# Supplemental Material

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

Marie-Theres Gansauge[1], Tobias Gerber[1], Isabelle Glocke[1], Petra Korlević[1], Laurin Lippik[1], Sarah Nagel[1], Lara Maria Riehl[2], Anna Schmidt[1], Matthias Meyer[1]

## 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<br>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<br>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>

<table>
<tr><td colspan="2">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.</td></tr>
<tr><td colspan="2">Heat denaturation, dephosphorylation and ligation of first adapter</td></tr>
<tr><td>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.</td><td>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.</td></tr>
<tr><td colspan="2">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.</td></tr>
<tr><td>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.</td><td>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.</td></tr>
</table>

***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.

<table>
<tr><td colspan="3">Primer annealing and extension</td></tr>
<tr><td>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).</td><td></td><td>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).</td></tr>
<tr><td colspan="3">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. .</td></tr>
<tr><td>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.</td><td></td><td>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</td></tr>
<tr><td colspan="3">Post-extension washes<br>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.</td></tr>
</table>

<table>
<tr><td colspan="3">Tubes were spun briefly in a microcentrifuge and placed into a magnetic rack. The supernatant was discarded and the ThermoMixer set to 25°C.<br>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.</td></tr>
<tr><td></td><td></td><td>Blunt-end repair<br>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.<br>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.<br>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.</td></tr>
<tr><td></td><td></td><td>Post-blunt-end-repair washes<br>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</td></tr>
</table>

| | | |
|---|---|---|
| | | 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.<br>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***

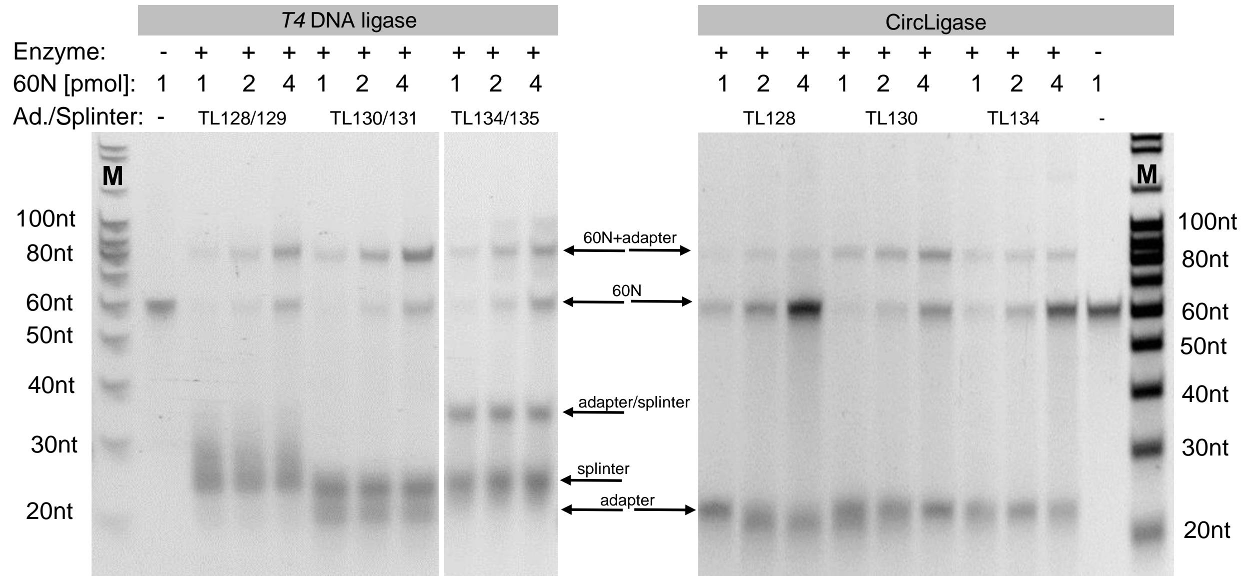
The following components were mixed to obtain reaction mixed with a total volume of 100 µl: 73.5 µl water, 10 µl 10x *T4* DNA ligase buffer (ThermoFisher Scientific), 10 µl 50 % PEG-4000, 2 µl CL53/73 (100 µM), 2.5 µl 1% Tween-20 and 2 µl *T4* DNA ligase (5 U/µl).

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

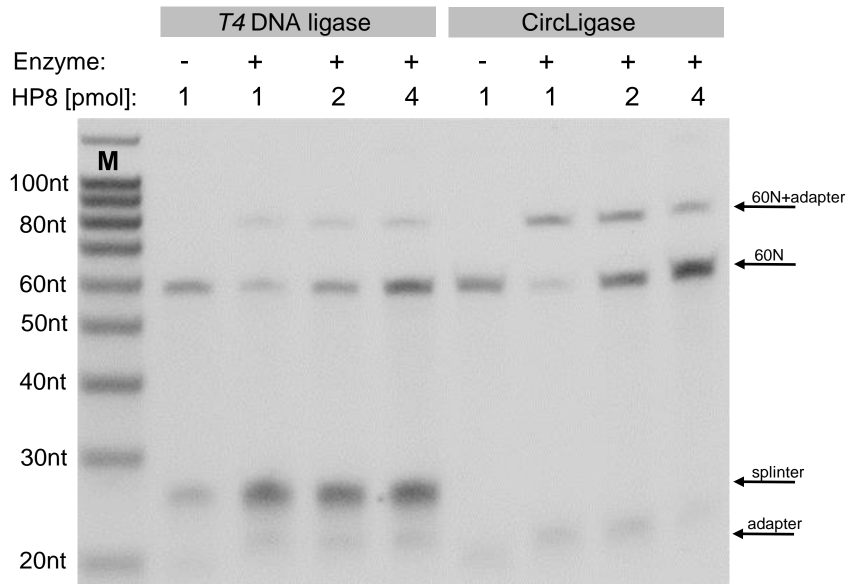
***Post-ligation washes***

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.

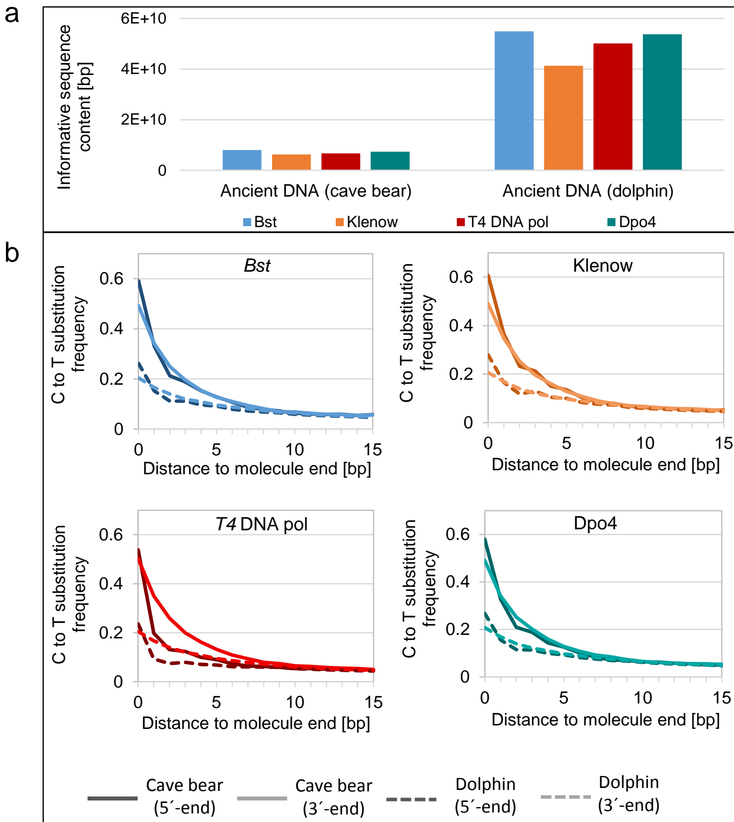
| Tubes were spun briefly in a microcentrifuge and placed into a magnetic rack. The supernatant was discarded and the ThermoMixer set to 25°C.<br>ThermoMixerBeads were pelleted using a magnetic rack and the supernatant was discarded. 200 µl 0.1xBWT were added, tubes were transferred to ThermoMixer (25°C)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

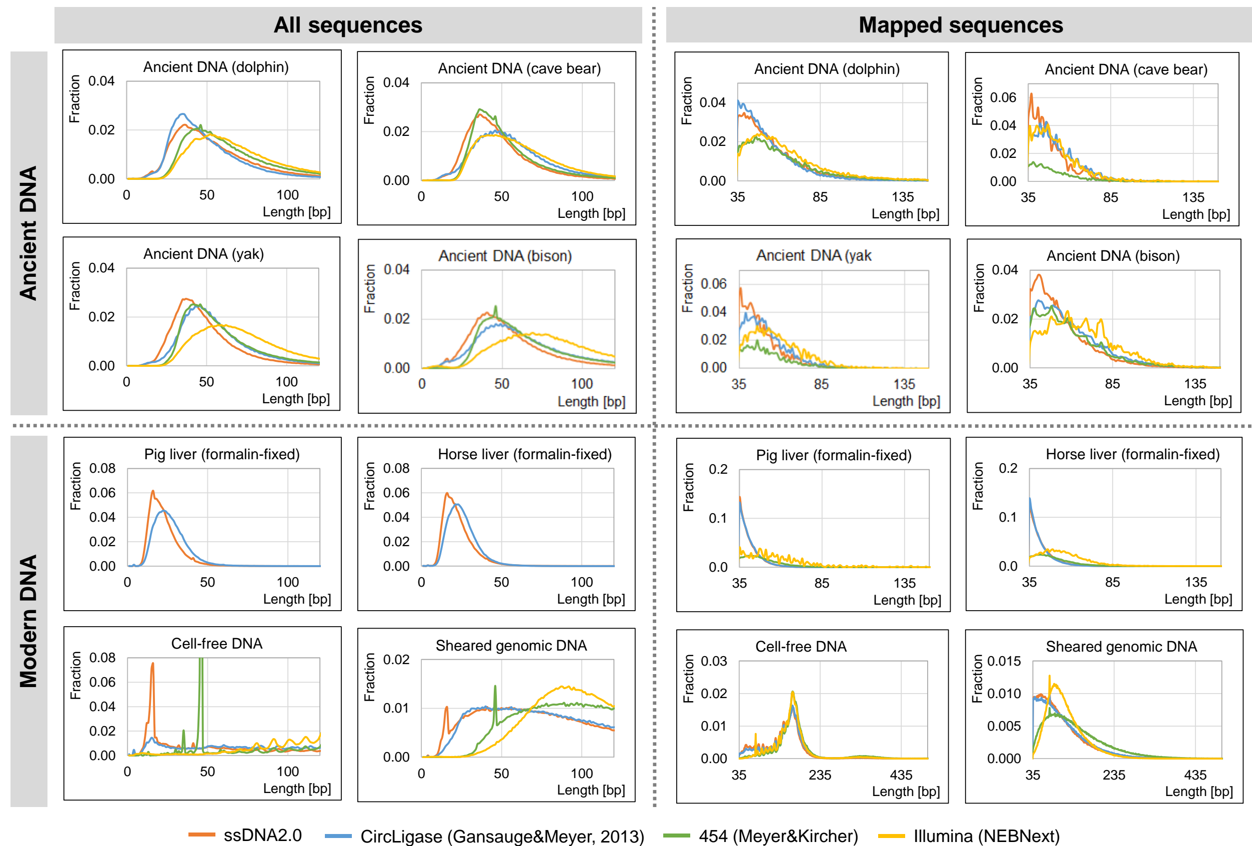



**Supplementary Figure 2. Single-stranded DNA ligation with *T4* DNA ligase and CircLigase using an acceptor with a specific sequence.** The 3´ biotinylated donor oligonucleotide ('CL78', migrating at approximately 20 nt) was ligated to different quantities of a 60 nt oligonucleotide ('HP8') using *T4* 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.

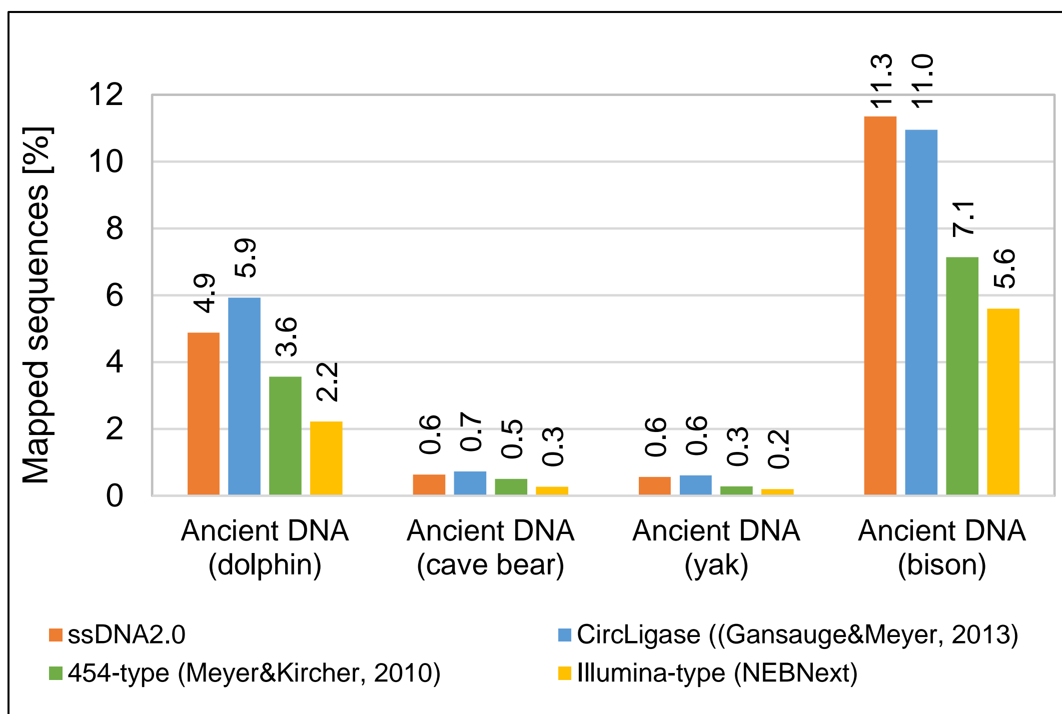



**Supplementary Figure 3. Effects of polymerase choice on library characteristics.**

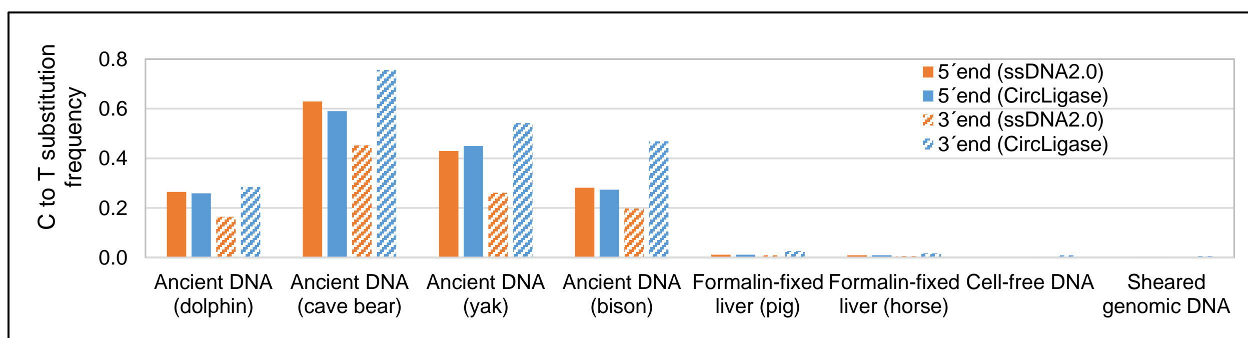
**(a)** Comparison of the informative sequence content in libraries prepared with four different polymerases from two ancient DNA extracts, **(b)** C to T substitution frequencies near the end of sequence alignments.



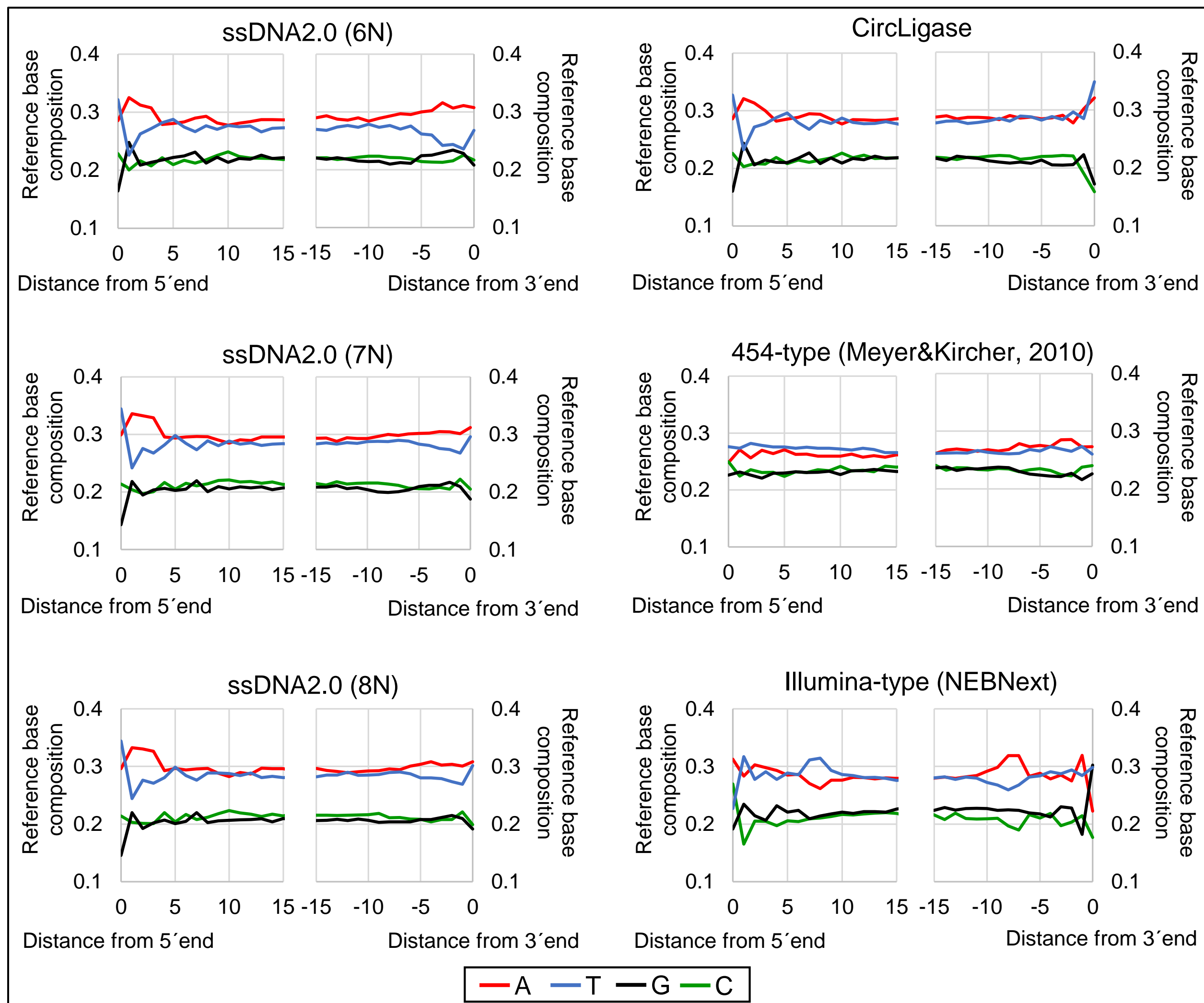

**Supplementary Figure 4. Size distribution of sequences obtained for 8 samples with single-stranded and double-stranded library preparation methods.** Size distributions are shown for all sequences (based on overlap-merged sequences only) as well as those mapping to the respective reference genome with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



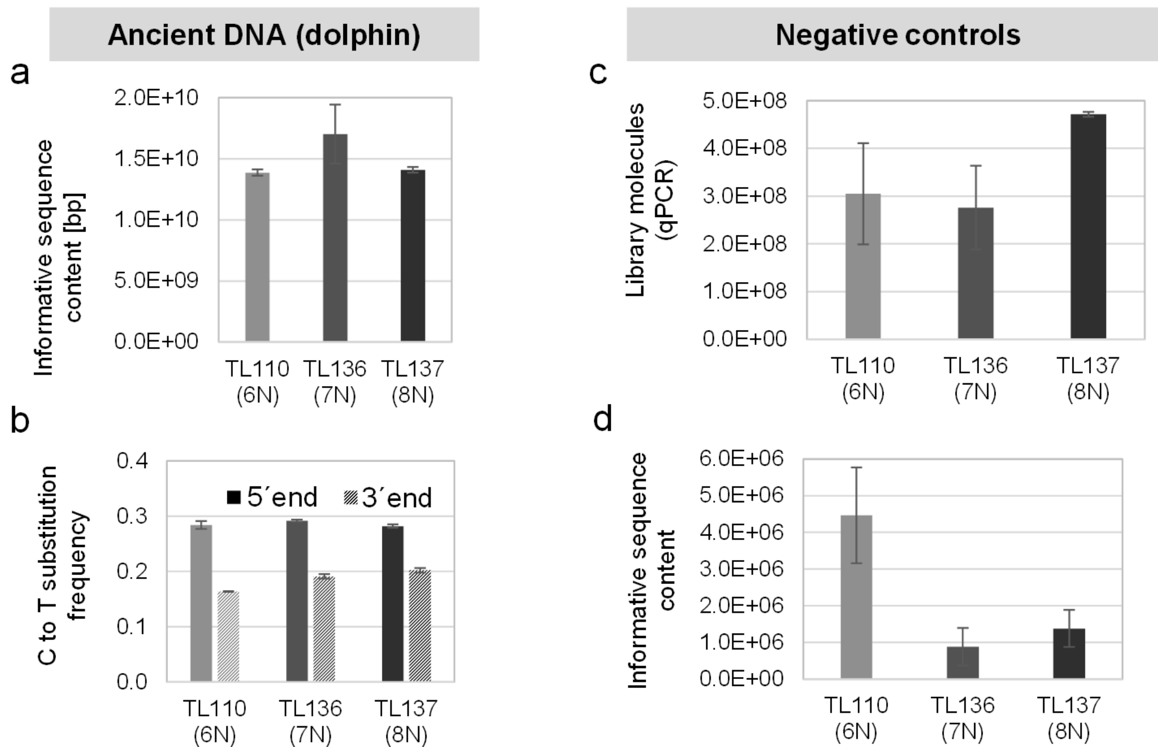

**Supplementary Figure 5. Percentage of sequences ≥35 bp mapping to the reference genome in libraries prepared with four different methods.** Values are denoted above the bars.




**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<br>(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]CTTCCGATCTNNNNNNN-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]CTTCCGATCTNNNNNNNN-AmC6 | desalted | Splinter |
| TL137 | SpacerC12-AA[SpacerC12]CTTCCGATCTNNNNNNNNN-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 | NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN | 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 |
| | | | | SP3571 (cave bear) | 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 |
| | | | | SP3571 (cave bear) | 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 |
| | | | | SP3571 (cave bear) | 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 |
| | | | | Negative control | - | 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 |
| | | | | SP3571 (cave bear) | 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 |
| | | | | SP3571 (cave bear) | 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 |
| | | | | SP3571 (cave bear) | 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 |
| | | | | Negative control | - | 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 | 174,766 | 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 | 7.13E+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 | 2,734,889 | 74.1 | 76,487 | 2.80 | 54.7 | 1.41E+10 | 6.1 | 29 | 16 |
| | | | | SP1060 (dolphin) | 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 (TL136) | | SP1060 (dolphin) | 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 |
| | | | | SP1060 (dolphin) | 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) | | SP1060 (dolphin) | 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 |
| | | | | SP1060 (dolphin) | 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 |
| | | | | Negative control | - | 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 (TL136) | | Negative control | - | 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 |
| | | | | Negative control | - | 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) | | Negative control | - | 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 |
| | | | | Negative control | - | 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 € | |