Supplemental Material

Single-stranded DNA library preparation from highly degraded DNA using *T4* DNA ligase

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Single-stranded library preparation

<table>
<tr><th>ssDNA2.0</th><th></th><th>CircLigase method</th></tr>
<tr><td colspan="3">Enzymatic purification of oligonucleotides involved in single-stranded DNA ligation
Purification of adapter oligonucleotide CL78 (e.g. Sigma-Aldrich or Eurogentec for oligonucleotide systhesis) was carried out in 20 µl reactions containing 2 µl of 10 µM oligonucleotide, 1 x T4 RNA ligase buffer (New England Biolabs (NEB)), 10 U Klenow fragment of E. coli DNA polymerase I (ThermoFisher Scientific) and 10 U T4 polynucleotide kinase (ThermoFisher Scientific). Reactions were incubated for 20 min at 37°C in a thermal cycler, followed by heat inactivation of the enzyme for 1 min at 95°C.</td></tr>
<tr><td>Splinter oligonucleotides were purified in 20 µl reactions containing 40 µM splinter (TL93, TL106, TL110, TL136 or TL137), 1x T4 RNA ligase buffer, 10 U Klenow fragment of E. coli DNA polymerase I and 10 U T4 polynucleotide kinase. Reactions were incubated for 20 min at 37°C in a thermal cycler, followed by heat inactivation of the enzyme for 1 min at 95°C. Purified adapter and splinter oligonucleotides were combined to a final reaction volume of 40 µl and hybridized by incubation at 95°C for 10 s in a thermal cycler, followed by a ramp to 10°C at 0.1°C/s. Final concentrations of the hybridized double stranded adapter oligonucleotides were 10 µM CL78 / 20 µM TLxx.</td><td></td><td>The purified adapter oligonucleotide CL78 was diluted to 10 µM by adding 20 µl TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).</td></tr>
<tr><td colspan="3">Hybridization of double-stranded adapter for 2nd ligation
In preparation of the second ligation step in library preparation, double-stranded adapter molecules were generated by combining 20 µl of 500 µM oligonucleotide CL53, 20 µl of 500 µM oligonucleotide CL73, 9.5 µl TE buffer and 0.5 µl 5 M NaCl in a 50 µl reaction. Oligonucleotides were hybridized by incubation the reaction mix in a thermal cycler at 95°C for 10 s and cooling</td></tr>
</table>

to 14°C at a rate of 0.1°C /s. 50 µl TE buffer were added to obtain CL53/73 adapter in a final concentration of 100 µM.	
Heat denaturation, dephosphorylation and ligation of first adapter	
Reaction mixes with the following components were prepared in 0.5 ml LoBind tubes (Eppendorf): 8 µl 10x *T4* RNA ligation buffer, 2 µl 2% Tween 20 (Sigma-Aldrich), 1 µl FastAP (1 U/µl; Thermo Fisher Scientific), varying volumes of DNA extract. Water was added to obtain a total volume of 45.6 µl.	Reaction mixes with the following components were prepared in 0.5 ml LoBind tubes (Eppendorf): 8 µl 10x CircLigase Buffer II (Epicentre), 4 µl 50 mM $MnCl_2$ (Epicentre), 1 µl FastAP (1 U/µl, ThermoFisher Scientific), varying volumes of DNA extract. Water was added to obtain a total volume of 43 µl.
Reactions were thoroughly mixed by flicking the tubes with a finger and spun down shortly in a microcentrifuge. Reactions were incubated for 10 min at 37°C in thermal cycler, heated to 95°C for 2 min and immediately placed into an ice water bath.	
The following components were added to obtain a total reaction volume of 80 µl (tubes were properly mixed by vortexing before adding the enzyme): 32 µl 50 % PEG-8000 (NEB), 0.4 µl 100 mM ATP (ThermoFisher Scientific), 1 µl CL78/TL110 (10/20 µM) and 1 µl *T4* DNA Ligase (30 U/µl; ThermoFisher Scientific). After adding the enzyme reactions were thoroughly mixed by flicking the tubes with a finger and briefly spun down in a microcentrifuge. Reactions were incubated for 1 h at 37°C and 1 min at 95°C. Reactions were held at 10°C afterwards and frozen at -20°C until proceeding with the next steps.	The following components were added to obtain a total reaction volume of 80 µl (tubes were properly mixed by vortexing before adding the enzyme): 32 µl 50 % PEG-4000 (Epicentre), 1 µl CL78 (10 µM) and 4 µl CircLigase (100 U/µl, Epicentre). After adding the enzyme reactions were thoroughly mixed by flicking the tubes with a finger and briefly spun down in a microcentrifuge. Reactions were incubated for 1 h at 60°C. 2 µl of stop solution (0.5 M EDTA) was added to each reaction mixture. Contents was mixed by vortexing, tubes were spun in a microcentrifuge and froze at -20°C before further use.

Immobilization of ligation products on beads

Stock solution of MyOne C1 beads (ThermoFisher Scientific) was resuspended by vortexing. For each reaction, 20 µl bead suspension were transferred into a 1.5 ml tube. Beads were washed twice with 500 µl 1xBWT+SDS (1 M NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 0.05% Tween-20 and 0.5% SDS) and resuspended in 250 µl 1xBWT+SDS (multiplied by the number of reactions, e.g. 1.5 ml for 6 reactions). Per sample, 250 µl beads were transferred to 1.5 ml LoBind tube.

Ligation mixes were thawn, incubated for 1 min at 95°C and immediately transferred to an ice-water bath. After 2 to 5 min of cooling, the ligation mix was added to the bead suspension. The bead suspension was briefly vortexed and tubes were rotated for 20 min at room temperature. Afterwards tubes were spun briefly in a microcentrifuge.

Beads were pelleted using a magnet rack and the supernatant was removed. 200 µl 0.1xBWT+SDS (0.1 M NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 0.05% Tween-20 and 0.5% SDS) were added, tubes were transferred to a ThermoMixer (set to 25°C; MKR 13, HLC/Ditabis) and vortexed for 8 s to resuspend the beads. Tubes were spun briefly in a microcentrifuge and placed into a magnetic rack. The supernatant was discarded.

Stringency wash for splinter removal

100 µl Stringency wash buffer (0.1xSSC and 0.1% SDS) were added, beads were resuspended by vortexing and transferred to the ThermoMixer, which was preheated to 45°C. Suspensions were incubated for 3 min at 45 °C with 3 s interval mixing at 1500 rpm every 30 s. Tubes were spun briefly in a microcentrifuge and placed into a magnetic rack. The supernatant was discarded and the ThermoMixer was set to 25°C.

Bead wash

200 µl 0.1xBWT (0.1 M NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0) and 0.05 % Tween-20) were added, tubes were transferred to the ThermoMixer and vortexed for 8 s.

Primer annealing and extension		
A 48 µl fill-in mix containing the following components was prepared for each reaction: 39.1 µl water, 5 µl 10x Klenow reaction buffer (ThermoFisher Scientific), 0.4 µl 25 mM dNTP (ThermoFisher Science), 2.5 µl 1% Tween-20 and 1 µl CL130 (100 µM).		A 47 µl fill-in mix containing the following components was prepared for each reaction: 40.5 µl water, 5 µl 10x Isothermal amplification buffer (NEB), 0.5 µl 25 mM dNTP´s (ThermoFisher Science) and 1 µl CL9 (100 µM).
The bead suspension was spun down in a microcentrifuge and placed into a magnetic rack. The supernatant was discarded and the beads were resuspended in the fill-in mix by vortexing. Tubes were transferred to a thermal cycler with open lid, incubated at 65°C for 2 min, immediately placed into an ice-water bath for 2 to 5 min and transferred to a tube rack at room temperature. .		
2 µl Klenow fragment (10 U/µl) were added, tubes were transferred to the ThermoMixer (set to 25°C) and the beads resuspended by mixing for 3 s at 1500 rpm. Suspensions were incubated first for 5 min at 25°C with 2 s interval mixing at 1500 rpm every 60 s and then for 25 min at 35°C with 2 s interval mixing every 60 s.		3 µl *Bst* polymerase 2.0 (8 U/µl, NEB) were added, tubes were transferred to the ThermoMixer (set to 15°C) and the beads resuspended by mixing for 3 s at 1500 rpm every 60 s and then for 1 min at 15°C followed by a 30 min ramp to 37°C (hold at 37°C for 5 min) with 2 s interval mixing every 60 s
Post-extension washes The ThermoMixer was set to 25°C. Tubes were spun briefly in a microcentrifuge. Beads were pelleted using a magnet rack and the supernatant was discarded. 200 µl 0.1xBWT+SDS were added, tubes were transferred to the ThermoMixer (set to 25°C) and vortexed for 8 s at 1500 rpm. Tubes were spun briefly in a microcentrifuge, placed into a magnetic rack, and the supernatant was discarded. 100 µl Stringency wash buffer were added, beads were resuspended by vortexing and transferred to the ThermoMixer pre-heated to 45°C. The bead suspensions were incubated for 3 min at 45 °C with 3 s interval mixing at 1500 rpm every 30 s.		

Tubes were spun briefly in a microcentrifuge and placed into a magnetic rack. The supernatant was discarded and the ThermoMixer set to 25°C. ThermoMixerBeads were pelleted using a magnetic rack and the supernatant was discarded. 200 µl 0.1xBWT were added, tubes were transferred to ThermoMixer (25°C) and vortexed for 8 s at 1500 rpm.		
		Blunt-end repair The following components were mixed to obtain reaction mixed with a total volume of 99 µl: 86.1 µl water, 10 µl 10x Tango buffer (ThermoFisher Scientific), 0.4 µl 25 mM dNTP, and 2.5 µl 1% Tween-20. The bead-suspensions were spun down in a microcentrifuge, the supernatant was discarded and beads were resuspended in the blunt-end repair mix by vortexing. To each reaction, 1 µl *T4* DNA polymerase (5 U/µl; ThermoFisher Scientific) was added, tubes were transferred to the ThermoMixer (set to 25°C) and vortexed for 3 s at 1500 rpm. Suspensions were then incubated for 15 min at 25°C with 2 s interval mixing at 1500 rpm every 60 s.
		Post-blunt-end-repair washes The ThermoMixer was set to 25°C. Tubes were spun briefly in a microcentrifuge. Beads were pelleted using a magnet rack and the supernatant was discarded. 200 µl 0.1xBWT+SDS were added, tubes were transferred to the ThermoMixer (set to 25°C) and vortexed for 8 s at 1500 rpm. Tubes were spun briefly in a microcentrifuge, placed into a magnetic rack, and the supernatant was

		discarded. 100 µl Stringency wash buffer were added, beads were resuspended by vortexing and transferred to the ThermoMixer preheated to 45°C. The bead suspensions were incubated for 3 min at 45 °C with 3 s interval mixing at 1500 rpm every 30 s. Tubes were spun briefly in a microcentrifuge and placed into a magnetic rack. The supernatant was discarded and the ThermoMixer set to 25°C. ThermoMixerBeads were pelleted using a magnetic rack and the supernatant was discarded. 200 µl 0.1xBWT were added, tubes were transferred to ThermoMixer (25°C) and vortexed for 8 s at 1500 rpm.

Ligation of second adapter, library elution

The following components were mixed to obtain reaction mixed with a total volume of 100 µl: 73.5 µl water, 10 µl 10x *T4* DNA ligase buffer (ThermoFisher Scientific), 10 µl 50 % PEG-4000, 2 µl CL53/73 (100 µM), 2.5 µl 1% Tween-20 and 2 µl *T4* DNA ligase (5 U/µl).

The bead-suspensions were spun down in a microcentrifuge, the supernatant was discarded and beads were resuspended in the ligation mix by vortexing. Tubes were transferred to the ThermoMixer (set to 22°C) and vortexed for 3 s at 1500 rpm. Suspensions were then incubated for 1 h at 22°C with 2 s interval mixing at 1500 rpm every 60 s

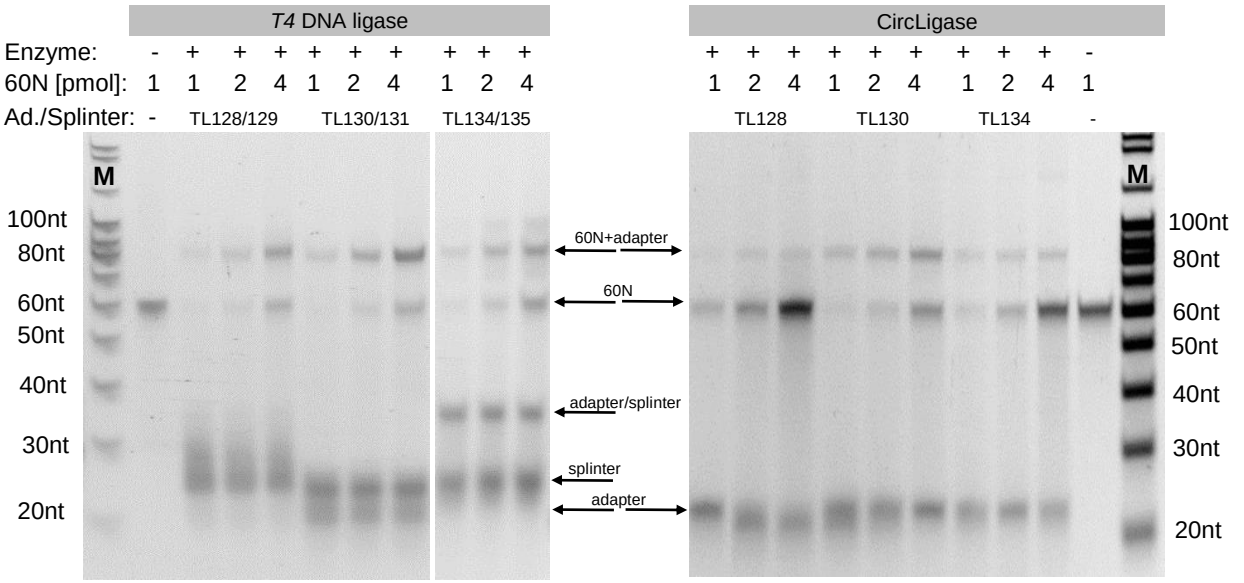
Post-ligation washes

The ThermoMixer was set to 25°C. Tubes were spun briefly in a microcentrifuge. Beads were pelleted using a magnet rack and the supernatant was discarded. 200 µl 0.1xBWT+SDS were added, tubes were transferred to the ThermoMixer (set to 25°C) and vortexed for 8 s at 1500 rpm. Tubes were spun briefly in a microcentrifuge, placed into a magnetic rack, and the supernatant was discarded. 100 µl Stringency wash buffer were added, beads were resuspended by vortexing and transferred to the ThermoMixer preheated to 45°C. The bead suspensions were incubated for 3 min at 45 °C with 3 s interval mixing at 1500 rpm every 30 s.

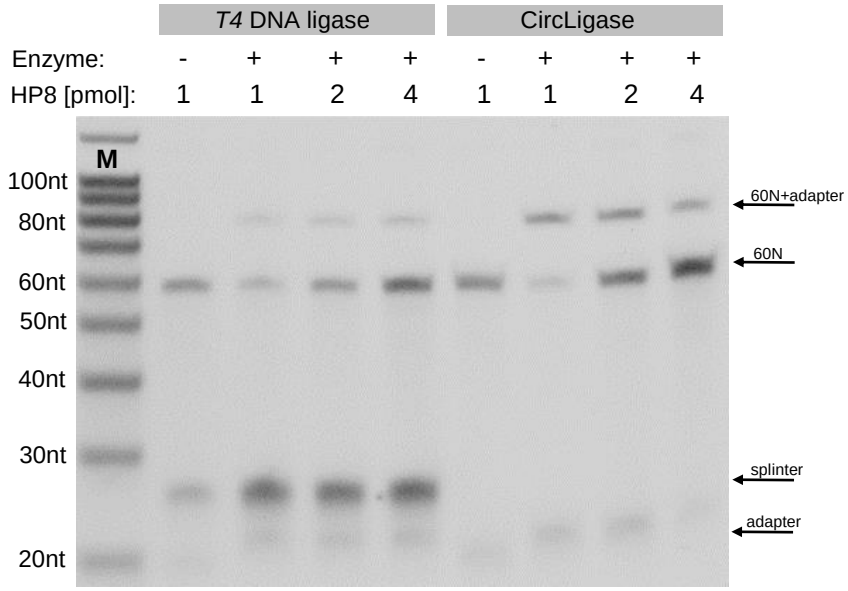
Tubes were spun briefly in a microcentrifuge and placed into a magnetic rack. The supernatant was discarded and the ThermoMixer set to 25°C.
ThermoMixerBeads were pelleted using a magnetic rack and the supernatant was discarded. 200 µl 0.1xBWT were added, tubes were transferred to ThermoMixer (25°C) and vortexed for 8 s at 1500 rpm

Elution of the final library

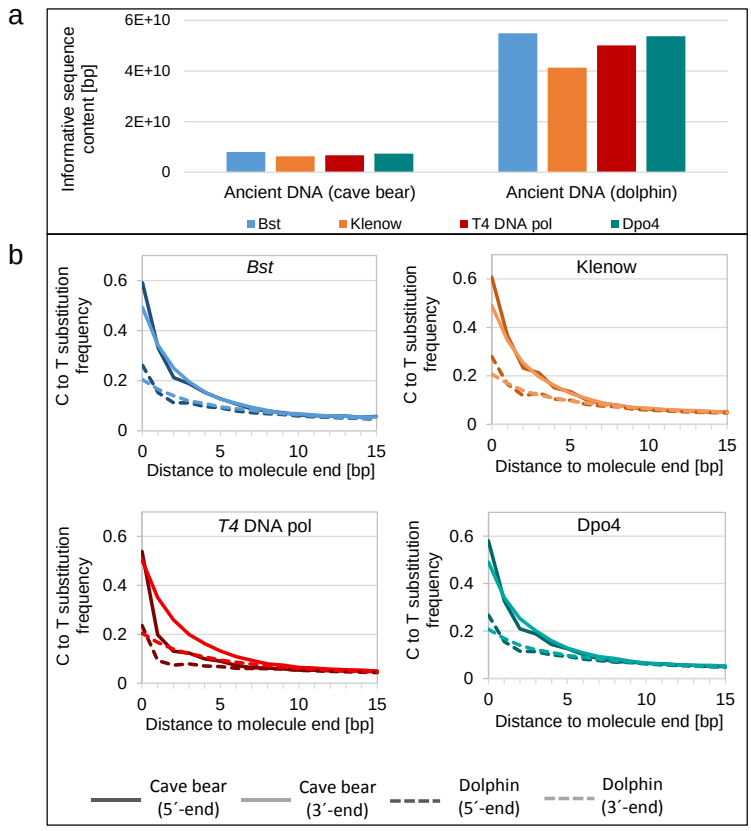
Tubes were spun briefly in a microcentrifuge. Beads were pelleted using a magnet rack and the supernatant was discarded. 50 µl EBT (10mM Tris-HCl (pH 8.0) and 0.05% Tween-20) were added, tubes were transferred to the ThermoMixer (set to 25°C) and vortexed for 8 s at 1500 rpm. Bead suspension was transferred to fresh 0.2 ml PCR tubes. Suspensions were then incubated for 1 min at 95°C. Tubes were placed into a magnetic rack, and the supernatant was transferred to fresh 0.5 ml LoBind tubes.



Supplementary Figure 1. Single-stranded DNA ligation with *T4* DNA ligase and CircLigase using random adapter/splinter sequences. The 3´ biotinylated donor oligonucleotides (migrating at approximately 20 nt) were ligated to different quantities of a 60 nt adapter oligonucleotide pool ('60N') using CircLigase and, in the presence of an additional splinter (migrating at approximately 22nt), using *T4* DNA ligase. Ligation products were visualized on a 10 % denaturing polyacrylamide gel stained with SybrGold. Band shifts from 60 nt to 80 nt indicate successful ligation. M: Single-stranded DNA size marker.

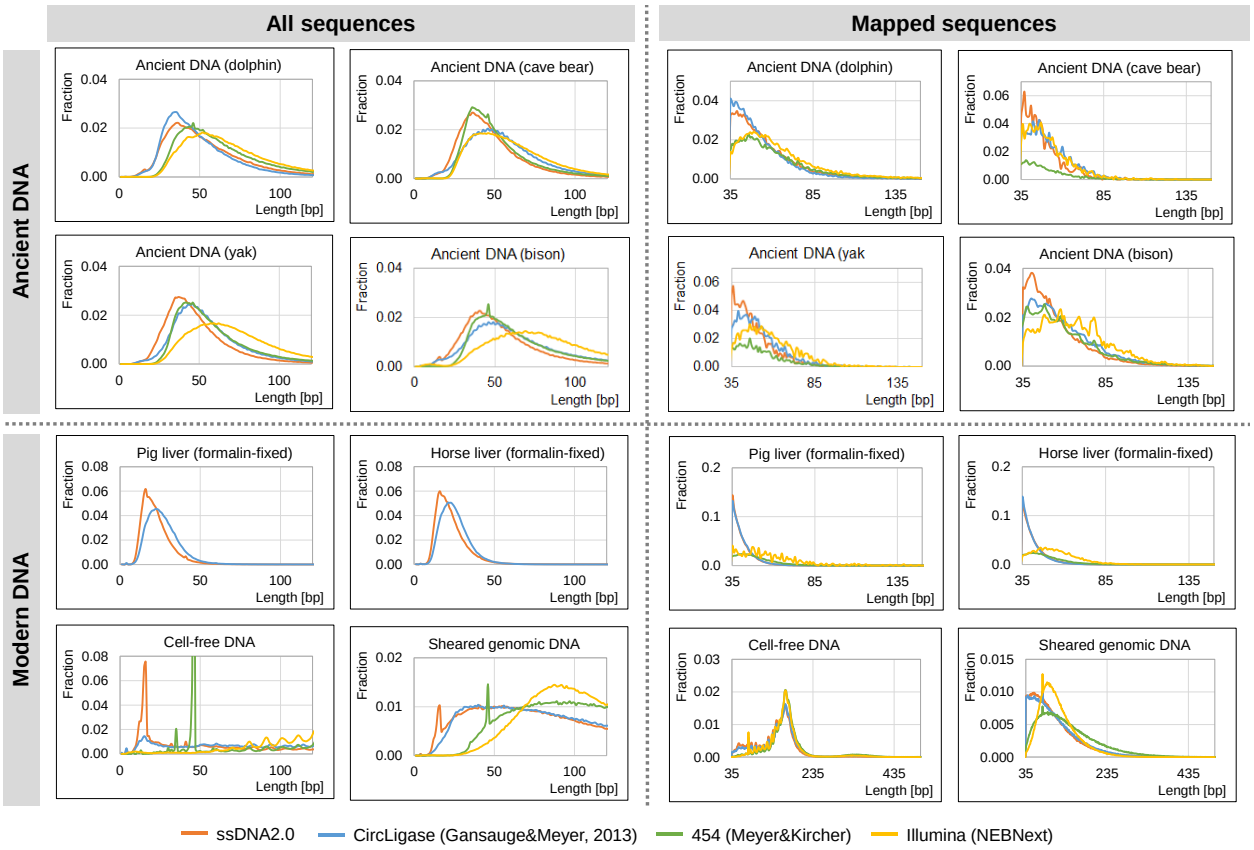


Supplementary Figure 2. Single-stranded DNA ligation with *T4* DNA ligase and CircLigase using an acceptor with a specific sequence. The 3´ biotinylated donor oligonucleotide (‘CL78’, migrating at approximately 20 nt) was ligated to different quantities of a 60 nt oligonucleotide (‘HP8’) using *T4* DNA ligase in the presence of a splinter (‘TL38’, migrating at approximately 25 nt) or CircLigase. Ligation products were visualized on a 10 % denaturing polyacrylamide gel stained with SybrGold. Band shifts from 60 nt to 80 nt indicate successful ligation. M: Single-stranded DNA size marker.

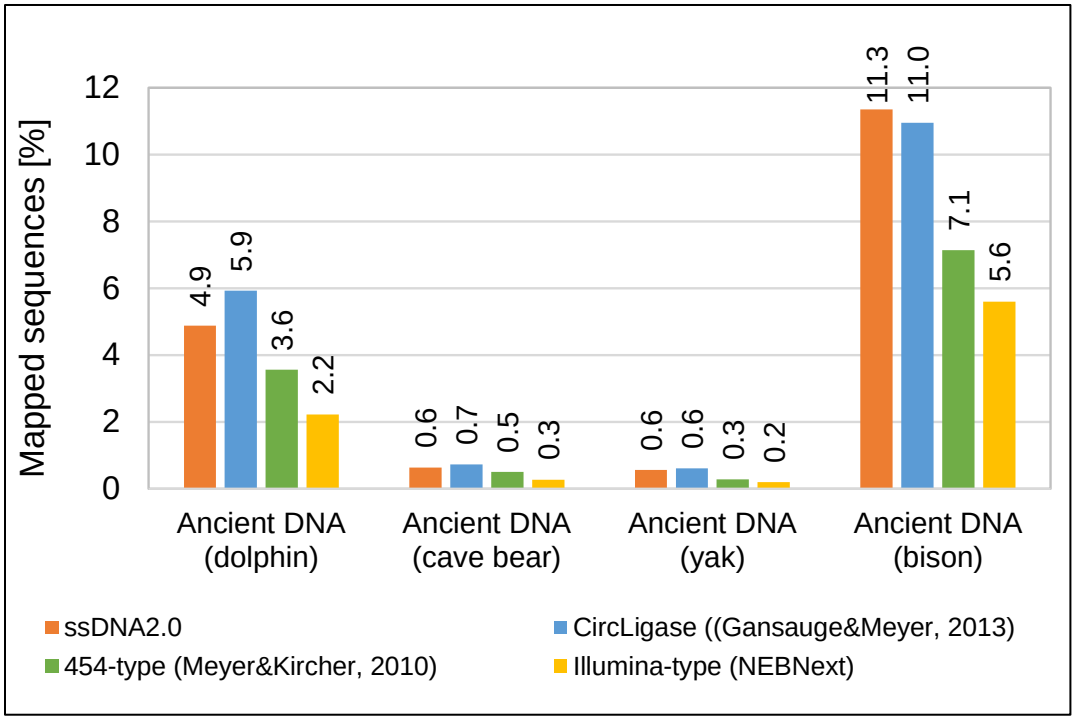


Supplementary Figure 3. Effects of polymerase choice on library characteristics.

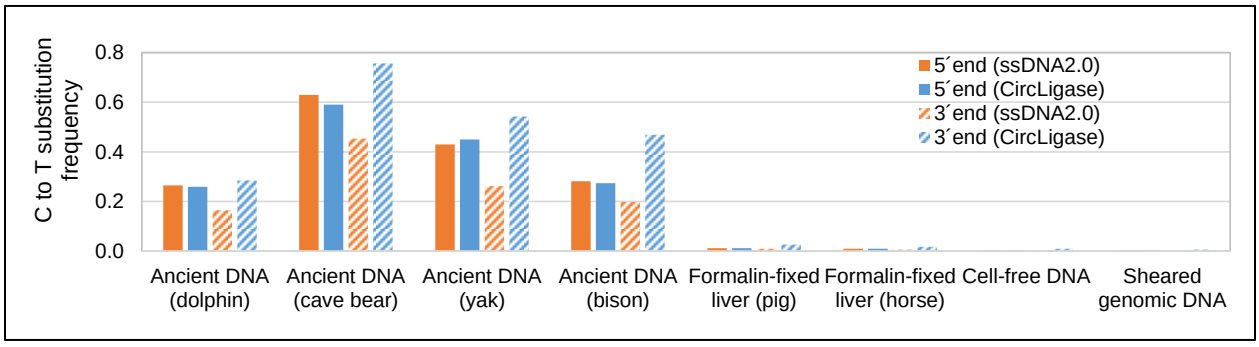
(a) Comparison of the informative sequence content in libraries prepared with four different polymerases from two ancient DNA extracts, **(b)** C to T substitution frequencies near the end of sequence alignments.



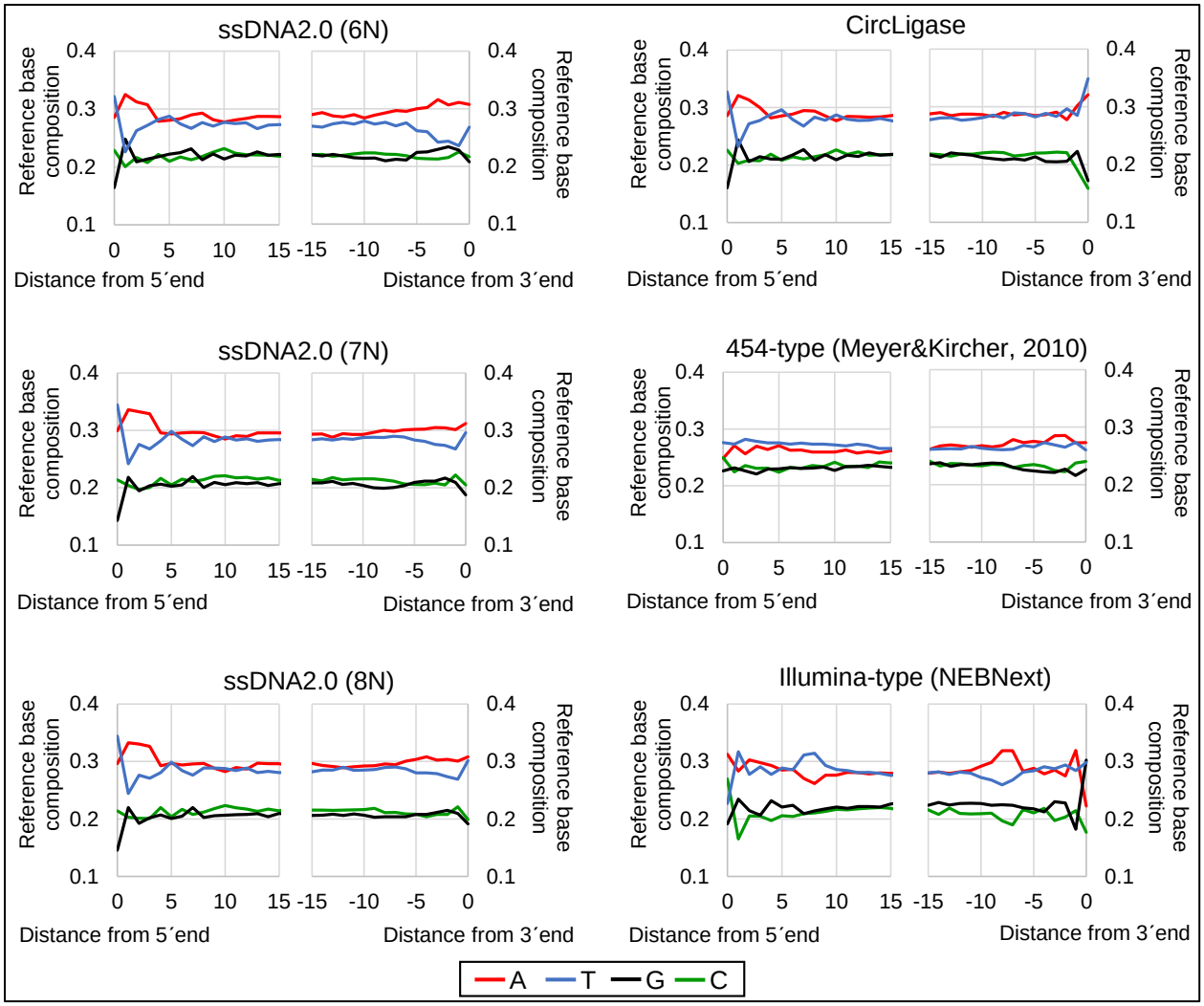
Supplementary Figure 4. Size distribution of sequences obtained for 8 samples with single-stranded and double-stranded library preparation methods. Size distributions are shown for all sequences (based on overlap-merged sequences only) as well as those mapping to the respective reference genome with a length cut-off of 35 bp. Double-stranded libraries produced from formalin-fixed DNA are not shown as they are dominated by artifacts.



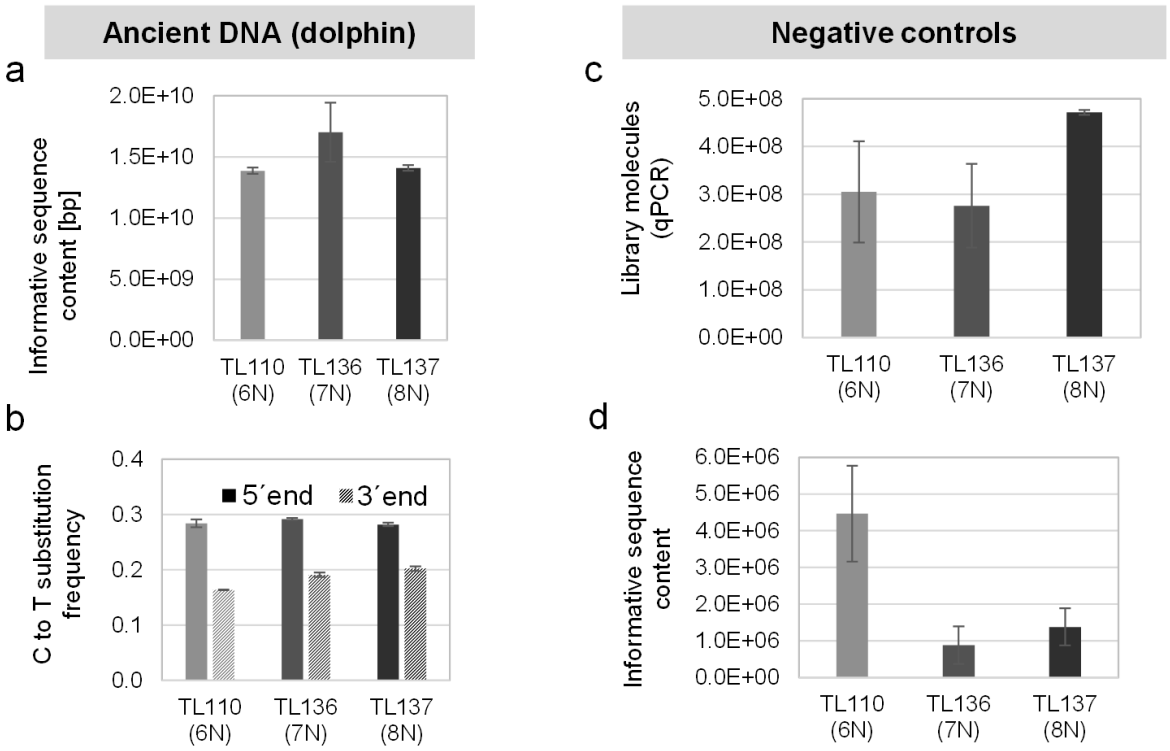
Supplementary Figure 5. Percentage of sequences ≥35 bp mapping to the reference genome in libraries prepared with four different methods. Values are denoted above the bars.



Supplementary Figure 6. Frequency of C to T substitutions at the 5' and 3' ends of sequence alignments obtained with the two single-stranded library preparation methods.



Supplementary Figure 7. Base composition near the 5' and 3' ends of sequence alignments in libraries obtained from an ancient dolphin bone. SsDNA2.0 libraries were prepared using splinter oligonucleotides with 6, 7 or 8 degenerate bases. To mitigate the effect of damage-induced C to T and G to A substitutions, the base composition of the reference genome is plotted and not that of the molecules sequenced. Only overlap-merged and mapped sequences ≥35 bp were used in this analysis.



Supplementary Figure 8. Characteristics of libraries prepared with splinter oligonucleotides carrying 6 ('6N'), 7 ('7N') or 8 ('8N') degenerate bases. (a) Informative sequence content of the libraries, **(b)** C to T substitution frequencies at the 5´ and 3´ ends of sequence alignments, **(c)** number of molecules in the respective negative controls as inferred by qPCR (predominantly caused by artifacts of library preparation), **(d)** Load of human contamination introduced during library preparation (i.e. the content of human-like sequences in the negative controls).

Error bars denote standard deviation of two technical replicates.

Supplementary Table 1: Information overview about used ancient samples

SP No.	Species	Age (C14 dated)	Location	Country
SP3571	Cave bear	>50,000 years	Schwabenreith Cave	Austria
SP2698	Cave bear	Not dated (layer G1, possibly 45,000-47,000)	Vindija Cave	Croatia
SP1060	Dolphin	5,600	North Sea, seabed	Netherlands
SP3391	Yak	>49,000	Denisova Cave	Russia
SP3424	Bison	Not dated	Yucon Territory, permafrost	Canada

Supplementary Table 2: Oligonucleotides used for library preparation

Name	Sequence 5´ -> 3´	Purification	Application
TL38	AmC12-TGTGCTCTTCCGATCTNNNNNN-AmC3	RP-HPLC	Splinter
TL93	AmC12-AACTTCCGATCTNNNNNN-AmC7	RP-HPLC	Splinter
TL106	AmC12-AACTTCCGATCTNNNNNN-AmC3	RP-HPLC	Splinter
TL110	SpacerC12-AA[SpacerC12]CTTCCGATCTNNNNNN-AmC6	RP-HPLC	Splinter
TL128	Pho-TGAGAGGGCAAATAGGCAGG-Pho	RP-HPLC	Adapter
TL129	AmC12-CCTATTTGCCCTCTCANNNNNN-AmC3	RP-HPLC	Splinter
TL130	Pho-ACTTGTGAAGTGTCTACTAT-Pho	RP-HPLC	Adapter
TL131	AmC12-TAGTCACTTCACAAGTNNNNNN-AmC3	RP-HPLC	Splinter
TL134	Pho-GCGCTATCCGACTATTTCCA-Pho	RP-HPLC	Adapter
TL135	AmC12-AATAGTCGGATAGCGCNNNNNN-AmC3	RP-HPLC	Splinter
TL136	SpacerC12-AA[SpacerC12]CTTCCGATCTNNNNNNN-AmC6	desalted	Splinter
TL137	SpacerC12-AA[SpacerC12]CTTCCGATCTNNNNNNNN-AmC6	desalted	Splinter
CL9†	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	RP-HPLC	Extension primer
CL53†	CGACGCTCTTC-ddC	RP-HPLC	Adapter oligo 1, double-stranded ligation
CL73†	Pho-GGAAGAGCGTCGTGTAGGGAAAGAG*T*G*T*A	RP-HPLC	Adapter oligo 2, double-stranded ligation
CL78†	Pho-AGATCGGAAG[C3Spacer]$_{10}$-TEG-biotin	dual purification by ion-exchange HPLC	Adapter, single-stranded ligation
CL104†	Pho-TCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGA-Pho	RP-HPLC	Positive control for library prep
CL130†	GTGACTGGAGTTCAGACGTGTGCTCTTCC*GA*TC*T	RP-HPLC	Extension primer
IS1‡	A*C*A*C*TCTTTCCCTACACGACGCTCTTCCG*A*T*C*T	RP-HPLC	Adapter oligo, double-stranded ligation
IS2‡	G*T*G*A*CTGGAGTTCAGACGTGTGCTCTTCCG*A*T*C*T	RP-HPLC	Adapter oligo, double-stranded ligation
IS3‡	A*G*A*T*CGGAA*G*A*G*C	RP-HPLC	Adapter oligo, double-stranded ligation
HP8†	GGCCCUAGGACGAGAGCAAGGTGAGACGTTGCTTGACTGCTTACAAGCTTCTACCTCGC	RP-HPLC	Insert
60N	NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN	RP-HPLC	Insert

† Sequences already published in Gansauge & Meyer 2013, ‡ Sequences already published in Meyer & Kircher 2010, AmC7: 3' amino linker C7 (Sigma-Aldrich); AmC6: 3' amino modifier C6 (Eurogentec); Pho: Phosphate; ddC: dideoxy cytosine; TEG: triethyleneglycol; *: phosphorothioate bonds; N: mixture of adenine, thymine, cytosine and guanine, RP-HPLC: Reverse Phase High Performance Liquid Chromatography

Supplementary Table 3: Summary of libraries and sequences generated in this study

Set		Method (splinter)	Polymerase	Sample	Input of extract [µl]	LibID	No. of library molecules (qPCR)	No. of unique library strands*	Contribution of artifacts based on qPCR [%]	Reference genome	Reference genome size [bp]	Raw sequences	Sequences ≥35bp†	Sequences ≥35bp [%]	Mapped sequences ≥35bp	Mapped sequences (≥35bp) [%]	Average size of mapped seq. ≥35bp [bp]‡	Informative sequence content [bp]	Genomes	C-to-T subst. frequency 5´ end [%]	C-to-T / G-to-A subst. frequency 3´ end [%] #
1	single-stranded	Circ	*Bst*	SP3571 (cave bear)	1	R7577	1.37E+09	1.37E+09	0.7	ursMar0	2.27E+09	1,181,536	692,196	58.6	9,648	1.39	49.9	5.58E+08	0.2	45	48
					3	R7578	5.20E+09	5.20E+09	0.2	ursMar0	2.27E+09	1,322,386	806,611	61.0	11,593	1.44	49.7	2.26E+09	1.0	46	48
					9	R7579	1.39E+10	1.39E+10	0.1	ursMar0	2.27E+09	1,042,630	697,738	66.9	10,997	1.58	49.8	7.30E+09	3.2	45	51
					27	R7580	2.22E+10	2.22E+10	0.0	ursMar0	2.27E+09	1,027,378	857,702	83.5	14,865	1.73	54.7	1.76E+10	7.7	47	71
				Positive control (0.1µM CL104)	1	R7586	5.14E+09	5.14E+09	0.2	-	-	-	-	-	-	-	-	-	-	-	-
				Negative control	-	R7587	1.05E+07	1.05E+07	-	ursMar0	2.27E+09	127,439	106,596	83.6	14	0.01	48.0	-	-	33	0
					-	R7588	9.93E+06	9.93E+06	-	ursMar0	2.27E+09	130,735	111,732	85.5	14	0.01	43.8	-	-	0	0
		T4 (TL106)	*Bst*	SP3571 (cave bear)	1	R7589	1.80E+09	1.80E+09	15.3	ursMar0	2.27E+09	1,016,469	525,472	51.7	8,669	1.65	48.9	7.51E+08	0.3	44	44
					3	R7590	5.44E+09	5.44E+09	5.1	ursMar0	2.27E+09	1,223,552	718,004	58.7	11,290	1.57	49.5	2.49E+09	1.1	44	45
					9	R7591	1.89E+10	1.89E+10	1.5	ursMar0	2.27E+09	1,380,538	815,988	59.1	13,586	1.66	49.3	9.17E+09	4.0	44	41
					27	R7592	4.78E+10	4.78E+10	0.6	ursMar0	2.27E+09	1,185,390	751,542	63.4	12,415	1.65	49.5	2.48E+10	10.9	46	43
				Positive control (0.1µM CL104)	1	R7598	5.95E+09	5.95E+09	4.6	-	-	-	-	-	-	-	-	-	-	-	-
				Negative control	-	R7599	3.22E+08	3.22E+08	-	ursMar0	2.27E+09	128,054	13,885	10.8	10	0.07	39.1	-	-	0	0
					-	R7600	2.30E+08	2.30E+08	-	ursMar0	2.27E+09	128,588	14,338	11.2	12	0.08	54.9	-	-	0	0
2	single-stranded	T4 (TL93)	*Bst*	SP2698 (cave bear)	5	A8936	2.09E+10	2.09E+10	1.2	ursMar0	2.27E+09	12,038,087	7,737,782	64.3	97,016	1.25	47.5	8.01E+09	3.5	59	49
				SP1060 (dolphin)	5	A8937	1.17E+10	1.17E+10	2.1	turTru1.75	2.30E+09	9,549,290	6,709,901	70.3	814,491	12.14	55.2	5.50E+10	23.9	26	20
				Positive control (0.1µM CL104)	1	A8938	7.97E+09	7.97E+09	3.1	-	-	-	-	-	-	-	-	-	-	-	-
				Negative control	-	A8939	2.47E+08	2.47E+08	-	hg19_evan	2.82E+09	2,372,510	135,588	5.7	462	0.34	55.7	2.68E+06	0.0	2	4
			Klenow	SP2698 (cave bear)	5	A8940	1.65E+10	1.65E+10	2.3	ursMar0	2.27E+09	15,099,400	9,604,468	63.6	122,781	1.28	47.2	6.31E+09	2.8	61	49
				SP1060 (dolphin)	5	A8941	8.21E+09	8.21E+09	4.6	turTru1.75	2.30E+09	12,287,647	8,517,001	69.3	1,119,535	13.14	55.2	4.13E+10	18.0	28	21
				Positive control (0.1µM CL104)	1	A8942	7.84E+09	7.84E+09	4.8	-	-	-	-	-	-	-	-	-	-	-	-
				Negative control	-	A8943	3.74E+08	3.74E+08	-	hg19_evan	2.82E+09	2,437,560	228,306	9.4	193	0.08	58.3	1.73E+06	0.0	2	2
			T4	SP2698 (cave bear)	5	A8944	1.73E+10	1.73E+10	0.3	ursMar0	2.27E+09	14,293,390	8,909,932	62.3	117,924	1.32	46.8	6.67E+09	2.9	54	50
				SP1060 (dolphin)	5	A8945	1.06E+10	1.06E+10	0.4	turTru1.75	2.30E+09	12,046,405	8,357,429	69.4	1,040,281	12.45	54.5	5.01E+10	21.8	24	21
				Positive control (0.1µM CL104)	1	A8946	6.42E+09	6.42E+09	0.7	-	-	-	-	-	-	-	-	-	-	-	-
				Negative control	-	A8947	4.72E+07	4.72E+07	-	hg19_evan	2.82E+09	1,921,542	174,756	9.1	1,019	0.58	56.4	1.41E+06	0.0	2	7
			Dpo4	SP2698 (cave bear)	5	A8948	2.06E+10	2.06E+10	0.9	ursMar0	2.27E+09	13,333,821	8,528,351	64.0	100,814	1.18	47.4	7.37E+09	3.2	58	49
				SP1060 (dolphin)	5	A8949	1.10E+10	1.10E+10	1.8	turTru1.75	2.30E+09	13,012,388	9,153,587	70.3	1,151,503	12.58	55.3	5.38E+10	23.4	27	21
				Positive control (0.1µM CL104)	1	A8950	8.28E+09	8.28E+09	2.3	-	-	-	-	-	-	-	-	-	-	-	-
				Negative control	-	A8951	1.94E+08	1.94E+08	-	hg19_evan	2.82E+09	2,275,890	147,838	6.5	441	0.30	55.2	2.08E+06	0.0	2	6
3	single-stranded	T4 (TL110)	Klenow	SP1060 (dolphin)	5	R7640	2.46E+10	2.46E+10	0.1	turTru1.75	2.30E+09	1,017,679	791,973	77.8	38,607	4.87	56.5	5.27E+10	22.9	27	16
				SP2698 (cave bear)	5	R7641	2.89E+10	2.89E+10	0.1	ursMar0	2.27E+09	469,692	348,263	74.1	2,212	0.64	47.8	6.49E+09	2.9	63	45
				SP3391 (yak)	5	R7642	3.55E+10	3.55E+10	0.1	bosTauUMD3.1	2.65E+09	1,013,594	772,679	76.2	4,326	0.56	49.0	7.41E+09	2.8	43	26
				SP3424 (bison)	5	R7643	9.27E+09	9.27E+09	0.4	bosTauUMD3.1	2.65E+09	1,012,477	822,923	81.3	93,394	11.35	54.4	4.65E+10	17.6	28	20
				Pig liver, formalin-fixed	10	R7644	1.57E+09	1.57E+09	2.3	susScrofa9	2.23E+09	1,268,114	157,081	12.4	80,146	51.02	41.1	4.07E+09	1.8	1	1
				Horse liver, formalin fixed	10	R7645	1.72E+09	1.72E+09	2.1	equCab2	1.43E+09	1,038,021	130,337	12.6	88,504	67.90	41.8	6.13E+09	4.3	1	1
				Cell-free DNA (human)	10	R7646	2.50E+09	2.50E+09	1.5	hg19_evan	2.82E+09	715,737	539,912	75.4	406,764	75.34	143.2	2.03E+11	72.2	0	0
				Sheared genomic DNA (human)	1	R7647	1.41E+10	1.41E+10	0.3	hg19_evan	2.82E+09	773,531	664,249	85.9	640,369	96.40	102.4	1.20E+12	425.0	0	0
				Positive control (0.1µM CL104)	1	R7648	3.19E+09	3.15E+09	1.1	-	-	-	-	-	-	-	-	-	-	-	-
				Negative control	-	R7649	3.78E+07	3.78E+07	-	hg19_evan	2.82E+09	263,401	70,487	26.8	496	0.70	46.2	3.29E+06	0.0	0	2
				Negative control	-	R7650	3.48E+07	3.48E+07	-	hg19_evan	2.82E+09	79,728	20,118	25.2	157	0.78	49.2	3.37E+06	0.0	0	0
		Circ	*Bst*	SP1060 (dolphin)	5	R7628	2.45E+10	2.45E+10	0.1	turTru1.75	2.30E+09	931,615	686,900	73.7	40,693	5.92	53.7	5.74E+10	25.0	26	29
				SP2698 (cave bear)	5	R7629	1.18E+10	1.18E+10	0.1	ursMar0	2.27E+09	913,115	776,627	85.1	5,604	0.72	51.8	3.74E+09	1.6	59	76
				SP3391 (yak)	5	R7630	2.21E+10	2.21E+10	0.1	bosTauUMD3.1	2.65E+09	1,071,890	941,252	87.8	5,684	0.60	52.2	6.10E+09	2.3	45	54
				SP3424 (bison)	5	R7631	4.16E+09	4.16E+09	0.4	bosTauUMD3.1	2.65E+09	909,517	802,609	88.2	87,888	10.95	59.5	2.39E+10	9.0	27	47
				Pig liver, formalin-fixed	10	R7632	9.27E+08	9.27E+08	1.8	susScrofa9	2.23E+09	959,739	211,786	22.1	127,694	60.29	41.3	5.10E+09	2.3	1	3
				Horse liver, formalin fixed	10	R7633	2.26E+09	2.26E+09	0.7	equCab2	1.43E+09	1,026,006	167,457	16.3	126,679	75.65	41.4	1.15E+10	8.0	1	2
				Cell-free DNA (human)	10	R7634	1.90E+09	1.90E+09	0.9	hg19_evan	2.82E+09	288,837	264,628	91.6	207,394	78.37	153.9	2.10E+11	74.4	0	1
				Sheared genomic DNA (human)	1	R7635	2.58E+10	2.58E+10	0.1	hg19_evan	2.82E+09	601,060	532,960	88.7	512,505	96.16	106.0	2.33E+12	826.6	0	1
				Positive control (0.1µM CL104)	1	R7636	6.22E+09	6.22E+09	0.3	-	-	-	-	-	-	-	-	-	-	-	-
				Negative control	-	R7637	1.57E+07	1.57E+07	-	hg19_evan	2.82E+09	134,397	64,757	48.2	67	0.10	41.6	3.25E+05	0.0	0	18
				Negative control	-	R7638	1.69E+07	1.69E+07	-	hg19_evan	2.82E+09	153,822	74,716	48.6	308	0.41	45.5	1.54E+06	0.0	1	6
	double-stranded	Meyer&Kircher 2010		SP1060 (dolphin)	5	R7652	6.54E+09	3.27E+09	0.5	turTru1.75	2.30E+09	912,332	840,092	92.1	29,907	3.56	62.8	6.74E+09	2.9	22	24
				SP2698 (cave bear)	5	R7653	3.40E+09	1.70E+09	0.9	ursMar0	2.27E+09	917,454	771,250	84.1	3,889	0.50	50.3	3.62E+08	0.2	57	58
				SP3391 (yak)	5	R7654	9.39E+09	4.69E+09	0.3	bosTauUMD3.1	2.65E+09	1,095,912	1,008,670	92.0	2,802	0.28	53.0	6.37E+08	0.2	36	38
				SP3424 (bison)	5	R7655	2.55E+09	1.27E+09	1.2	bosTauUMD3.1	2.65E+09	1,126,336	1,052,655	93.5	75,095	7.13	59.8	5.07E+09	1.9	26	27
				Pig liver, formalin-fixed	10	R7656	4.10E+07	2.05E+07	76.2	susScrofa9	2.23E+09	854,365	784,253	91.8	1,439	0.18	47.5	1.64E+06	0.0	2	1
				Horse liver, formalin fixed	10	R7657	3.37E+07	1.68E+07	92.8	equCab2	1.43E+09	1,091,878	999,589	91.5	53,984	5.40	48.8	4.06E+07	0.0	1	1
				Cell-free DNA (human)	10	R7658	9.37E+08	4.69E+08	3.3	hg19_evan	2.82E+09	545,698	538,960	98.8	437,238	81.13	174.3	6.55E+10	23.2	0	0
				Sheared genomic DNA (human)	1	R7659	1.69E+10	8.46E+09	0.2	hg19_evan	2.82E+09	624,264	621,633	99.6	595,090	95.73	136.7	1.10E+12	390.6	0	0
				Negative control	-	R7660	2.70E+07	1.35E+07	-	hg19_evan	2.82E+09	152,398	134,597	88.3	29	0.02	67.4	1.73E+05	0.0	0	0
				Negative control	-	R7661	3.54E+07	1.77E+07	-	hg19_evan	2.82E+09	138,652	124,750	90.0	36	0.03	58.8	2.70E+05	0.0	0	0
		NEBNext Kit		SP1060 (dolphin)	5	R7663	1.64E+09	1.64E+09	0.7	turTru1.75	2.30E+09	1,538,144	1,453,694	94.5	32,200	2.22	65.3	2.24E+09	1.0	2	17
				SP2698 (cave bear)	5	R7664	9.27E+08	9.27E+08	1.2	ursMar0	2.27E+09	1,454,314	1,295,742	89.1	3,517	0.27	51.5	1.15E+08	0.1	5	43
				SP3391 (yak)	5	R7665	1.82E+09	1.82E+09	0.6	bosTauUMD3.1	2.65E+09	1,910,508	1,842,405	96.4	3,681	0.20	58.0	2.04E+08	0.1	2	30
				SP3424 (bison)	5	R7666	9.65E+08	9.65E+08	1.1	bosTauUMD3.1	2.65E+09	335,395	323,742	96.5	18,135	5.60	66.6	3.48E+09	1.3	1	15
				Pig liver, formalin-fixed	10	R7667	7.69E+06	7.69E+06	142.2	susScrofa9	2.23E+09	459,207	404,122	88.0	715	0.18	59.5	1.13E+05	0.0	0	1
				Horse liver, formalin fixed	10	R7668	1.70E+07	1.70E+07	64.2	equCab2	1.43E+09	379,790	339,558	89.4	16,949	4.99	54.5	4.14E+07	0.0	0	1
				Cell-free DNA (human)	10	R7669	9.83E+08	9.83E+08	1.1	hg19_evan	2.82E+09	606,174	601,656	99.3	540,065	89.76	166.0	1.45E+11	51.6	0	0
				Sheared genomic DNA (human)	1	R7670	1.57E+10	1.57E+10	0.1	hg19_evan	2.82E+09	930,087	926,876	99.7	889,608	95.98	113.2	1.70E+12	602.0	0	0
				Negative control	-	R7671	1.09E+07	1.09E+07	-	hg19_evan	2.82E+09	45,937	39,804	86.6	48	0.12	74.0	8.46E+05	0.0	0	0
				Negative control	-	R7672	1.09E+07	1.09E+07	-	hg19_evan	2.82E+09	17,951	15,169	84.5	47	0.31	109.5	3.14E+06	0.0	0	0
4	single-stranded	T4 (TL110)	Klenow	SP1060 (dolphin)	5	D5871	1.24E+10	1.24E+10	2.46	turTru1.80	2.30E+09	3,692,344	2,734,899	74.1	76,487	2.80	54.7	1.41E+10	6.1	29	16
		T4 (TL110)			5	D5879	1.21E+10	1.21E+10	2.52	turTru1.81	2.30E+09	4,091,632	3,035,972	74.2	84,761	2.79	54.7	1.37E+10	6.0	28	16
		T4 (TL106)			5	D5877	1.64E+10	1.64E+10	1.68	turTru1.82	2.30E+09	4,371,267	3,186,942	72.9	90,953	2.85	54.9	1.87E+10	8.2	29	19
		T4 (TL106)			5	D5869	1.29E+10	1.29E+10	2.14	turTru1.83	2.30E+09	3,412,415	2,498,865	73.2	73,339	2.93	55.2	1.53E+10	6.7	29	19
		T4 (TL137)			5	D5878	1.24E+10	1.24E+10	3.80	turTru1.84	2.30E+09	2,578,484	1,850,629	71.8	54,063	2.92	54.9	1.43E+10	6.2	28	20
		T4 (TL137)			5	D5870	1.21E+10	1.21E+10	3.90	turTru1.85	2.30E+09	3,820,485	2,759,634	72.2	79,088	2.87	55.6	1.39E+10	6.1	28	21
		T4 (TL110)		Negative control	-	D5839	3.80E+08	3.80E+08	-	turTru1.75	2.30E+09	81,266	18,710	23.0	26	0.14	44.3	5.39E+06	0.0	0	0
		T4 (TL110)			-	D5847	2.30E+08	2.30E+08	-	turTru1.75	2.30E+09	51,458	10,880	21.1	18	0.17	44.0	3.54E+06	0.0	0	0
		T4 (TL106)			-	D5845	2.14E+08	2.14E+08	-	turTru1.76	2.30E+09	56,567	7,679	13.6	2	0.03	68.5	5.18E+05	0.0	0	0
		T4 (TL136)			-	D5837	3.38E+08	3.38E+08	-	turTru1.77	2.30E+09	53,097	7,452	14.0	2	0.03	97.5	1.24E+06	0.0	0	0
		T4 (TL137)			-	D5846	4.75E+08	4.75E+08	-	turTru1.78	2.30E+09	63,939	6,271	9.8	4	0.06	58.5	1.74E+06	0.0	0	0
					-	D5838	4.68E+08	4.68E+08	-	turTru1.79	2.30E+09	80,660	8,659	10.7	3	0.03	58.7	1.02E+06	0.0	0	0

Supplementary Table 4: Comparison of reagent costs for CircLigase-based library preparation and ssDNA2.0. Costs for buffers and oligonucleotides were disregarded as they contribute very little to the costs of a single reaction.

Prep	Consumables	Cost/reaction	Sum
CircLigase	FastAP Thermosensitive Alkaline Phosphatase (1 U/µL)	0.05 €	54.80 €
	CircLigase II ssDNA Ligase (100 U/µL)	48.64 €	
	Dynabeads MyOne Streptavidin C1	3.23 €	
	Bst 2.0 DNA Polymerase (8 U/µL)	0.80 €	
	T4 DNA Polymerase (5 U/µL)	1.62 €	
	T4 DNA Ligase (5 U/µL)	0.46 €	
T4 DNA ligase	T4 DNA Ligase, HC (30 U/µL)	1.37 €	8.49 €
	FastAP Thermosensitive Alkaline Phosphatase (1 U/µL)	0.05 €	
	Dynabeads MyOne Streptavidin C1	3.23 €	
	Klenow Fragment (10 U/µL)	3.39 €	
	T4 DNA Ligase (5 U/µL)	0.46 €	