp-content/uploads/2021/03/myBaits_Expert_HumanAffinities_v1.0_Manual.pdf)
517 [content/uploads/2021/03/myBaits_Expert_HumanAffinities_v1.0_Manual.pdf](https://arborbiosci.com/wp-content/uploads/2021/03/myBaits_Expert_HumanAffinities_v1.0_Manual.pdf)).

518
519 **Twist Ancient DNA.** We explored a range of probe lengths, reagent volumes and temperature
520 settings to optimize performance for unmultiplexed low-complexity single stranded ancient DNA
521 libraries. The experimental conditions which used here (which are substantially different from
522 the protocol optimized by Twist for in-solution enrichment products applied to multiplex modern
523 DNA) are as follows. We used 1 g of dried library and reconstituted in 7 µl of Universal
524 Blockers and 5 µl Blocker Solution. In a second plate, we combined 5 µl of Hybridization mix
525 (standard protocol is 20 µl) with 1 µl of Twist Ancient DNA probes (this is an optimized volume
526 based on our testing; the standard protocol from Twist for modern high quality DNA specifies 4
527 µl). We melted the (double-stranded) probes for 5 min at 95 °C and cooled to 4 °C for 5 min.
528 During the 4 °C cooling of the probes, we incubated libraries and blockers for 5 min at 95 °C. We
529 next equilibrated both plates to room temperature for 5 min. We added the 6 µl of probe (6.167
530 µl if mitochondrial DNA probes were added) and hybridization buffer to the 12 µl library and
531 blocker, mixed, and overlaid with 30 µl Hybridization Enhancer and incubated at 62 °C (standard
532 is 70 °C) in a thermal cycler for at least 16 h. We used 300 µl Streptavidin beads (standard is 100
533 µl) and bound for 30 min at room temperature. In manual processing, we next washed beads 4
534 times with 2 different wash buffers; of these, 3 were stringent washes at 49 °C (standard is 48 °C)
535 (in automated processing, we performed 7 washes of which 6 were stringent washes at 49 °C; the
536 automation protocol is available from Twist Biosciences). We amplified from 50% of the bead
537 slurry with Kapa HiFi HotStart ReadyMix for 23 cycles (standard is fewer cycles) with the
538 provided primers (ILMN) for single-stranded libraries or indexing primer for double-stranded
539 libraries in an off-bead PCR. We finished by purifying the PCRs with 1.8x Purification Beads
540 (standard is 1x) and eluted in 50 µl TE.

541
542 **Sequencing.** We sequenced enriched and shotgun libraries on HiSeqX10 instruments with 2x101
543 cycles, and either 2x7 cycles (double-stranded libraries) or 2x8 cycles (single-stranded libraries)
544 to read the index sequences.

545
546 **Bioinformatic data processing.** Because the enriched ancient DNA libraries were sequenced in
547 pools, we needed to demultiplexed sequences which we did based on two different types of
548 oligonucleotide tags: library-specific barcode pairs (for double-stranded libraries) and index pairs
549 (for all libraries). We merged paired-end sequences requiring either a minimum of 15 base pair
550 overlap (with at most one mismatch, base quality \geq 20) or up to three mismatches of lower base
551 quality. We mapped these sequences to the human genome (*hg19*) using *samse* from *bwa-v0.6.1*

552 (49). We restricted analysis to merged sequences of at least 30 base pairs. For analyses in which
553 we were interested in relative efficiency of retrieval of molecules at different targeted locations,
554 we measured coverage prior to removal of PCR duplicated molecules; for other analyses, we
555 assessed coverage after removal of PCR duplicates. To represent each nucleotide position for
556 analyses that required SNP genotype calls (Principal Component Analysis and f_4 -statistics), we
557 chose a random sequence at each location, requiring a mapping and base quality of 10 and 20.

558

559 **Fraction of published ancient DNA data produced by in-solution enrichment:** To compute
560 the proportion of genome-wide ancient human DNA data for which data had been generated by
561 1240k enrichment (>70%), we used all published data from version v51 of the Allen Ancient
562 DNA Resource ([https://reich.hms.harvard.edu/allen-ancient-dna-resource-aadr-downloadable-](https://reich.hms.harvard.edu/allen-ancient-dna-resource-aadr-downloadable-genotypes-present-day-and-ancient-dna-data)
563 [genotypes-present-day-and-ancient-dna-data](https://reich.hms.harvard.edu/allen-ancient-dna-resource-aadr-downloadable-genotypes-present-day-and-ancient-dna-data)), consisting of compiled records of published
564 genome-wide ancient human DNA data as of December 22, 2021.

565

566 **Distribution of endogenous DNA proportion in published ancient DNA data:** To compute
567 the fraction of individuals with proportions of endogenous DNA below different thresholds, we
568 restricted to published data from our laboratory for which we had at least 15,000 SNPs on
569 chromosomes 1-22 present targeted by the 1240k reagent, and assessed as passing quality control
570 either fully ('PASS') or with minor concerns ('QUESTIONABLE'). We restricted to individuals
571 for which we had an endogenous DNA proportion estimate for at least one library, and
572 represented each individual by the library with the highest proportion of endogenous DNA.

573

574

575 **Data Availability Statement.** The aligned sequences are available through the European
576 Nucleotide Archive, accession [to be made available upon publication].

577

578

579 **ACKNOWLEDGMENTS.** We thank Kim Callan, Elizabeth Curtis Lora Iliev, Lijun Qiu, Noah
580 Workman, and Fatma Zalzala for support in the wet laboratory. We are grateful to Mark
581 Consugar, Ellie Juarez, Paul Frere, Keith McKenna and Frank Capriglione at Twist Biosciences
582 who supported the development of the Twist Ancient DNA reagent. We thank Ryan Doan, Steve
583 Horvath, Iosif Lazaridis, Alissa Mitnik, Vagheesh Narasimhan, and Iñigo Olalde, who advised
584 on choice of additional SNPs and targeted regions for the Twist reagent, and Ali Akbari who
585 created the imputed dataset that made it possible to identify SNPs with reduced susceptibility to
586 capture bias. We thank Jacob Enk and Alison Default at Daicel Arbor who drove the
587 development of the myBaits Expert Human Affinities capture reagents; and Pontus Skoglund and
588 Yassine Souilme who advised on SNP choice for that reagent (none of these colleagues had input
589 into the manuscript). We thank Songül Alpaslan-Roodenberg, Ian Armit, Nihat Erdogan, Julian
590 Jansen van Rensburg, Carles Lalueza-Fox, Benjamin Neil, Ron Pinhasi, Mary Prendergast, Bob
591 Sattler and Irina Shingiray for the collaborations that produced the ancient DNA data samples
592 used for the technical comparisons reported in this study; this paper does not provide information
593 on archaeological context of the analyzed libraries, although such analyses were previously
594 reported for some individuals (Table 1). This research was funded by NIH grants GM100233 and
595 HG012287, by the Allen Discovery Center program, a Paul G. Allen Frontiers Group advised
596 program of the Paul G. Allen Family Foundation, by John Templeton Foundation grant 61220,
597 and by the Howard Hughes Medical Institute.

598 **Supplementary Information Summary**

599

600 ***Supplementary Tables***

601 Supplementary Table 1 Sequencing results on all 27 libraries

602

603 ***Supplementary Figures***

604 Supplementary Figure 1 10 library downsampling experiment using coverage as output

605

606 ***Supplementary Information***

607 Supp. Information section 1 Content added to Twist Ancient DNA Reagent beyond 1240k

608 Supp. Information section 2 EM Algorithm to Correct for Binomial Sampling Variance

609

610 ***Online Tables (large text files, all compressed)***

611 Can be accessed through the following Dropbox link:

612 <https://www.dropbox.com/sh/h024odwt5w1yc37/AAC9jCMhhOncXQRBaWMWOzPla?dl=0>

613

614	Online Table 1	Twist SNP targets	1,352,529 rows	SNPs
615	Online Table 2	Methylation CpG targets	80,000 rows	bases
616	Online Table 3	Human Accelerated Regions (HAR)	857,339 rows	bases
617	Online Table 4	Gene Resequencing Regions	2,577 rows	bases
618	Online Table 5	Mappable Y Chromosome	10,446,037 rows	bases
619	Online Table 6	Mitochondrial DNA	16,569 rows	bases
620	Online Table 7	Statistics at 1000 Genomes SNPs	81,286,436 rows	SNPs

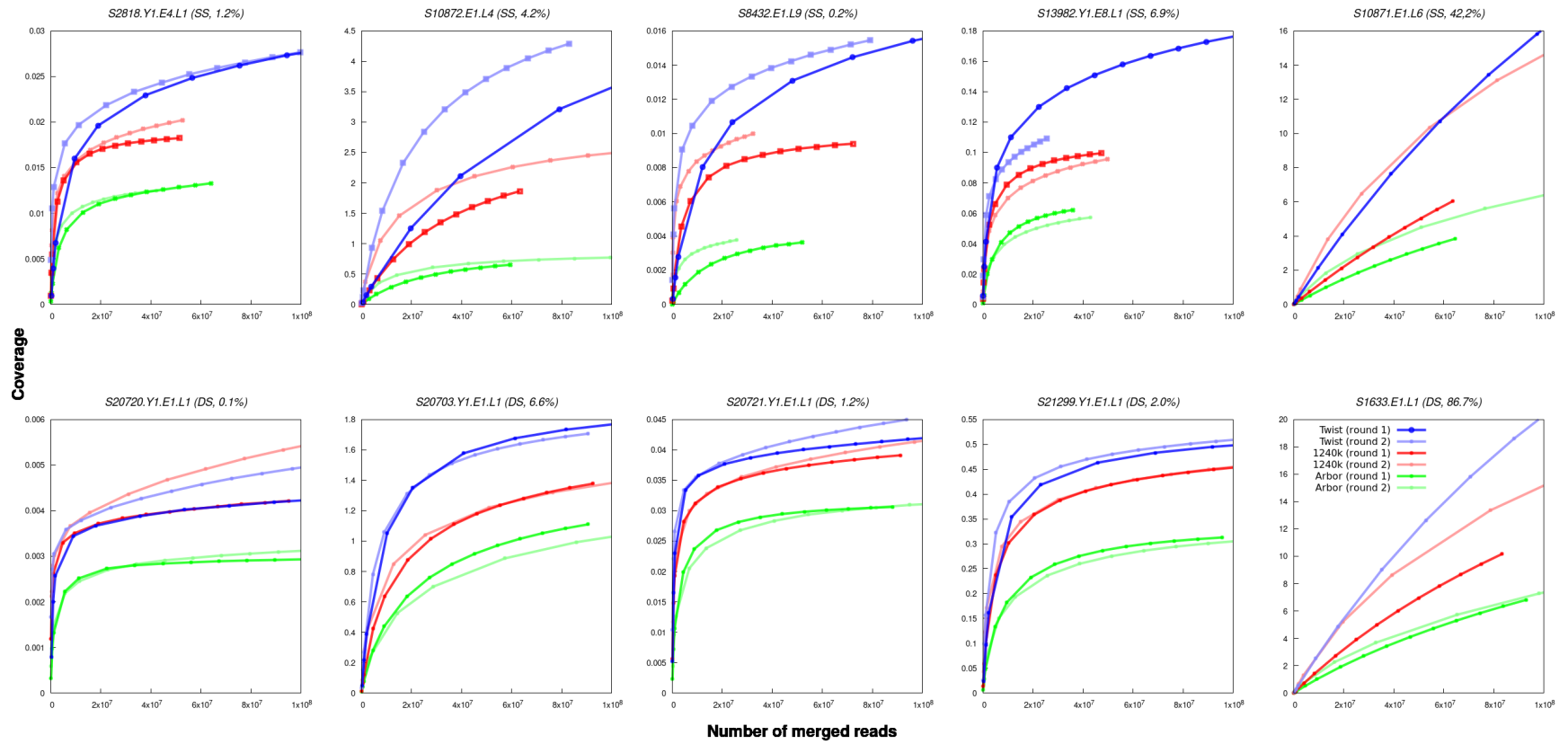
621
622
623

Supplementary Table 1: Sequencing results on all 27 libraries

A total of 10 libraries were sequenced after both the first and second round of enrichment (except for S1633.E1.L1 and S10871.E1.L6 which were not sequenced after a second Twist round). The bottom 17 libraries reflect 2, 2 and 1 rounds of enrichment for 1240k, Arbor and Twist respectively. DS - double-stranded, SS - single-stranded.

Library ID	% aligning to human lib. in shotgun sequencing	Merged reads prior to removal of PCR duplicates				Mean length of merged reads				Percentage of merged reads overlapping core set of 1,150,639 autosomal SNPs prior to removal of PCR duplicates (this does not include sequences that land close to but not overlapping the targets, or sequences successfully enriched for targets outside the core set)			Number of the core set of 1,150,639 autosomal SNPs covered at least once				Mean coverage after duplicate removal on core set of autosomal SNPs (unique sequences overlapping the 1,150,639 autosomal SNPs targeted by all three reagents, divided by number of targets)				
		Shotgun	1240k	Arbor	Twist	Shot.	1240k	Arbor	Twist	1240k	Arbor	Twist	Shotgun	1240k	Arbor	Twist	Shotgun	1240k	Arbor	Twist	
10 library set - 1 round of enrichment for all data types except shotgun																					
S20720.Y1.E1.L1	DS	0.10%	251,053	95,278,044	119,451,860	178,421,670	44	48	46	43	3.17%	0.27%	0.60%	35	4,010	3,826	4,351	0.000030	0.004	0.003	0.004
S20721.Y1.E1.L1	DS	1.2%	156,117	91,159,969	97,037,453	104,752,984	44	47	47	47	8.9%	2.4%	6.0%	159	38,937	37,271	41,516	0.000133	0.036	0.033	0.039
S21299.Y1.E1.L1	DS	2.0%	48,278	102,561,843	83,445,818	229,480,365	53	61	61	56	15.4%	5.8%	11.4%	47	373,893	311,833	419,480	0.000041	0.425	0.328	0.493
S20703.Y1.E1.L1	DS	6.6%	219,514	92,428,434	94,887,900	204,399,952	58	66	67	63	16.0%	9.2%	25.9%	584	773,139	725,363	916,293	0.000489	1.290	5.826	1.757
S1633.E1.L1	DS	86.7%	2,727,670,965	83,318,054	100,160,597	176,327,313	44	53	53	50	19.2%	9.6%	31.9%	1,147,352	994,422	1,025,646	1,125,216	27.572747	9.528	5.826	27.187
S8432.E1.L9	SS	0.17%	65,834	72,216,321	49,468,219	240,555,004	42	40	40	36	0.32%	0.09%	0.15%	5	9,980	7,414	18,747	0.000004	0.009	0.006	0.017
S2818.Y1.E4.L1	SS	1.2%	70,741	51,539,481	49,474,365	188,783,289	53	44	43	40	2.1%	0.59%	0.52%	191	18,937	19,906	30,797	0.000160	0.017	0.018	0.028
S13982.Y1.E8.L1	SS	6.9%	70,180	47,411,908	37,978,331	111,587,418	38	40	40	37	8.9%	2.4%	5.8%	63	99,090	94,349	168,002	0.000054	0.092	0.087	0.164
S10872.E1.L4	SS	4.2%	1,862,592	63,248,591	42,084,693	395,280,379	51	58	50	48	8.6%	0.50%	8.3%	1,755	766,012	145,853	1,108,683	0.001506	1.742	0.148	5.109
S10871.E1.L6	SS	42.2%	531,724,501	63,585,236	55,434,463	194,734,351	49	53	54	48	12.8%	8.3%	24.1%	1,123,329	984,211	874,574	1,132,162	4.050870	5.635	2.995	22.876
10 library set - 2 rounds of enrichment for all data types except shotgun																					
S20720.Y1.E1.L1	DS	0.10%	251,053	154,968,445	50,881,006	120,715,793	44	50	48	44	18.0%	4.4%	4.0%	35	4,046	3,567	4,270	0.000030	0.006	0.003	0.005
S20721.Y1.E1.L1	DS	1.2%	156,117	138,240,603	105,047,509	93,769,358	44	48	48	49	29.9%	11.9%	19.6%	159	38,877	36,345	40,495	0.000133	0.040	0.034	0.042
S21299.Y1.E1.L1	DS	2.0%	48,278	150,336,633	108,058,253	103,616,402	53	62	62	60	41.3%	24.2%	29.0%	47	376,547	316,466	404,683	0.000041	0.444	0.340	0.478
S20703.Y1.E1.L1	DS	6.6%	219,514	255,052,779	111,154,612	90,643,234	58	66	66	65	42.3%	23.3%	40.6%	584	817,446	692,005	877,949	0.000489	1.481	1.057	1.605
S1633.E1.L1*	DS	86.7%	2,727,670,965	393,161,016	94,405,383	NA	44	55	55	n/a	38.7%	22.7%	n/a	1,147,352	1,065,225	942,538	n/a	27.572747	26.654	6.664	n/a
S8432.E1.L9	SS	0.17%	65,834	32,205,778	41,587,887	104,852,445	42	42	42	43	13.2%	3.4%	2.2%	5	9,839	8,116	15,901	0.000004	0.009	0.007	0.015
S2818.Y1.E4.L1	SS	1.2%	70,741	52,678,133	63,282,613	110,858,903	53	45	44	44	23.4%	14.9%	7.7%	191	18,870	20,663	25,591	0.000160	0.019	0.020	0.026
S13982.Y1.E8.L1	SS	6.9%	70,180	49,807,292	59,662,915	25,380,559	38	41	40	40	32.9%	23.9%	22.7%	63	91,750	94,644	104,093	0.000054	0.088	0.091	0.099
S10872.E1.L4	SS	4.2%	1,862,592	150,903,215	61,320,864	83,020,755	51	60	61	50	36.5%	16.4%	31.0%	1,755	863,816	534,501	1,057,659	0.001506	2.469	1.120	3.995
S10871.E1.L6*	SS	42.2%	531,724,501	271,351,127	65,680,438	NA	49	57	59	n/a	37.4%	28.0%	n/a	1,123,329	1,080,929	863,274	n/a	4.050870	21.284	5.728	n/a
17 library set - 2 rounds of enrichment for 1240k, 2 rounds of enrichment for Arbor Complete, 1 round of enrichment for Twist Ancient DNA																					
S2949.E1.L7	DS	1.7%	355,389,471	115,165,304	104,071,862	121,477,955	45	46	47	52	20.2%	3.2%	11.2%	9,157	8,233	8,404	8,305	0.007933	0.011	0.008	0.012
S11857.E1.L1	DS	7.5%	325,565,070	104,040,047	97,458,534	122,812,661	43	44	44	48	25.9%	7.0%	21.3%	36,112	30,035	32,008	31,342	0.031811	0.034	0.030	0.039
S10871.E1.L1	DS	52.6%	3,392,817,802	121,068,282	116,546,266	86,963,332	43	53	50	45	42.7%	25.7%	27.3%	1,099,029	864,395	861,995	1,000,935	5.291361	3.324	2.555	3.846
S1734.E1.L1	DS	73.9%	2,659,971,741	119,325,041	102,138,788	114,955,866	47	54	56	51	33.5%	23.6%	32.2%	1,148,681	988,673	975,842	1,128,780	24.002465	14.997	7.888	21.993
S1583.E1.L1	DS	68.7%	3,389,551,748	111,077,550	105,916,375	114,884,025	43	55	55	51	40.0%	23.7%	29.3%	1,144,814	955,084	955,462	1,112,846	28.168891	15.903	7.888	20.676
S5950.E1.L1	DS	69.6%	3,134,086,352	104,660,609	106,370,574	100,976,181	44	58	61	55	40.8%	24.3%	32.9%	1,149,674	960,933	983,961	1,127,994	29.167912	16.330	9.070	21.185
S4795.E1.L1	DS	79.3%	2,139,845,680	122,810,057	102,313,347	75,602,282	50	58	58	52	39.5%	19.7%	30.9%	1,149,061	991,301	960,201	1,115,350	24.278570	17.828	7.643	15.476
S1965.E1.L1	DS	78.3%	2,629,697,020	109,876,861	109,704,294	119,062,251	45	56	56	51	42.9%	24.3%	31.7%	1,148,250	976,230	984,875	1,125,607	26.989401	19.947	9.226	24.820
S4532.E1.L1	DS	69.1%	2,577,523,845	78,884,451	99,141,301	110,043,936	46	62	63	54	41.7%	18.7%	34.4%	1,148,250	932,718	959,501	1,130,902	20.690906	17.284	8.494	26.114
S2514.E1.L1	DS	75.8%	2,527,210,551	113,661,363	99,289,207	120,124,073	44	56	56	51	39.6%	21.2%	27.6%	1,149,061	926,540	924,542	1,100,117	26.029809	21.351	8.164	22.906
S1960.E1.L1	DS	93.2%	1,725,743,223	114,318,024	98,726,011	102,690,235	50	62	63	58	43.9%	26.2%	36.0%	1,144,945	987,361	989,363	1,123,767	26.379657	23.066	10.555	25.417
S1496.E1.L1	DS	85.5%	2,516,632,984	110,844,132	116,688,408	104,487,273	44	58	59	54	34.8%	24.7%	33.3%	1,148,075	982,715	1,007,662	1,125,313	33.817423	20.338	11.077	24.524
S2861.E1.L1	DS	94.9%	1,581,288,485	95,125,912	98,601,383	102,898,166	49	56	60	53	21.2%	22.2%	35.6%	1,149,674	963,971	973,089	1,124,139	27.212571	15.530	13.007	28.835
S1507.E1.L1	DS	66.6%	2,190,377,154	112,632,143	92,203,232	122,428,470	46	60	62	55	36.0%	24.2%	34.1%	1,145,533	986,514	962,047	1,127,321	25.511422	24.653	10.813	30.646
S1961.E1.L1	DS	76.2%	2,005,096,673	114,032,076	107,798,886	132,005,549	49	60	63	54	43.0%	25.6%	32.8%	1,144,017	974,391	989,221	1,126,761	25.828512	28.049	12.580	31.813
S2520.E1.L1	DS	87.3%	2,014,245,352	117,091,275	105,749,641	110,176,205	45	58	59	53	40.7%	23.4%	29.5%	1,149,058	936,241	956,714	1,104,061	27.544326	28.105	11.492	24.415
S5319.E1.L1	DS	95.5%	1,630,628,900	112,717,831	96,926,398	99,049,210	43	60	62	53	42.3%	21.9%	34.6%	1,149,058	975,859	972,853	1,125,249	29.167912	28.373	11.294	25.987

624 **Supplementary Figure 1: 10 library downsampling experiment using coverage as output.**



625 **Supplementary Section 1: Content added to Twist Ancient DNA Reagent beyond 1240k**

626
627 *(1a) Adding 94,586 polymorphisms on chromosomes 1-22 and X*

628
629 For the Twist Ancient DNA reagent, we began by attempting to bait all 1,233,013 SNPs in the 1240k
630 reagent. We then added additional content to target SNPs of phenotypic significance or SNPs
631 improving characterization of variation on the Y chromosome.

- 632
- 633 • “*GWAS*” SNPs (*SNPs associated with phenotypes in Genome-Wide Association Studies*)
634 We used a list of 236,638 SNPs that are genome-wide significant in one of 4,155 GWAS’s on 558
635 traits in a diverse set of populations (32). In contrast to the GWAS catalog database (50), this list
636 only includes SNPs identified in GWAS of 50,000 individuals or more.
 - 637
 - 638 • “*RELATE*” SNPs
639 We included SNPs estimated to have been under recent selection in any of 26 diverse modern
640 populations from the 1000 Genomes Project (38) based on distortions in coalescent tree shapes
641 (33). We selected all 61,308 SNPs with selection p-values $< 10^{-5}$ in any population.
 - 642
 - 643 • “*Clinvar*” SNPs
644 We included 32,689 SNPs from the Clinvar database by selecting all variants where the highest
645 reported allele frequency is $>1\%$ (34) (<https://www.ncbi.nlm.nih.gov/clinvar/>). These SNPs are
646 highly enriched for coding, non-synonymous variants.
 - 647
 - 648 • “*Polyfun*” SNPs
649 We included 75,592 fine-mapped SNPs falling in regions with functional annotations that are
650 enriched for heritability for a range of complex traits, specifically all SNPs with Posterior Inclusion
651 Probability of >0.1 (35).

652
653 *(b) Linkage disequilibrium (LD) pruning to remove genetically correlated SNPs*

654
655 We pruned the selected SNPs for linkage disequilibrium in 2,261 individuals from the 1000 Genomes
656 Project. For pruning, we use the PLINK (51) command `--indep-pairwise 1000 100 0.9`.

657
658 We computed LD for each of the remaining SNPs to the core set of 1240k SNPs using the command `--`
659 `r2 --ld-window-r2 0.2 --ld-window 10 --ld-window-kb 1000`. We excluded all SNPs with LD greater
660 than 0.9 to any 1240k SNP.

661
662 *(c) Quality control*

663
664 We characterized SNPs from all sources by their dbSNP reference numbers (rs-IDs) as well as their
665 reference and variant alleles. We filtered out insertion/deletion polymorphisms. We mapped rs-IDs to
666 chromosome and position and determined alleles using the Ensembl database for genome build
667 GRCh37 (hg19), accessed through biomaRt (<http://www.biomart.org/>). The hg19 reference sequence
668 (“hg19_1000g.fa.gz”) was then used to obtain 52 bp flanking either side. For multi-allelic sites, the
669 two variants identified in the original sources were kept. Alleles in the hg19 reference sequence were
670 designated as “ref”, and the alternative alleles as “alt”.

671

672 Table S1.1 shows a record of the SNPs deriving from each of these four methodologies, including the
673 number retained after the different pruning steps; this identified 94,586 SNPs.

674 **Table S1.1: SNPs selected from each source (there is some overlap, so total is not the sum)**

Name	Initial	Not in 1240k	After pruning	R ² <0.9	Would keep	Mean allele frequency	Mean R ² (>0.2)	Mean R ² (≤0.2)
Clinvar	32705	27495	20544	17262	17601	0.167	0.7	0.337
GWAS	236638	160819	66857	38540	38478	0.401	0.79	0.012
Polyfun	75592	59500	42088	32430	33145	0.279	0.72	0.174
Relate	61308	49701	23228	14579	14428	0.419	0.78	0.008
Total	375408	276824	140520	93812	94586	0.361	0.77	0.066

675 Note: “Would keep” includes SNPs not in the 1000 Genomes Project and with unclear LD, and excludes SNPs with
676 mismatching alleles or positions.

677

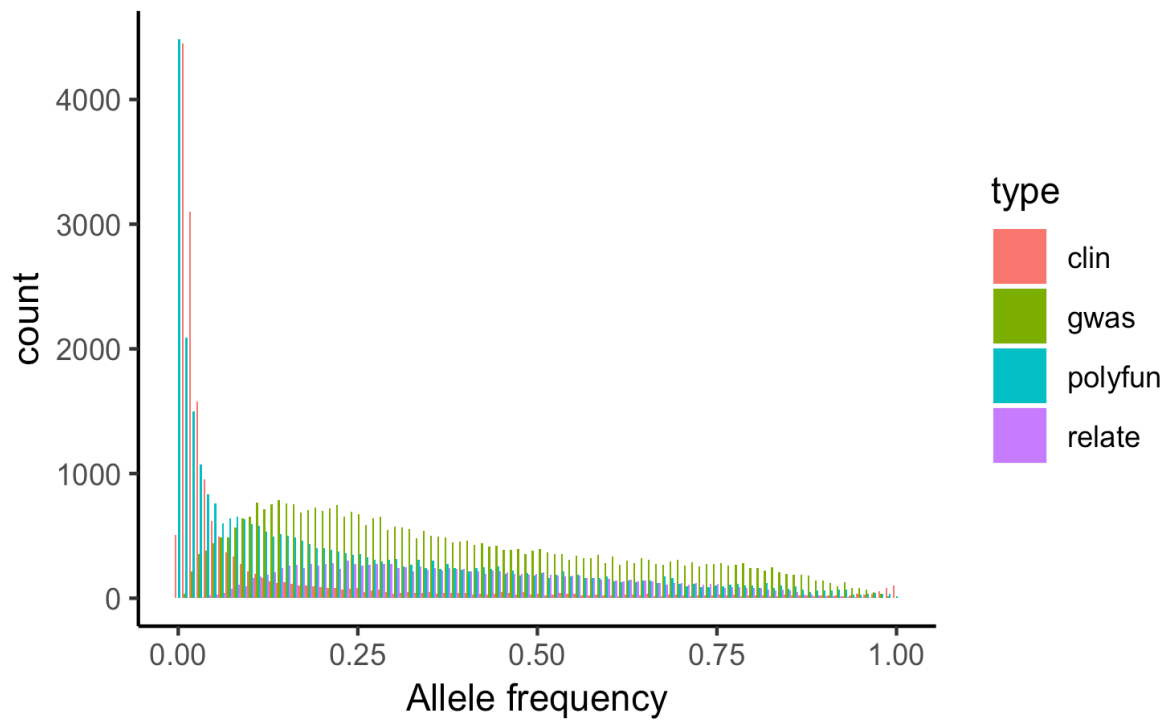
678 We sought to understand the genomic distribution and other characteristics of the newly added SNPs.
679 Table S1.2 shows the distribution across chromosomes for each of the four methodologies. Figure S1.1
680 shows the allele frequency distribution of the variant allele. Figure S1.2 shows the distribution of
681 maximum R² to any 1000 Genomes Project SNPs.

682

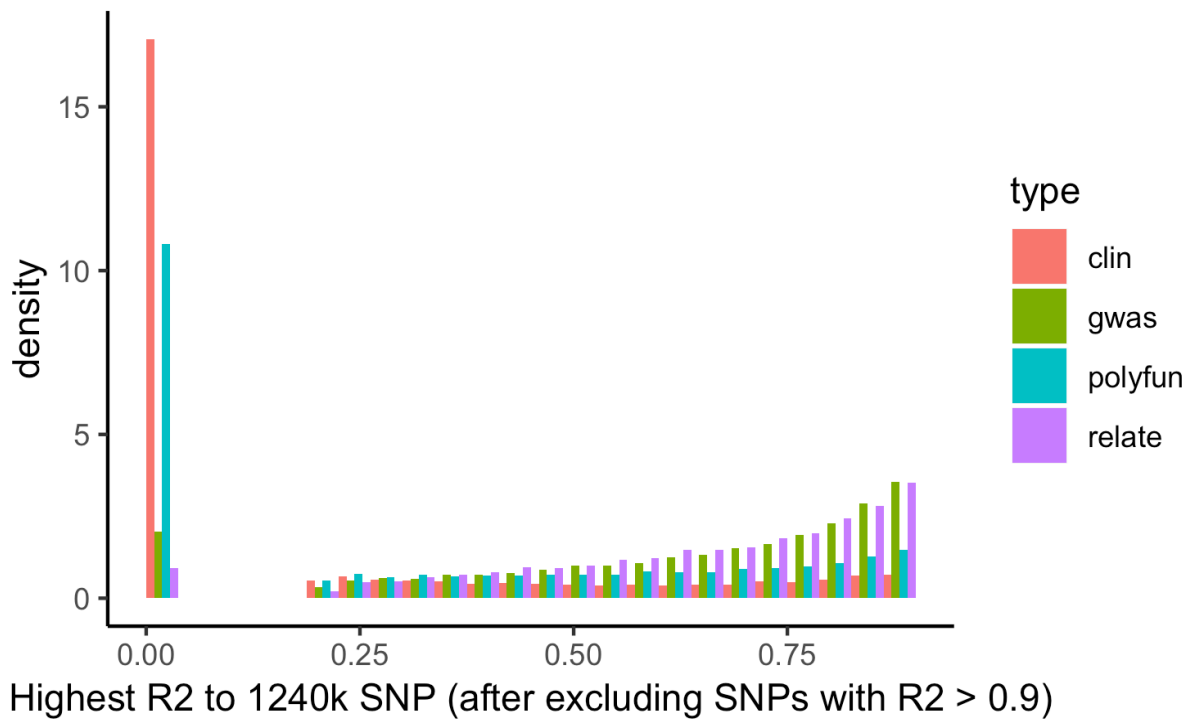
683 **Table S2: Number of newly targeted SNPs by chromosome**

Chromosome	Clinvar	GWAS	Polyfun	Relate
1	1496	2932	3053	1052
2	1612	4291	2773	1348
3	890	2775	2094	989
4	693	1902	1497	922
5	922	2486	1826	830
6	908	3020	1903	764
7	741	2030	1883	800
8	668	2300	1279	820
9	907	2158	1391	907
10	684	1549	1479	702
11	1031	2066	1677	714
12	888	2019	1841	644
13	392	999	909	458
14	533	1036	910	527
15	613	1517	1149	476
16	1042	1081	1433	713
17	1095	1156	1646	380
18	341	785	771	379
19	936	607	1557	307
20	464	1204	1098	301
21	324	80	410	192
22	378	485	567	203
X	43	NA	NA	NA
Total	17601	38478	33146	14428

684 **Figure S1.1: Allele frequency distribution by source of newly added SNPs**



685 **Figure S1.2: Linkage disequilibrium distribution by source. All SNPs with highest LD<0.2 set to 0.**



686 Finally, we manually added in 15 phenotypically important multi-allelic polymorphisms and 6
 687 insertion/deletion targets where we tiled both alternative alleles (Table S1.3).
 688
 689

Table S1.3: Manual addition of 15 multiallelic SNPs and 6 insertion/deletion targets

Target	Ch r	Ascertainment	Target type	Position of site in hg19 (start for Indel)	Beginning of targeted sequence in hg19	End of targeted sequence in hg19	Ref	Var(s)	Tiled Oligo-nucleotide
rs77931234	1	Medium-chain acyl-CoA dehydrogenase deficiency	Multiallelic position (design reference)	76226846	76226794	76226898	A	C,G,T	TTTTAATTCTAGC ACCAAGCAATATC ATTATGCTGGCTG AAATGGCAATGTA AGTTGAACAGCT AGAATGAGTTACC AGAGAGCAGCTTG GGAGGTTGATTG
snp_2_136608745	2	lactase persistence	Multiallelic position (design reference)	136608745	136608693	136608797	A	C,T	TTGTAGGGTCTAAG TACATTTTCTCTGA ATGAAAGGTATTA AATGGTAACCTTCG TCTTTATGCACTCT ATAAACTATGACG TGATCGTCTCCGTC TAACAACTA
rs75030631	5	Spinal Muscular Atrophy	Multiallelic position (design reference)	70220935	70220883	70220987	C	G,A	ACTCTTAAGAAGG GACGGGGCCCCAC GCTGCGCACCCCG GGGTTTGGTATGGA GATGAGCAGCGGC GGCAGTGGTGCGG GGTCCCGGAGCA GGAGGATTCGGTG
rs1800562	6	Hereditary Hemochromatosis	Multiallelic position (design reference)	26093141	26093089	26093193	G	A,T	CAGGGCTGGATAA CCTTGGCTGTACCC CTGGGGAAGAGC AGAGATATACGTT CCAGGTGGAGCAC CCAGGCCTGGATC AGCCCTCATTGTG ATCTGGGGTATG
rs111033171	9	Familial Dysautonomia	Multiallelic position (design reference)	111662096	111662044	111662148	A	G,T	ATTGCTTCACACA TAAATCACAAGCT AACTAGTCGCAAA CAGTACAATGGCT CTTACTTGCCAAC CACTTCCGAATCTG AGCTAAAACCGAG GCTCGATGATG
rs33985472	11	β -Thalassemia	Multiallelic position (design reference)	5246715	5246663	5246767	T	C,G	TAAAAATTTTCAGA AATAATTTAAATAC ATCATTGCAATGA AAATAAATGTTTGT TATTAGGCAGAAT CCAGATGCTCAAG GCCCTTATAATAT CCCCAGTTTA
rs35004220	11	β -Thalassemia	Multiallelic position (design reference)	5248050	5247998	5248102	C	T,A	ACCTCTGGGTCCAA GGGTAGACCACCA GCAGCCTAAGGGT GGGAAAAATAGACA AATAGGCAGAGAG AGTCAGTGCTATC AGAAACCCAAGAG TCTTCTGTCT
rs80338863	11	Smith-Lemli-Opitz syndrome	Multiallelic position (design reference)	71148990	71148938	71149042	C	G,T	TGCTTTCAGGTAC CAGGTTTGGTTCCA GAAGAAGTCAATC ACGTAGATGGCTT GCAAGACAGAAGC AGCCGTGACCAC CCCCGCCCTCCTG GGGCCCCATG
rs5030858	12	Phenylketonuria	Multiallelic position (design reference)	103234271	103234219	103234323	G	A,C	TCCAAGACCTCAAT CCTTGGGTGTATG GGTCTAGCGAAC TGAGAAGGGCCA GGTATTGTGGCAG CAAAGTTCCTAAG ACCAAAACACAG GCTTGAGTGAAG
snp_15_28496195	15	pigmentation	Multiallelic position (design reference)	28496195	28496143	28496247	A	G,C	ATGTCCATACAG GACCCACGTGCC ACAGGAACCAAAA AGTCACATGAGC CAGGATGAAGACA CAGGAGACAACCT GTGTGGACAGCAC AGAGCCACCTGCC G
snp_16_89383725	16	pigmentation	Multiallelic position (design reference)	89383725	89383673	89383777	T	C,G	ACAGGAATGGCAG CTTTGAGCAGGAA GGAGAACAGAGAA GGGTCAAGCACTT GGTAGTGGCAGAA AGGACGCATGGC

									CTAGGGTGTGGCT GTGTTCTGGGTGGC
rs3212355	16	pigmentation	Multiallelic position (design reference)	89984378	89984326	89984430	C	T,G	GAGTGAACCCAGG AAGATGCCTGCAG TGGGTGCCAGGGC CCCTCTCCACCCTG CCTGCTGGGCTTCG GGGCCACGCCCGA CTGCTGGAACGG CCTGCGGAGCAC
snp_16_89986122	16	pigmentation	Multiallelic position (design reference)	89986122	89986070	89986174	C	A,T	TGGGCGCCATCGC CGTGGACCGCTAC ATCTCCATCTCTA CGCACTGCGCTATC ACAGCATCGTGAC CCTGCGCGGGCG CGGGAGCCGTGG CGCCATCTGGG
snp_16_90024206	16	pigmentation	Multiallelic position (design reference)	90024206	90024154	90024258	A	G,T	CTCTCTAGCGGT GGTCTCTCTCGG CCTCAGGGGCTGA GGTAGAAGGGCTC GAGACAGCCAGGG TGAAGAGCGGGC CTCACCACCTGG GGAGGTTTCCC
snp_20_32665748	20	pigmentation	Multiallelic position (design reference)	32665748	32665696	32665800	A	G,T	GTTCACATTFTA CCCTGTAGGAAA TCGAGGCTCAGAA AGCTGAGTGGCT TGCTCAGGGATC AGCTCGTAGGGAC TGAGCCAGGGTTG GAGTCCAGACTGA
rs333	3	HIV-AIDS immunity	Insertion/deletion (design both versions)	46414947	46414908	46415012	GTC AGT ATC AAT TCT GGA AGA ATT TCC AGA CA	deletion	AAGGTCTTCATTAC ACCTGCAGCTTCA TTTTCCATACAGTC AGTATCAAATCTGG AAGAATTCAGCA CATTAAGATAGT CATCTTGGGGCTGG TCCTGCCGC
rs333.deletion	3	HIV-AIDS immunity	Insertion/deletion (design both versions)	46414947	46414893	46415029	GTC AGT ATC AAT TCT GGA AGA ATT TCC AGA CA	deletion	CCAGATCTCAAAA AGAAGGTCTTCATT ACACCTGCAGCTCT CATTTTCCATACAT TAAAGATAGTATC CTTGGGGCTGGTCC TGCCGCTGGTTGTC ATGGTCATC
rs113993960	7	Cystic Fibrosis	Insertion/deletion (design both versions)	117199646	117199594	117199698	CTT	deletion	TCTGTCTCAGTTT TCCTGGATTATGCC TGCCACCATAAA GAAAATATCATCTT TGGGTGTTCTATG ATGAATATAGATA CAGAAGCGTCTATC AAAGCATGCC
rs113993960.deletion	7	Cystic Fibrosis	Insertion/deletion (design both versions)	117199646	117199593	117199700	CTT	deletion	TTCTGTTCTCAGTT TTCTGGATTATGC CTGGACCATTAA AGAAAATATCAAT GGTGTTCCTATGA TGAATATAGATAC AGAAGCGTCTATCA AAGCATGCCAA
rs387906309	15	Tay-Sachs	Insertion/deletion (design both versions)	72638921	72638870	72638974	insertion	GATA	TCAAATGCCAGGG GTTCCACTATGTAG AAATCTTCCAGTC AGGGCCATAGGAT ATACGGTTCAGGT ACCAGGGGCGAGA GAGAAGGCCCGG AAGCCGGCCTTG
rs387906309.insertion	15	Tay-Sachs	Insertion/deletion (design both versions)	72638921	72638872	72638972	insertion	GATA	AAATGCCAGGGGT TCCACTATGTAGAA ATCTTCCAGTCAG GGCCATAGGATAG ATATACGGTTCAG GTACCAGGGGCGA GAGAGAAGGGCCC GGAAGCCGGCCT
rs41474145	16	α -Thalassemia	Insertion/deletion (design both versions)	223008	222956	223060	TGA GG	deletion	GGTAAGGTCCGC GCGCAGCTGGCG AGTATGGTGCAG GGCCCTGGAGAGG TGAGGCTCCCTCCC CTGCTCCGACCCGG GCTCCTGCCCGCC CGGACCCACAG

rs41474145.deletion	16	α -Thalassemia	Insertion/deletion (design both versions)	223008	222953	223062	TGA GG	deletion	CTGGGGTAAGTTC GGCGGCACGCTG GGAGTATGGTGC GGAGGCCCTGGAG AGGCTCCCTCCCT GCTCCGACCCGGG CTCCTCGCCCGCC GGACCCACAGGC
rs63751471	16	α -Thalassemia	Insertion/deletion (design both versions)	223510	223463	223567	CTC CCC GCC GAG	deletion	CTGCACAGCTCCTA AGCCTGCTGCTGCT GGTACCTGGCC GCCACTCCCGC CGAGTTCACCCCTG CGGTGCAGCCTCC CTGGACAAGTCTCT GGCTTCTG
rs63751471.deletion	16	α -Thalassemia	Insertion/deletion (design both versions)	223510	223463	223579	CTC CCC GCC GAG	deletion	CTGCACAGCTCCTA AGCCTGCTGCTGCT GGTACCTGGCC GCCACTTCACCC TGCGGTGCAGCCT CCCTGGACAAGTTC CTGGCTTCTGTGAG CACCGTGC
rs587776730	X	Favism	Insertion/deletion (design both versions)	153761232	153761189	153761293	C	deletion	ACGGCTGAAAAG TGGCGGTGGTGA CCCGGGGGCACC GTGGGGTGTCCA GGTACCCTTTGGTG GCCTCGCCCTCTCC ATCGGGGTTCCCA CGTACTGGCCC
rs587776730.deletion	X	Favism	Insertion/deletion (design both versions)	153761232	153761177	153761305	C	deletion	ACATAGAGGACGA CGGTGCAAAAGT GGCGGTGGTGGAC CCCGGGGGACCG TGCCCTGCCCCTCT CCATCGGGGTTCC CACGTAAGTGGCC AGGACCACATTG

690 (1b) Targeting 81,925 polymorphisms on chromosome Y

691

692 To identify Y chromosome targets, we started with 32,670 chromosome Y SNPs from the 1240k
693 reagent. These had been identified by starting with ISOGG 9.77 SNPs (<https://isogg.org/>), and then
694 merging with SNPs identified as polymorphic in the Simons Genome Diversity Panel (52, 53).

695

696 For our redesign, we added in 69,991 Y SNPs from the ISOGG Y SNP index version 14.199
697 downloaded Nov. 5 (<https://isogg.org/>). To obtain this list, we started with 88,795 polymorphisms in
698 the download, removed ones with duplicate positions, and restricted to true SNPs that are biallelic for
699 the alleles A/C/G/T.

700

701 After merging and removing duplicates, this generated 88,023 SNPs. We reduced this to 81,925 by
702 removing SNPs monomorphic in the existing 1240k enrichment dataset, or that had coverage counts in
703 that dataset of <10%.

704

705 In contrast to the 94,586 SNPs identified in Section 1a which represent a supplement to the 1240k
706 content on chromosomes 1-22 and X, for the Y chromosome the 81,925 SNPs we discuss are a
707 replacement of the 1240k content on chromosome Y.

708

709 (1c) Final count of SNPs

710

711 The total number of SNPs targeted for the reagent is:

712

713	1,200,343	1240k content on chromosomes 1-22 and Y
714	94,586	Newly designed phenotypic discussed in Section 1a
715	81,925	Fully redesigned Y chromosome content discussed in Section 1b
716	1,376,854	Total

717

718 For each targeted SNP, we randomly selected a third allele to represent each position and flanked it
719 52bp on either side according to the sequence from the hg19 reference genome. We then mapped the
720 sequence to hg19. After removing oligonucleotides that mapped unreliably with a score of MAPQ<23,
721 or that mapped to a location that disagreed with the recorded positions, or that was duplicated in its
722 sequence compared to another in the dataset, or that failed other quality controls, our design file
723 targeted 1,352,535 SNPs.

724

725 *(1d) Tiled regions (with either 1x or 2x tiling)*

726

727 Beyond SNP targeting, we also added in probes to bait additional genomics regions.

728

729 • *“Methylation” targets*

730 We are grateful to Steve Horvath and Vagheesh Narasimhan for providing us with the coordinates
731 of 40,000 CpG dinucleotides chosen to be locations where methylation rates are correlated to the
732 skeletally determined ages of ancient individuals. These CpG dinucleotides are also ones where
733 methylation rates have been shown to be well-correlated to the ages of living individuals. Of these
734 targets, we successfully designed single probes for 39,886 (we did not design probes for the others
735 due to repetitive flanking sequence).

736

737 • *“Human Accelerated Region (HAR)” targets*

738 We are grateful to Ryan Doan for sharing with us a list of 3,171 Human Accelerated Regions
739 (HARs) spanning 857,339 nucleotides. We tiled each of these regions twice (with 80bp probes
740 overlapping every 40bp).

741

742 • *“Gene resequencing” targets*

743 This includes 9 contiguous regions in 3 genes, specified in hg19 coordinates. The segments target
744 SNPs believed to contribute to β -thalassemia (chr. 11: 5247022-5247193 and 5248114-5248429),
745 α -thalassemia (chr. 16: 222873-223052 and 223469-223733), and favism (chr. X: 153220145-
746 153220335, 153760378-153761377, 153761761-153761889, 153763362-153763532, 153764171-
747 153764423, and 153774226-153774316). The SNPs are rs34690599, rs34451549, rs35724775,
748 rs33915217, rs33971440, rs33960103, rs33986703, rs34716011, rs63750783, rs334,
749 rs34598529, rs33944208, rs111033603, rs281864819, rs41474145, rs63750404, rs63751471,
750 rs33987053, rs41397847, rs41464951, rs63751269, rs137852348, rs137852344, rs72554664,
751 rs72554665, rs72554665, rs137852324, rs137852317, rs137852337, rs2230037, rs137852336,
752 rs137852323, rs137852335, rs137852316, rs137852316, rs137852321, rs137852334, rs137852320,
753 rs137852322, rs2230036, rs387906468, rs137852329, rs137852345, rs137852333, rs137852342,
754 rs5030869, rs587776730, rs76723693, rs137852347, rs137852339, rs137852327, rs74575103,
755 rs137852318, rs137852346, rs137852328, rs137852328, rs137852319, rs137852326, rs137852332,
756 rs137852332, rs137852330, rs5030868, rs267606836, rs5030872, rs5030872, rs137852343,
757 rs137852331, rs137852314, rs2515904, rs137852313, rs137852341, rs1050829, rs137852349,
758 rs1050828, rs137852315, rs76645461, and rs78478128. We tiled segments with 80bp probes
759 staggered every 40bp.

760 Supplementary Section 2: EM Algorithm to Correct for Binomial Sampling Variance

761
762 The problem we wish to solve is that we have empirical counts of reference and variant alleles for
763 large numbers of known or highly probable heterozygous positions. Here we describe how we
764 deconvolve the noise to learn the underlying distribution of reference bias.

765
766 We consider a set of reference and variance counts (typically summing to 100 or more). At SNP k we
767 observe a_k reference and b_k variant alleles. We suppose the ‘true’ allele frequency of reference is $z_k = z$
768 which we can think of as the frequency we would observe if the coverage were infinite. We wish to
769 learn the probability distribution of z . We will ignore (in this note) the case that the observed counts
770 are not polymorphic, so we assume $a_k, b_k \geq 1$.

771
772 Let us model z_k as lying on a mesh; for instance, $z_k = i/100$ for some $i = 1 \dots 99$. We propose to estimate
773 $p_i = (z_k = i/100)$. Write $\alpha_i = i/100$; $\beta_i = (100-i)/100$. We see that the log likelihood of our observation
774 for SNP k is:

$$775 \quad \mathcal{L}(k) = \log \left(\sum_i \alpha_i^{a_k} \beta_i^{b_k} + (a_k + b_k) \log 2 \right)$$

776
777 The last term is not essential, but good technique is to score against some random model; here that a_k is
778 from tossing a fair coin toss (50% probability heads). The overall log likelihood is:

$$779 \quad \mathcal{L} = \mathcal{L}(\mathbf{p}) = \sum_i \mathcal{L}(k)$$

780
781 \mathcal{L} is easily maximized by an EM algorithm. Write:

$$782 \quad l(i,k) = \log p_i + a_k \log \alpha_i + b_k \log \beta_i$$

$$783 \quad lmax_k = \max_i l(i,k)$$

$$784 \quad \theta(i,k) = \exp(l(i,k) - lmax_k)$$

$$785 \quad \gamma(i,k) = \frac{\theta(i,k)}{\sum_j \theta(j,k)}$$

786
787 Thus, $\gamma(i,k)$ is the posterior probability that $z_k = \alpha_i$. Reestimates are now simply:

$$788 \quad \hat{p}_i = \sum_k \gamma(i,k) / N$$

789
790 where N is the number of SNPs. Standard EM shows that:

$$791 \quad \mathcal{L}(\hat{\mathbf{p}}) \geq \mathcal{L}(\mathbf{p})$$

792
793 We iterate until convergence. We implemented this in C to produce the inferences in Figure 5.

Literature Cited (Main manuscript)

1. H. A. Burbano *et al.*, Targeted investigation of the Neandertal genome by array-based sequence capture. *Science* **328**, 723-725 (2010).
2. A. Gnirke *et al.*, Solution hybrid selection with ultra-long oligonucleotides for massively parallel targeted sequencing. *Nat Biotechnol* **27**, 182-189 (2009).
3. J. K. Teer, J. C. Mullikin, Exome sequencing: the sweet spot before whole genomes. *Hum Mol Genet* **19**, R145-151 (2010).
4. M. L. Carpenter *et al.*, Pulling out the 1%: whole-genome capture for the targeted enrichment of ancient DNA sequencing libraries. *Am J Hum Genet* **93**, 852-864 (2013).
5. Q. Fu *et al.*, DNA analysis of an early modern human from Tianyuan Cave, China. *Proc Natl Acad Sci U S A* **110**, 2223-2227 (2013).
6. T. Maricic, M. Whitten, S. Paabo, Multiplexed DNA sequence capture of mitochondrial genomes using PCR products. *PLoS One* **5**, e14004 (2010).
7. S. Castellano *et al.*, Patterns of coding variation in the complete exomes of three Neandertals. *Proc Natl Acad Sci U S A* **111**, 6666-6671 (2014).
8. Q. Fu *et al.*, An early modern human from Romania with a recent Neanderthal ancestor. *Nature* **524**, 216-219 (2015).
9. W. Haak *et al.*, Massive migration from the steppe was a source for Indo-European languages in Europe. *Nature* **522**, 207-211 (2015).
10. I. Mathieson *et al.*, Genome-wide patterns of selection in 230 ancient Eurasians. *Nature* **528**, 499-503 (2015).
11. I. Lazaridis *et al.*, Genomic insights into the origin of farming in the ancient Near East. *Nature* **536**, 419-424 (2016).
12. M. Lipson *et al.*, Ancient DNA and deep population structure in sub-Saharan African foragers. *Nature In press* (2022).
13. M. Lipson *et al.*, Ancient West African foragers in the context of African population history. *Nature* **577**, 665-670 (2020).
14. I. Mathieson *et al.*, The genomic history of southeastern Europe. *Nature* **555**, 197-203 (2018).
15. I. Olalde *et al.*, The genomic history of the Iberian Peninsula over the past 8000 years. *Science* **363**, 1230-1234 (2019).
16. N. Patterson *et al.*, Ancient admixture in human history. *Genetics* **192**, 1065-1093 (2012).
17. N. Nakatsuka *et al.*, The promise of discovering population-specific disease-associated genes in South Asia. *Nat. Genet.* **49**, 1403-1407 (2017).
18. I. Lazaridis *et al.*, Ancient human genomes suggest three ancestral populations for present-day Europeans. *Nature* **513**, 409-413 (2014).
19. S. Lopez *et al.*, Evidence of the interplay of genetics and culture in Ethiopia. *Nat Commun* **12**, 3581 (2021).
20. P. Flegontov *et al.*, Paleo-Eskimo genetic legacy across North America. *bioRxiv* (2017).
21. C. Jeong *et al.*, The genetic history of admixture across inner Eurasia. *Nat Ecol Evol* **3**, 966-976 (2019).
22. C. C. Wang *et al.*, Genomic insights into the formation of human populations in East Asia. *Nature* **591**, 413-419 (2021).
23. P. Skoglund *et al.*, Genomic insights into the peopling of the Southwest Pacific. *Nature* **538**, 510-513 (2016).
24. W. Kutanan *et al.*, Reconstructing the Human Genetic History of Mainland Southeast Asia: Insights from Genome-Wide Data from Thailand and Laos. *Mol Biol Evol* **38**, 3459-3477 (2021).
25. J. K. Pickrell *et al.*, The genetic prehistory of southern Africa. *Nat Commun* **3**, 1143 (2012).
26. M. Lipson *et al.*, Population Turnover in Remote Oceania Shortly after Initial Settlement. *Curr Biol* **28**, 1157-1165 e1157 (2018).

27. P. Qin, M. Stoneking, Denisovan Ancestry in East Eurasian and Native American Populations. *Mol Biol Evol* **32**, 2665-2674 (2015).
28. C. Barbieri *et al.*, The Current Genomic Landscape of Western South America: Andes, Amazonia, and Pacific Coast. *Mol Biol Evol* **36**, 2698-2713 (2019).
29. A. Bergstrom *et al.*, Insights into human genetic variation and population history from 929 diverse genomes. *Science* **367** (2020).
30. D. M. Behar *et al.*, A "Copernican" reassessment of the human mitochondrial DNA tree from its root. *Am J Hum Genet* **90**, 675-684 (2012).
31. R. E. Green *et al.*, A complete Neandertal mitochondrial genome sequence determined by high-throughput sequencing. *Cell* **134**, 416-426 (2008).

Literature Cited (Supplementary Sections)

32. K. Watanabe *et al.*, A global overview of pleiotropy and genetic architecture in complex traits. *Nat Genet* **51**, 1339-1348 (2019).
33. L. Speidel, M. Forest, S. Shi, S. R. Myers, A method for genome-wide genealogy estimation for thousands of samples. *Nat Genet* **51**, 1321-1329 (2019).
34. M. J. Landrum *et al.*, ClinVar: improvements to accessing data. *Nucleic acids research* **48**, D835-D844 (2020).
35. O. Weissbrod *et al.*, Functionally informed fine-mapping and polygenic localization of complex trait heritability. *Nat Genet* **52**, 1355-1363 (2020).
36. P. Flegontov *et al.*, Palaeo-Eskimo genetic ancestry and the peopling of Chukotka and North America. *Nature* **570**, 236-240 (2019).
37. D. Gokhman *et al.*, Differential DNA methylation of vocal and facial anatomy genes in modern humans. *Nat Commun* **11**, 1189 (2020).
38. A. Auton *et al.*, A global reference for human genetic variation. *Nature* **526**, 68-74 (2015).
39. C. Fowler *et al.*, A high-resolution picture of kinship practices in an Early Neolithic tomb. *Nature* 10.1038/s41586-021-04241-4 (2021).
40. T. Gunther, C. Nettelblad, The presence and impact of reference bias on population genomic studies of prehistoric human populations. *PLoS Genet* **15**, e1008302 (2019).
41. R. Martiniano, E. Garrison, E. R. Jones, A. Manica, R. Durbin, Removing reference bias and improving indel calling in ancient DNA data analysis by mapping to a sequence variation graph. *Genome Biol* **21**, 250 (2020).
42. S. Rubinacci, D. M. Ribeiro, R. J. Hofmeister, O. Delaneau, Efficient phasing and imputation of low-coverage sequencing data using large reference panels. *Nat Genet* **53**, 120-126 (2021).
43. N. Patterson *et al.*, Large-scale migration into Britain during the Middle to Late Bronze Age. *Nature* 10.1038/s41586-021-04287-4 (2021).
44. J. Dabney *et al.*, Complete mitochondrial genome sequence of a Middle Pleistocene cave bear reconstructed from ultrashort DNA fragments. *Proc Natl Acad Sci U S A* **110**, 15758-15763 (2013).
45. P. Korlevic *et al.*, Reducing microbial and human contamination in DNA extractions from ancient bones and teeth. *Biotechniques* **59**, 87-93 (2015).
46. N. Rohland, I. Glocke, A. Aximu-Petri, M. Meyer, Extraction of highly degraded DNA from ancient bones, teeth and sediments for high-throughput sequencing. *Nat Protoc* **13**, 2447-2461 (2018).
47. M.-T. Gansauge, A. Aximu-Petri, S. Nagel, M. Meyer, Manual and automated preparation of single-stranded DNA libraries for the sequencing of DNA from ancient biological remains and other sources of highly degraded DNA. *Nature Protocols* **15**, 2279-2300 (2020).
48. N. Rohland, E. Harney, S. Mallick, S. Nordenfelt, D. Reich, Partial uracil-DNA-glycosylase treatment for screening of ancient DNA. *Philos Trans R Soc Lond B Biol Sci* **370**, 20130624 (2015).

49. H. Li, R. Durbin, Fast and accurate long-read alignment with Burrows-Wheeler transform. *Bioinformatics* **26**, 589-595 (2010).
50. D. Welter *et al.*, The NHGRI GWAS Catalog, a curated resource of SNP-trait associations. *Nucleic acids research* **42**, D1001-1006 (2014).
51. S. Purcell *et al.*, PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet* **81**, 559-575 (2007).
52. Q. Fu *et al.*, An early modern human from Romania with a recent Neanderthal ancestor. *Nature* **524**, 216-219 (2015).
53. S. Mallick *et al.*, The Simons Genome Diversity Project: 300 genomes from 142 diverse populations. *Nature* **538**, 201-206 (2016).