Chapter 9

A Method for Single-Stranded Ancient DNA Library Preparation

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Abstract

Genomic library preparation from highly degraded DNA is more efficient when library molecules are prepared separately from the complementary strands of DNA fragments. We describe a protocol in which libraries are constructed from single DNA strands in a three-step procedure: single-stranded ligation of the first adapter with *T4* DNA ligase in the presence of a splinter oligonucleotide, copying of the DNA strand with a proofreading polymerase, and blunt-end ligation of the second double-stranded adapter with *T4* DNA ligase.

Key words NGS, Library preparation, Ancient DNA, Single-stranded ligation, *T4* DNA ligase

1 Introduction

High-throughput sequencing requires the preparation of DNA libraries. This process involves the ligation of short, known adapter sequences to both ends of DNA molecules, enabling their amplification and readout. Commonly used double-stranded library preparation methods are not ideally suited for recovering highly degraded DNA from ancient specimens. In 2012, we described a single-stranded library preparation technique that increased the sequence information retrieved from a small sample of finger bone from an extinct Denisovan individual by approximately one order of magnitude, allowing its genome to be sequenced to high coverage [1]. The method has since been used to generate genome-wide sequence data from many other ancient specimens at various levels of coverage depth (e.g., [2–4]), including some samples of extraordinary old age [5]. Comparisons to double-stranded library preparation have confirmed that the single-stranded method greatly increases sequence complexity in libraries [6, 7] and in many cases improves the ratio of endogenous to environmental sequences [6].

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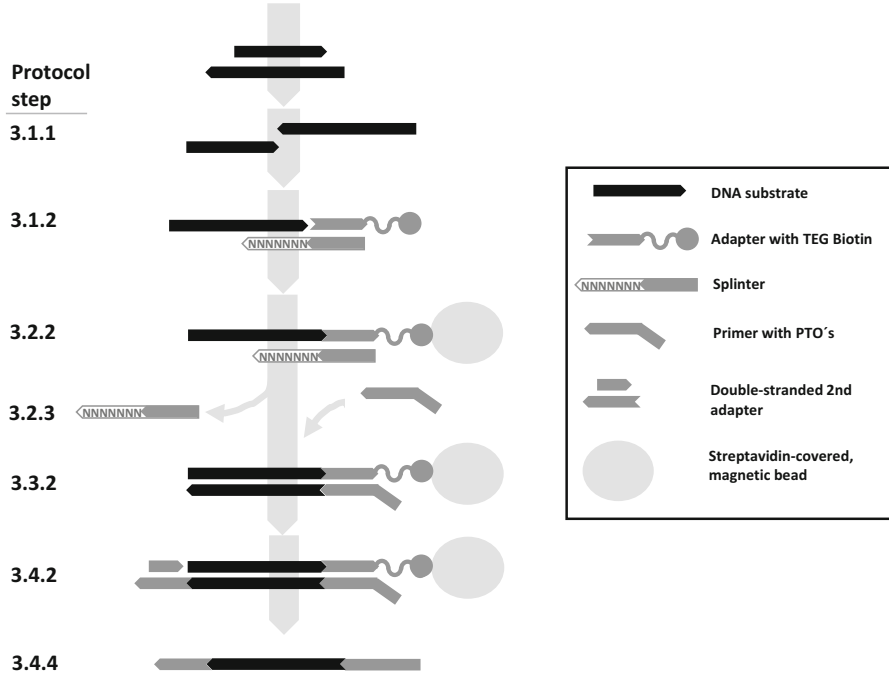

Fig. 1 Single-stranded library preparation. In the protocol provided here, DNA fragments are dephosphorylated at the 5′ and 3′ ends and separated into single strands by heat denaturation. 3′ biotinylated adapter molecules are attached to the 3′ ends of the DNA fragments using *T4* DNA ligase and a splinter oligonucleotide carrying a stretch of six random nucleotides (marked as "N"). Following the immobilization of the ligation products on streptavidin-coated beads, the splinter oligonucleotide is removed by a bead wash at elevated temperature. Synthesis of the second strand is carried out using the Klenow fragment of *E. coli* DNA polymerase I. Not incorporated primers are removed through a bead wash at elevated temperature. Following the blunt-end ligation of the second adapter, the final library strand is released from the beads by heat denaturation

The single-stranded library preparation method that we described in 2012 has been refined in recent years [8, 9]. In its most recent implementation [10], single-stranded DNA ligation is performed using *T4* DNA ligase in combination with a "splinter" oligonucleotide [11]. This replaces CircLigase, which is an expensive RNA ligase that is available from only one supplier. This modification has helped reduce costs and improve the robustness of the protocol (*see* Fig. 1 for an overview of the reaction steps). Nevertheless, single-stranded library preparation remains more time-consuming than double-stranded methods and requires higher initial investments in reagents due to expensive oligonucleotide modifications (*see* Table 1 for oligonucleotide sequences). In addition, a change to one of the Illumina adapter sequences necessitates the use of a nonstandard primer in sequencing (Fig. 2). These aspects should be considered when deciding whether to implement the method described below.

Table 1
Overview of the oligonucleotides required for single-stranded library preparation, amplification, and sequencing

Name	Sequence (5′–3′)	Application
CL78	Pho-AGATCGGAAG(C3Spacer)$_{10}$-TEG-biotin	Adapter, single-stranded ligation
TL136	SpacerC12-AA(SpacerC12)CTTCCGATCTNNNNNNN-AmC6	Splinter, single-stranded ligation
CL130	GTGACTGGAGTTCAGACGTGTGCTCTTCC*GA*TC*T	Extension primer
CL53	CGACGCTCTTC-ddC	Adapter oligo 1, double-stranded ligation
CL73	Pho-GGAAGAGCGTCGTGTAGGGAAAGAG*T*G*T*A	Adapter oligo 2, double-stranded ligation
CL104	Pho-TCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGA-Pho	Positive control oligo
CL105	ACACTCTTTCCCTACACGACGCTCTTCCTCGTCGTTTGGTATGGCTTC	Primer for qPCR standard
CL106	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTCATGTAACTCGCCTTGATCGT	Primer for qPCR standard
IS7	ACACTCTTTCCCTACACGAC	qPCR primer
IS8	GTGACTGGAGTTCAGACGTGT	qPCR primer
IS5	AATGATACGGCGACCACCGA	Primer for reamplification
IS6	CAAGCAGAAGACGGCATACGA	Primer for reamplification
CL72	ACACTCTTTCCCTACACGACGCTCTTCC	Sequencing primer

All oligonucleotides should be purified by reverse-phase HPLC with the exception of CL78, which requires dual purification by ion-exchange HPLC. Possible suppliers include Sigma-Aldrich and Eurogentec

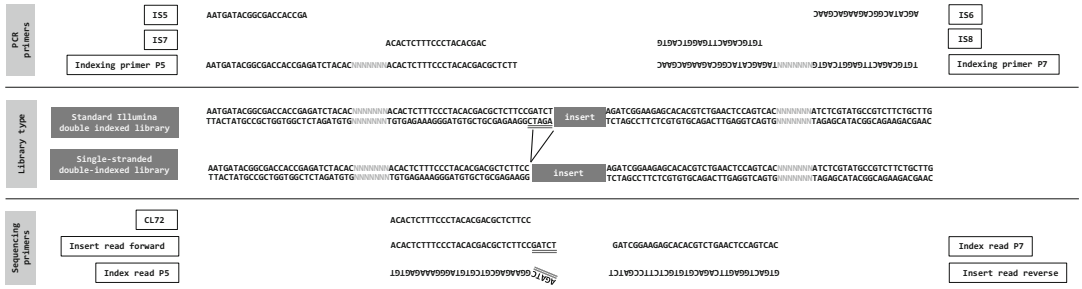

Fig. 2 Schematic overview of adapter sequences and primers used in library amplification and sequencing. Sequences are shown in the 5′–3′ direction. "N's" denote sample-specific index sequences (*see* **Note 9**). Standard Illumina adapter sequences are shown for comparison. For the forward insert read, single-stranded libraries require a nonstandard sequencing primer (CL72). Primers IS5 and IS6 can be used for library reamplification if it becomes necessary

2 Materials

Incubation steps involving bead suspensions are best carried out in a thermoshaker with an interval mixing option (e.g., Cooling ThermoMixer MKR13, HLC/Ditabis) to avoid bead settling. Manual mixing is possible but substantially increases hands-on time. Mixing intervals may be extended to 5 min in the case of manual mixing. It is recommended to prepare master mixes for all reaction steps. Enzymes may be included in the master mixes. Keep the master mixes on ice until used.

2.1 Preparation of Buffers (Prepare 50 mL Each, Store at Room Temperature for up to 6 Months)

1. 0.1× BWT + SDS buffer: 0.1 M NaCl, 10 mM Tris–HCl, 1 mM EDTA, 0.05% Tween 20, 0.5% SDS (pH 8.0).
2. 0.1× BWT buffer: 0.1 M NaCl, 10 mM Tris–HCl, 1 mM EDTA, 0.05% Tween 20 (pH 8.0).
3. Stringency wash buffer: 0.1× SSC (Sigma-Aldrich), 0.1% SDS.
4. TT buffer: 10 mM Tris–HCl, 0.05% Tween 20 (pH 8.0).
5. TE buffer: 10 mM Tris–HCl, 1 mM EDTA (pH 8.0).
6. TET buffer: 10 mM Tris–HCl, 1 mM EDTA, 0.05% Tween 20 (pH 8.0).

2.2 Adapter Decontamination and Hybridization

1. CL78 adapter decontamination: In a 0.2 mL PCR tube, combine 12 μL water, 4 μL 100 μM CL78, 2 μL 10× *T4* RNA ligation buffer (New England Biolabs), 1 μL 10 U/μL Klenow fragment (ThermoFisher Scientific), and 1 μL 10 U/μL *T4* polynucleotide kinase (ThermoFisher Scientific). Total reaction volume is 20 μL. Mix the reagents properly, spin the tube briefly in a microcentrifuge, and incubate the reaction in a thermal cycler for 20 min at 37 °C followed by 1 min at 95 °C (enzyme inactivation). The final concentration of CL78 is 20 μM.

2. TL136 splinter decontamination: In a 0.2 mL PCR tube, combine 8 μL water, 8 μL 100 μM TL136, 2 μL 10× *T4* RNA ligation buffer, 1 μL 10 U/μL Klenow fragment, and 1 μL 10 U/μL *T4* polynucleotide kinase. Total reaction volume is 20 μL. Mix reagents properly, spin the tube briefly in a microcentrifuge, and incubate the reaction in a thermal cycler for 20 min at 37 °C followed by 1 min at 95 °C (enzyme inactivation). The final concentration of TL136 is 40 μM.
3. Adapter/splinter hybridization: In a 0.2 mL PCR tube, combine 20 μL purified adapter CL78 with 20 μL purified splinter TL136 for a total volume of 40 μL. Heat the reaction mix for 10 s to 95 °C in a thermal cycler and cool down to 10 °C. The final concentration of hybridized adapter CL78/TL136 is 10/20 μM. Store at −20 °C until used.
4. Preparation of double-stranded adapter CL53/73: In a 0.2 mL PCR tube, combine 9.5 μL TE, 0.5 μL 5 M NaCl, 20 μL 500 μM CL53, and 20 μL 500 μM CL73. Total volume is 50 μL. Mix and incubate the reaction for 10 s at 95 °C in a thermal cycler, and cool down to 14 °C at a rate of 0.1 °C/s. Add 50 μL TE to obtain 100 μM CL53/73 in a final volume of 100 μL.

3 Methods

3.1 Heat Denaturation, Dephosphorylation, and Ligation of First Adapter

1. For each sample, combine the following reagents in a 0.5 mL tube (*see* **Note 1**): 8 μL 10× *T4* RNA ligation buffer, 2 μL 2% Tween 20, 1 μL 1 U/μL FastAP (ThermoFisher Scientific), and sample DNA supplemented with TT buffer to 34.6 μL. Total reaction volume is 45.6 μL. Mix properly and briefly spin the tubes in a microcentrifuge. Incubate for 10 min at 37 °C and 2 min at 95 °C in a thermal cycler. Transfer the tubes directly from the thermal cycler into an ice water bath, and let them stand for 2 min.
2. Add the following components to the reaction mix to obtain a total reaction volume of 80 μL: 32 μL 50% PEG-8000, 0.4 μL 100 mM ATP, 1 μL 10/20 μM CL78/TL136, and 1 μL 30 U/μL *T4* DNA ligase (ThermoFisher Scientific) (*see* **Notes 2** and **3**). Mix the reactions properly (*see* **Note 4**), and spin the tubes briefly in a microcentrifuge. Incubate for 1 h at 37 °C and 1 min at 95 °C in a thermal cycler. Transfer the tubes directly from the thermal cycler into an ice water bath. Proceed to the next step immediately or freeze the tubes at −20 °C.

3.2 Immobilize Ligation Products on Beads

1. Resuspend the stock solution of MyOne C1 beads (ThermoFisher Scientific) by vortexing. For each reaction, transfer 20 μL of the bead suspension into a 1.5 mL tube (multiply by

the number of samples, e.g., 120 μL for six samples). Wash beads twice with 500 μL 0.1× BWT + SDS. Resuspend the beads in 250 μL 0.1× BWT + SDS (multiply by the number of samples, e.g., 1.5 mL for six samples). Per sample, transfer 250 μL beads to a 1.5 mL tube.

2. If the reactions were frozen at the end of **step 2**, Subheading 3.1, thaw the tubes and incubate them for 1 min at 95 °C in a thermal cycler. Transfer the tubes directly from the cycler into an ice water bath, and let them stand for 2 min. Add the reaction mix to the bead suspension, vortex and rotate the bead suspension for 20 min at room temperature. Spin the tubes briefly in a microcentrifuge.
3. Pellet the beads using a magnet rack. Pipette off and discard the supernatant. Add 200 μL 0.1× BWT + SDS and resuspend the beads by vortexing. Spin the tubes briefly in a microcentrifuge, place on magnetic rack and discard the supernatant. Add 100 μL Stringency wash buffer and resuspend the beads by vortexing. Incubate the tubes for 3 min at 45 °C with interval mixing every 30 s. Spin the tubes briefly in a microcentrifuge, place them on a magnetic rack, and discard the supernatant. Add 200 μL 0.1× BWT and resuspend the beads by vortexing.

3.3 Primer Annealing and Extension

1. For each reaction, combine the following reagents in a 1.5 mL tube: 39.1 μL water, 5 μL 10× Klenow reaction buffer (ThermoFisher Scientific), 0.4 μL 25 mM dNTP, 2.5 μL 1% Tween 20, and 1 μL 100 μM CL130. Total volume is 48 μL.
2. Spin the tubes with the bead suspension in a microcentrifuge, and place them on a magnetic rack. Discard the supernatant, and resuspend the beads in the reaction mix from **step 1**, Subheading 3.3 by vortexing. Incubate the tubes for 2 min in a thermoshaker pre-heated to 65 °C. Place the tubes into an ice water bath. Add 2 μL 10 U/μL Klenow fragment (ThermoFisher Scientific), and briefly mix the reactions by vortexing. Incubate the tubes in a thermoshaker for 5 min at 25 °C, followed by 25 min at 35 °C with interval mixing every 30 s.
3. Spin the tubes in a microcentrifuge. Perform three bead washes exactly as described in **step 3**, Subheading 3.2.

3.4 Ligation of Second Adapter, Library Elution

1. For each reaction, combine the following reagents in a 1.5 mL tube: 73.5 μL water, 10 μL 10× *T4* DNA ligase buffer (ThermoFisher Scientific) (*see* **Note 5**), 10 μL 50% PEG 4000, 2.5 μL 1% Tween 20, 2 μL 100 μM CL53/73, and 2 μL 5 U/μL *T4* DNA ligase (ThermoFisher Scientific). Total reaction volume is 100 μL.
2. Spin the tubes with the bead suspension in a microcentrifuge. Place the tubes on a magnetic rack and discard the supernatant.

Resuspend the beads in the ligation mix from **step 1**. Incubate the reaction for 1 h at 22 °C with interval mixing/vortexing every 30 s.

3. Spin the tubes in a microcentrifuge. Perform three bead washes exactly as described in **step 3**, Subheading 3.2.
4. Spin the tubes briefly in a microcentrifuge, place them on a magnetic rack, and discard the supernatant. Resuspend the beads in 50 μL TT buffer by vortexing, and transfer the bead suspension to 0.2 mL PCR strip tubes. Spin the tubes briefly in a microcentrifuge. Incubate the bead suspension for 1 min at 95 °C. Immediately transfer the PCR strip tubes to a magnetic rack, and transfer the supernatant (the library) to fresh 1.5 mL tubes.

3.5 Library Quantification by qPCR

1. Dilute 1 μL of each library 50-fold in TET buffer (*see* **Note 6**). For each reaction (samples and qPCR standard, *see* **Note 7**), combine the following reagents in the wells of a 96-well PCR plate: 10.5 μL water, 12.5 μL 2× Maxima SYBR Green qPCR Master Mix (ThermoFisher Scientific), 0.5 μL 10 μM primer IS7, 0.5 μL 10 μM primer IS8, and 1 μL of diluted library/qPCR standard. Total reaction volume is 25 μL. Close the wells with optical caps, and briefly spin the PCR plate in a centrifuge at 1000 × *g*. Place the plate into a qPCR cycler, and incubate the reactions at 95 °C for 10 min, followed by 40 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. Carry out fluorescence measurements at the end of each extension step.
2. Use the software provided with the qPCR system to calculate the number of molecules in each library (*see* **Note 8**).

3.6 Amplification and Indexing of the Sequencing Libraries

1. For each reaction, combine the following reagents in 0.2 mL PCR tubes: 20 μL water, 10 μL 10× AccuPrime *Pfx* buffer (ThermoFisher Scientific), 10 μL 10 μM P7 indexing primer, 10 μL 10 μM P5 indexing primer (*see* **Note 9**), 49 μL library, and 1 μL 2.5 U/μL AccuPrime *Pfx* DNA polymerase (ThermoFisher Scientific). Total reaction volume is 100 μL. Mix the reaction properly and spin the tubes in a microcentrifuge. Incubate the reactions in a thermal cycler at 95 °C for 2 min, followed by an appropriate number of cycles (*see* **Note 10**) at 95 °C for 20 s, 60 °C for 30 s, and 68 °C for 1 min, with a final extension step of 5 min at 68 °C.
2. Purify the amplified libraries using the MinElute PCR purification kit (Qiagen). Elute the purified products in 30 μL TE buffer. Determine the concentration using a DNA-1000 chip on Bioanalyzer 2100 (Agilent Technologies) and sequence the libraries on an Illumina MiSeq or HiSeq instrument using CL72 as the primer for the forward insert read (*see* Fig. 2).

4 Notes

1. Choose 0.2 mL tubes if the thermal cycler cannot fit 0.5 mL tubes. At least one negative control containing water instead of sample DNA should be included in each experiment. We also recommend the addition of a positive control, e.g., using 0.1 pmol of oligonucleotide CL104.
2. PEG-8000 is highly viscous and requires very thorough mixing when preparing a master mix.
3. *T4* DNA ligase is used in two different concentrations throughout this protocol: 30 U/μL for single-stranded ligation **step 2**, Subheading 3.1 and 5 U/μL for double-stranded ligation **step 4**, Subheading 3.4.
4. Proper mixing in this step is critical to the success of the library preparation. Vortexing is ineffective due to the high viscosity of PEG-8000. Mix by flicking the tubes with a finger several times, and control the success of mixing by eye.
5. White precipitate may be present in the ligation buffer after thawing. Heat the buffer vial briefly to 37 °C and vortex until the precipitate has dissolved.
6. Save the library dilution for repeated measurements in case they become necessary. Store at −20 °C.
7. For the preparation of a qPCR standard with Illumina adapters, use pUC19 DNA and the primer pair CL105/CL106 to generate a 122-bp PCR product. Purify the product using the MinElute PCR Purification Kit. Determine its concentration and prepare a tenfold dilution series of the PCR product in TET buffer, ranging from 10^9 to 10^2 copies of the PCR product per microliter [8].
8. Library negative controls are used to infer the number of artifact molecules that have formed during library preparation (typically less than 3×10^8 molecules). Order newly synthesized batches of CL78 and TL136 if artifacts are formed in greater number.
9. Sequences of indexing primers are published in Gansauge and Meyer [8].
10. Optimal cycle numbers for amplification can be inferred individually for each sample using the qPCR amplification plots. Cycling into PCR plateau leads to the formation of undesired heteroduplexes and should be avoided [8].

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