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Blunt-End-Single-Tube (BEST): Double-Stranded Ancient DNA Library Protocol

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Protocol status: Working
We use this protocol and it's working

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Abstract

This prococol describes the process of Blunt-End-Single-Tube (BEST) library build for ancient DNA extractions. It includes USER II treatment of the DNA extract, end-repair, and adapter ligation. The expected outcome of this procotol is double-stranded libraries, which will need to be indexed before sequencing.

This protocol is originally written by Michael V. Westbury, edited by Alba Rey-Iglesia, and subsequently converted to a protocols.io version by Vanssy Li and Deon de Jager.

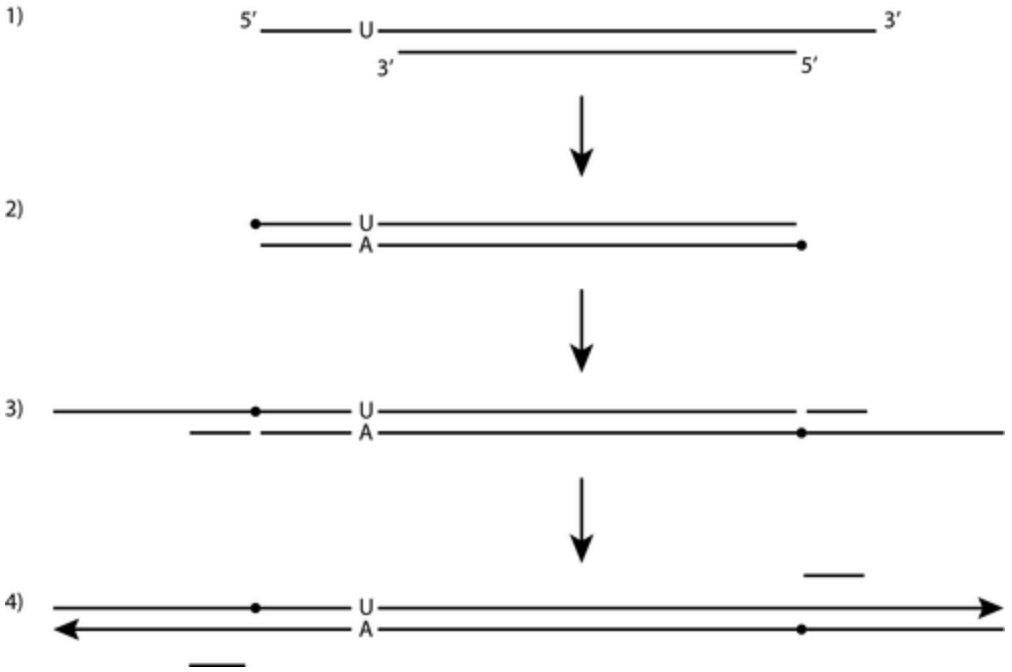

Figure caption from original publication: Overview of the steps in the basic library method used for all libraries in this paper. (1) Double-stranded DNA fragments are used as input. Here, a fragment containing 3' and 5' overhangs is presented with a uracil (U) base contained in the 5' overhang. (2) Through end-repair, 3' overhangs are digested by the T4 DNA Polymerase and 5' overhangs are filled in. If the 5' overhangs contains a uracil base, an adenosine nucleotide will be inserted on the opposite strand. Further, 3' phosphates are removed and 5' OH groups phosphorylated by the T4 Polynucleotide Kinase (dots). (3) Double-stranded adapters are ligated to the fragment by T4 DNA Ligase. These do not contain 5' phosphates and therefore only one strand is ligated to the target DNA. (4) The ligated adapter is filled in and the small guiding oligo is displaced using Bst DNA Polymerase.
Carøe et al. (2018) Methods Ecol Evol, Volume: 9, Issue: 2, Pages: 410-419, First published: 17 August 2017, DOI: (10.1111/2041-210X.12871).

Always cite the original paper (Carøe et al. 2018) when using this protocol, in addition to citing this protocols.io protocol:

- Carøe C, Gopalakrishnan S, Vinner L, et al. Single-tube library preparation for degraded DNA. *Methods Ecol Evol*. 2018; 9: 410–419. https://doi.org/10.1111/2041-210X.12871

Materials

Reaction enhancer (for End-Repair reaction)

- PEG 4000 (Poly(ethylene glycol)) **Bio Basic Inc.** **Catalog #**PB0431.SIZE.500g or 50% PEG 4000 (Mix 1g PEG4000 and 1.1 mL H20)
- BSA 20ng/mL **New England Biolabs** **Catalog #**B9000S
- NaCl (5 M), RNase-free **Thermo Fisher Scientific** **Catalog #**10609823
- H2O

Enzymes for USER treatment

- Thermolabile USER II Enzyme - 250 units **New England Biolabs** **Catalog #**M5508L

Reagents for end-repair and adapter ligase

- T4 DNA Ligase Reaction Buffer **New England Biolabs** **Catalog #**B0202S
- T4 DNA Ligase - 100,000 units **New England Biolabs** **Catalog #**M0202L
- T4 DNA polymerase - 750 units **New England Biolabs** **Catalog #**M0203L
- T4 PNK **New England Biolabs** **Catalog #** M0201L
- Isothermal Amplification Buffer - 6.0 ml **New England Biolabs** **Catalog #**B0537S
- Bst 2.0 WarmStart DNA Polymerase - 8,000 units **New England Biolabs** **Catalog #**M0538L
- dNTP Set 100 mM Solutions **Thermo Fisher Scientific** **Catalog #**R0181

Buffers

- Modified Qiagen PB buffer (aka "modified binding buffer) from Allentoft et al. 2015 (DOI: **10.1038/nature14507**) - see our **ancient DNA extraction protocol** for the recipe.
- PE buffer **Qiagen** **Catalog #**19065
- Buffer EB **Qiagen** **Catalog #**19086

Protocol materials

- PE buffer **Qiagen** **Catalog #**19065
- Buffer EB **Qiagen** **Catalog #**19086
- T4 DNA Ligase - 100,000 units **New England Biolabs** **Catalog #**M0202L
- T4 DNA polymerase - 750 units **New England Biolabs** **Catalog #**M0203L
- PEG 4000 (Poly(ethylene glycol)) **Bio Basic Inc.** **Catalog #**PB0431.SIZE.500g
- BSA 20ng/mL **New England Biolabs** **Catalog #**B9000S
- dNTP Set 100 mM Solutions **Thermo Fisher Scientific** **Catalog #**R0181
- NaCl (5 M), RNase-free **Thermo Fisher Scientific** **Catalog #**10609823
- Thermolabile USER II Enzyme - 250 units **New England Biolabs** **Catalog #**M5508L
- T4 PNK **New England Biolabs** **Catalog #** M0201L
- T4 DNA Ligase Reaction Buffer **New England Biolabs** **Catalog #**B0202S
- Isothermal Amplification Buffer - 6.0 ml **New England Biolabs** **Catalog #**B0537S
- Bst 2.0 WarmStart DNA Polymerase - 8,000 units **New England Biolabs** **Catalog #**M0538L
- 50% w/v PEG 4000 **Molecular Dimensions** **Catalog #**MD2-250-11
- 10% Tween-20 Solution **Teknova** **Catalog #**T0710
- BSA 20 mg/ml **NEB** **Catalog #**B9000S

Before start

- This protocol should be done in an ancient DNA clean lab facility.
- Clean all surfaces with 5% bleach solution followed by 70% ethanol before and after use. Clean all equipment with 70% ethanol before and after use.
- UV-treat buffers PE, PB, and elution buffer for 10 min in a UV cross-linker before use.
- DO NOT UV enzymes or Eppendorf/PCR tubes.

- Make sure the **Adapter Hybridization** has been done (this only needs to be done once whenever adapters are ordered).
- UDG/USER-treat your ancient DNA extractions before starting this protocol, if this is desired. See the protocol here: **USER II treatment of ancient DNA extracts**.

General comments:

- When referring to "adapters" in the protocol below, we are referring to the double stranded adapter hybrid oligos (P5+P7) prepared in the adapter hybridization protocol linked above.
- Remember to include **negative controls**! A library build negative control, and your DNA extraction negative control.
- Keep enzymes and Master Mix in cooling block.
- Never vortex enzymes vigorously.
- Do not vortex reactions (apart from when mixing adapter and sample). Instead of vortexing, either mix by pipetting the samples 10-15 time or flick the tube with your finger 5-10 times. When handling small volumes, flicking is easier. Remember to spin down briefly prior to incubation.
- Remember to keep the master mix in a cooling block in all the time.

Buffer and adapter preparation

1 **Reagents List:**

Reaction enhancer (for end-repair reaction)

- PEG 4000 (Poly(ethylene glycol)) **Bio Basic Inc. Catalog #**PB0431.SIZE.500g or 50% w/v PEG 4000 **Molecular Dimensions Catalog #**MD2-250-11
- BSA 20 mg/ml **NEB Catalog #**B9000S
- NaCl (5 M), RNase-free **Thermo Fisher Scientific Catalog #**10609823
- H2O

Reagents for end-repair and adapter ligation master mixes (only those not already listed above)

- T4 DNA Ligase Reaction Buffer **New England Biolabs Catalog #**B0202S
- T4 DNA Ligase - 100,000 units **New England Biolabs Catalog #**M0202L
- T4 DNA polymerase - 750 units **New England Biolabs Catalog #**M0203L
- T4 PNK **New England Biolabs Catalog #** M0201L
- Isothermal Amplification Buffer - 6.0 ml **New England Biolabs Catalog #**B0537S
- Bst 2.0 WarmStart DNA Polymerase - 8,000 units **New England Biolabs Catalog #**M0538L
- dNTP Set 100 mM Solutions **Thermo Fisher Scientific Catalog #**R0181

Buffers

- Modified Qiagen PB buffer (aka "modified binding buffer") from **Allentoft et al. 2015** - see our **ancient DNA extraction protocol** for the recipe.
- PE buffer **Qiagen Catalog #**19065
- Buffer EB **Qiagen Catalog #**19086 or **EBT buffer**: 5 mL Buffer EB + 2.5 µL Tween-20 (10% Tween-20 Solution **Teknova Catalog #**T0710)

2 **Adapters:**

- The amount of adapter (P5+P7 hybrid oligos) used is adjusted depending on the absolute amount of input DNA used in **Step 5**.
- Thus, ensure you have enough volume of the specific adapter concentration(s) you need before starting (1 µL needed per sample).

- Dilute the adapters as necessary from the 200 µM stock prepared in the **adapter hybridization protocol** if you do not have enough.

	DNA input amount (ng)	Corresponding adapter concentration (µM)
	100-400*	50
	50-100	20
	30-50	10
	20-30	5
	10-20	3
	5-10	2
	<5**	1

Note

**100-400 ng:* Rather than using 1 µL of 50 µM adapter, it is recommended to dilute the DNA extraction to 50-100 ng and use the 20 µM adapter. The BEST protocol is optimal for DNA input between 0.1 and 200 ng.
***<5 ng:* Too little DNA.
- You will use a maximum of 16 µL of your DNA extraction (Step 5), so: **16 µL x DNA concentration in ng/µL = ng of DNA used**.
- If your DNA extract is too concentrated (i.e. you have >400 ng in 16 uL, then dilute your DNA with EB buffer (also see * above).

3 **Reaction enhancer (for end-repair reaction):**

- Prepare in advance. The mixture can be frozen and mixed with the master mix buffer in the end-repair reaction (Step 4).

	Reagent	Stock Conc.	Volume	Final Conc.
	50% PEG 4000*	50%	500 uL	25%
	BSA	20 mg/mL	100 µL	2 mg/mL
	NaCl	5 M	80 µL	400 mM
	H2O	/	up tp 1 mL	/
	Final	/	1 mL	/

*Or 0.25 g of powdered PEG4000.

End-repair

4 Prepare the end-repair master mix.

	Reagent	Stock Conc.	Final Conc.*	Volume	N samples
	T4 DNA polymerase	3 U/µL	0.03 U/µL	0.2 µL	0.2 µL* (N+1)
	T4 PNK	10 U/µL	0.25 U/µL	0.5 µL	0.5 µL* (N+1)
	dNTP	25 mM	0.25 mM	0.2 µL	0.2 µL* (N+1)
	T4 DNA ligase buffer	10x	1x	2 µL	2 µL*(N+1)
	Reaction enhancer	/	/	1.1 µL	1.1 µL*(N+1)
	Final	/	/	4 µL	4 µL*(N+1)

***Note:** This final concentration refers to the concentration in the total reaction in Step 5 (final volume of 20 µL), and not the final concentration in the end-repair master mix itself.

5 In PCR tubes (individual or a strip), add **4 µL** of the **end-repair master mix** to **16 µL DNA** (may or may not be USER-treated) for each sample, for a **total reaction volume of 20 µL**.

6 In a thermocycler, incubate the reaction for 00:30:00 min at 20 °C followed by 00:30:00 min at 65 °C and then cool to 4 °C

> Note
>
> It is important that the reaction is cooled to 4°C for complete deactivation.

During this incubation (~1 h), prepare the ligation master mix (Step 8, below), and put it in a cold rack or on ice before using it.

7 Once the end-repair incubation is complete, put the PCR tubes in a cold rack (or ice) and add **1 µL adapter to each reaction,** ensuring to use the concentration that corresponds to the amount of input DNA in the reaction (see **Step 2**). **The reaction volume is now 21 µL**.

- Mix thoroughly by pipetting or by flicking and spinning down in a microcentrifuge several times.

Adapter ligation

8 Prepare the ligation master mix

	Reagent	Stock Conc.	Final Conc.*	Volume	N samples
	T4 DNA ligase buffer	10x	0.2x	0.5 µL	0.5 µL* (N+1)
	50% PEG 4000	50%	6%	3 µL	3 µL*(N+1)
	T4 DNA ligase	400 U/µL	8 U/µL	0.5 µL	0.5 µL* (N+1)
	Final	/	/	4 µL	4 µL*(N+1)

***Note:** This final concentration refers to the concentration in the total reaction in Step 9 (final volume of 25 µL), and not the final concentration in the ligation master mix itself.

9 Add **4 µL** of the **ligation master mix** to each sample to make a **25 µL reaction**.

10 Incubate for 00:30:00 min at 20 °C followed by 00:10:00 min at 65 °C and then cool to 4 °C.

During this incubation (~40 min), prepare the adapter fill-in master mix (Step 11, below), and put the master mix in a cold rack or on ice until it is needed.

Adapter fill-in

11 Prepare the adapter fill-in master mix.

	Reagent	Stock Conc.	Final Conc.*	Volume	N samples
	Isothermal amp. buffer	10x	0.33x	1 µL	1 µL*(N+1)
	dNTP	25 mM	0.33 mM	0.4 µL	0.4 µL* (N+1)
	Bst 2.0 Warmstart pol	8 U/µL	0.21 U/µL	0.8 µL	0.8 µL* (N+1)
	H2O	/	/	2.8 µL	2.8 µL* (N+1)
	Final			5 µL	5 µL*(N+1)

***Note:** This final concentration refers to the concentration in the total reaction in Step 12 (final volume of 30 µL), and not the final concentration in the adapter fill-in master mix itself.

12 Add **5 µL** of the **adapter fill-in master mix** to each sample to make a **30 µL reaction**.

13 Incubate for 00:15:00 min at 65 °C followed by 00:15:00 min at 80 °C and then cool to 4 °C.

During this incubation (~30 min), prepare the tubes for the DNA clean-up. Label Monarch columns (temporary) and one set of final 1.5 mL LoBind Eppendorf tubes. Label appropriately with sample code, date, your name etc. Also prepare a 50 mL Falcon tube and label it as "Qiagen waste" and with your name and the date.

Proceed to clean-up (Step 14) immediately after incubation.

Clean up using monarch spin columns

14

Note

REMINDER: UV-treat buffers for 10 min in a UV cross-linker before use, if not already done.

After the adapter fill-in incubation is complete, add **450 µL modified PB** (binding) **buffer** to the Monarch spin columns (one for each sample).

15 **Add the entire adapter fill-in reaction (30 µL)** to the modified PB (binding) buffer in the spin column and mix by pipetting.

16 Centrifuge at 8.000 rpm for 00:01:00 min.

- Prepare a square piece of paper towel/Kim Wipe by folding it in half twice (so there are four layers).
- Discard the **flow through** into the 50 mL Falcon tube labelled as "Qiagen waste".
- Dry the collection tube by tapping it upside down on the folded piece of paper towel before putting the column back. Ensure that you tap on a different piece of the paper towel for each sample, to avoid cross-contamination.

17 Add **650 µL PE buffer** to each spin column and centrifuge at 8.000 rpm for 00:01:00 min.

Discard the **flow through** and dry the collection tube as before.

18 Centrifuge at **maximum speed** for 00:01:00 min to dry the spin column of any excess PE buffer.

19 DNA (library) elution: Place spin column in a **fresh 1.5 mL LoBind Eppendorf tube** and add **16 µL EBT buffer (or EB buffer)** to the center of the spin column membrane.

Note

If LoBind tubes are not available, it is essential to use EBT or TET buffer (see the **SCR protocol**) in this step, as this helps to prevent the binding of DNA to the plastic of the tube.

It is obviously crucial to retain as much DNA as possible when working with aDNA libraries due to the low concentration and complexity of the libraries and the fact the source of the DNA is usually finite, so every step should limit DNA loss as much as possible.

When using LoBind tubes, standard Buffer EB can be used (without Tween-20), as the LoBind tubes are designed to reduce the binding of DNA. Although note that a recent study (**Gilardet et al. 2025**) showed that using TET (which includes Tween-20) for elution of aDNA extractions increases complexity of the final library - granted, that was for extractions (and used TET and not EBT), but it should be effective for libraries too.

20 Incubate for 00:05:00 and then centrifuge at **maximum speed** for 00:01:00 min to collect the eluted DNA in the Eppendorf tube.

21 Repeat steps 19 and 20 to give **a final library volume of 32 µL**.

22 Store libraries at -20°C.

Protocol references

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