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

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Library preparation (dsDNA single indexing, full-UDG, no split)

Forked from Library preparation (dsDNA single indexing, non-UDG, no split)

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ABSTRACT

Protocol for the preparation of single indexed double-stranded DNA libraries for Illumina sequencing, optimized for ultra-short ancient DNA molecules, modified from Meyer & Kircher (2010) Cold Spring Harb. Protoc. (doi: 10.1101/pdb.prot5448). This protocol does include treatment with UDG (USER) to remove DNA damage in form of deaminated cytosines.

GUIDELINES

Please read the general guidelines for working in the Ancient DNA protocol collection – University of Tartu, Institute of Genomics.

MATERIALS

Reagents:

	A	B	C	D	E	F	G
	Step	Reagents	Conc.	Unit	Manufacturer	Kit/full description	Product number
	End repair 1	Water			Fisher chemicals	Water for HPLC 1L	10367171
	End repair 1	NEB Buffer 2	10	X	New England Biolabs		B7002S
	End repair 1	dNTPs	25	mM	Thermo Fisher		R1121
	End repair 1	BSA	20	mg/ml	Thermo Fisher		B14
	End repair 1	ATP	10	mM	New England Biolabs		P0756S

Keywords: ancient DNA, aDNA, archeogenetics, archaeogenetics, paleogenetics, palaeogenetics, library preparation

	A	B	C	D	E	F	G
	End repair 1	T4 PNK	10	U/µl	New England Biolabs		M0201L
	End repair 1	USER enzyme	1	U/µl	New England Biolabs	NEBNext Multiplex Oligos for Illumina (Index Primers Set 1-4)	E7335S, E7500S, E7710S, E7730S
	End repair 2	T4 Polymerase	10	U/µl	New England Biolabs		M0201L
	Adapter Ligation	Quick Ligation Buffer	5.0	X	New England Biolabs	NEBNext Quick Ligation Module	E6056L
	Adapter Ligation	Adapter Mix (2.5 µM)	2.5	µM		*Custom made from Eurofins adapters	n/a
	Adapter Ligation	Quick T4 Ligase	1.0	U	New England Biolabs	NEBNext Quick Ligation Module	E6056L
	Fill in	Water			Fisher chemicals	Water for HPLC 1L	10367171
	Fill in	Thermopol Buffer 10X	10	X	New England Biolabs	Bst DNA Polymerase, Large Fragment	M0275
	Fill in	dNTP 25mM	25	mM	Thermo Fisher		R1121
	Fill in	Bst DNA polymerase	8	U/µl	New England Biolabs	Bst DNA Polymerase, Large Fragment	M0275
	PCR	Water			Fisher chemicals	Water for HPLC 1L	10367171
	PCR	10x PCR buffer	10	X	Eurogentec	HGS Taq Diamond set	TAQ-I011-1000+
	PCR	MgCl2	25	mM	Eurogentec	HGS Taq Diamond set	TAQ-I011-1000+
	PCR	BSA 20 mg/ml	20	mg/ml	Thermo Fisher		B14
	PCR	dNTPs (10 mM)	40	mM	Thermo Fisher		R0191
	PCR	HGS Taq Diamond	1	U	Eurogentec	HGS Taq Diamond set	TAQ-I011-1000+
	PCR	Universal Primer	10	µM	New England Biolabs	NEBNext Multiplex Oligos for Illumina (Index Primers Set 1-4)	E7335L, E7500L, E7710L, E7730L
	PCR	Indexing Primer	10	µM	New England Biolabs	NEBNext® Multiplex Oligos for Illumina® (Index Primers Set 1-4)	E7335L, E7500L, E7710L, E7730L
	Purification	PB Buffer			Qiagen	MinElute PCR Purification Kit	19066

	A	B	C	D	E	F	G
	Purification	PE Buffer			Qiagen	MinElute PCR Purification Kit	19065
	Purification	EB Buffer			Qiagen	MinElute PCR Purification Kit	28006

Equipment and consumables:

	A	B
	Number	Equipment and consumables
	1	0.2 ml tube rack
	2	1.5 ml tube rack
	1	1.5 ml cool block
	1	0.2 ml cool block
		10 µl filter tips
		20 µl filter tips
		100 µl filter tips
		200 µl filter tips
		1000 µl filter tips
	[# of samples]×4 (tubes)	0.2 µl PCR strips (8 tubes)
	[# of samples]×2+4	1.5 ml tubes
	1	5 ml tube
	[# of samples]×2	MinElute columns
	1	50 ml Falcon (waste)

Lab equipment:
Laminar flow hood
Centrifuge (2/1.5 ml)
Heat block
Mini table centrifuge/vortexer
Thermocycler
10 µl pipette
20 µl pipette
100 µl pipette
200 µl pipette
1000 µl pipette

Other consumables:

DNA ExitusPlus
Paper towels

SAFETY WARNINGS

Reagents

NaOCl (bleach) solution (6%)
- H290 May be corrosive to metals.
- H314 Causes severe skin burns and eye damage.
- H411 Toxic to aquatic life with long lasting effects.
- EUH206 Warning! Do not use together with other products. May release dangerous gases (chlorine). Remove from surface after recommended incubation time with water-soaked tissue.

DNA ExitusPlus
H319 Causes serious eye irritation.

Guanidinium hydrochloride (GuHCl) (in PB buffer of Qiagen MinElute kit)
- H302 Harmful if swallowed.
- H332 Harmful if inhaled.
- H315 Causes skin irritation.
- H319 Causes serious eye irritation.

Ethanol
- H225 Highly flammable liquid and vapor.
- H319 Causes serious eye irritation.

Equipment

UV radiation

- UV radiation can damage eyes and can be carcinogenic in contact with skin. Do not look directly at unshielded UV radiation. Do not expose unprotected skin to UV radiation.
- UV emitters generate ozone during operation. Use only in ventilated rooms.

BEFORE START INSTRUCTIONS

Previous step:
This protocol follows the extract purification protocol.

Following step:
This protocol ends with the PCR setup. Proceed with the PCR and library purification protocol.

Preparations:
Get the following consumables and equipment:

	A	B
	Numbers	Equipment and consumables
	1	0.2 ml tube rack
	2	1.5 ml tube rack
	1	1.5 ml cool block
	1	0.2 ml cool block
		10 µl filter tips
		20 µl filter tips
		100 µl filter tips
		200 µl filter tips
		1000 µl filter tips
	[# of samples]×4 (tubes)	0.2 µl PCR strips (8 tubes)
	[# of samples]×2+4	1.5 ml tubes
	1	5 ml tube
	[# of samples]×2	MinElute columns
	1	50 ml Falcon (waste)

Preparation

1 Turn the hood on full power and open the glass.

2 Spray hood and table bench surfaces with DNA Exitus, let sit a minute and wipe down with paper towels.

3 Wipe down outside surfaces of reagents/tips with DNA Exitus and place in the hood.

4 Label the following tubes:
5×1.5 ml tubes: ER, AL, FI, EB-1, EB-2
1×5 ml tube: PCR
0.2 ml PCR strips: ER, AL, FI, PCR
Label the 50 ml waste tube, PB tube and PE tube.

5 Aliquot EB buffer in tubes EB-1 and EB-2: each [# of samples]x30 µl plus 10%

6 Aliquot water for Master Mixes:
ER: [# of samples]×25.85 µl plus 10%
FI: [# of samples]×12.20 µl plus 10%
PCR: [# of samples]×17.00 µl plus 10%

7 Aliquot PB buffer to a 50 ml tube: [# of samples]x1000 µl plus 10%

8 Prepare PE (wash) buffer by adding ethanol.

9 Aliquot PE buffer to a 50 ml tube: [# of samples]x1380 µl plus 10%

10 Take DNA extracts out of the freezer to thaw at room temperature. Change gloves.

Blunt End Repair 1

11 Use 1.5 ml ER tube to set up the Blunt End Repair Master Mix On ice .

	A	B	C	D	E	F
	REAGENT	STOCK	FINAL	UNIT	1× VOL (µl)	NOTE
	Water [already added]				25.85	
	NEB Buffer 2	10	1.0	X	7.50	vortex
	dNTPs	25	0.30	mM	0.90	vortex
	BSA	20	0.20	mg/ml	0.75	vortex
	ATP	10	1.00	mM	7.50	vortex
	T4 PNK	10	0.40	U/µl	3.00	on ice
	USER enzyme	1	0.06	U/µl	4.50	on ice
	Master Mix total				50.00	
	Template DNA or water				25.00	
	REACTION TOTAL				75.00	

Calculate +10% for all Master Mix components.

12 Add 20 µl Master Mix to each tube of the ER strip.

13 Vortex and spin down DNA extracts, add 25 µl of template DNA or water to each tube.

14 Mix carefully by resuspending, remove bubbles and spin down.

15 Incubate at 37 °C for 03:00:00. 3h

Note

Use free time for preparing Master Mixes for the following steps. Master Mixes should be stored in the fridge.

Blunt End Repair 2

16 Add 3 µl T4 pol to each reaction.

	A	B	C	D	E	F
	REAGENT	STOCK	FINAL	UNIT	1× VOL (µl)	NOTE
	T4 DNA Polymerase	3	0.038	U/µl	3.00	on ice
	REACTION TOTAL				78.00	

17 Incubate at 25 °C for 00:30:00, then at 10 °C for 00:05:00 35m

Note

Use free time for preparing Master Mixes for the following steps. Master Mixes should be stored in the fridge.

MinElute Purification 1

18 Take MinElute columns out of the fridge.

19 Turn on heat block 37 °C for elution.

20 Label columns and tubes with sample ID numbers.

21 Add 500 µl PB buffer (binding buffer) to MinElute column. You can use the same tip.

22 Add end-repair reaction mix to the PB buffer inside the MinElute columns and mix by resuspending.

23 Spin at 13000 rpm, 00:01:00, discard supernatant, change gloves. 1m

24 Add 690 µl PE buffer (wash buffer), change tip for every sample.

25 Spin at 13000 rpm, 00:01:00, discard supernatant, change gloves. 1m

26 Spin at 13000 rpm, 00:01:00 (dry spin). 1m

27 Put column in new tube, change gloves.

28 Elute in 30 µl EB buffer (elution buffer), change tip for every sample.

29 Incubate at 37 °C for 00:10:00 . 10m

30 Spin at 13000 rpm, 00:02:00 . 2m

31 Discard the silica column and close the lid.

32

Note

Potential stopping point, place end-repaired DNA in freezer until ready for next step.

Adapter ligation

33 Use 1.5 ml AL tube to set up the Adapter Ligation Master Mix On ice .

	A	B	C	D	E	F
	REAGENT	STOCK	FINAL	UNIT	1× VOL (µl)	NOTE
	Quick Ligation Buffer	5.0	1.00	×	10	vortex
	Adapter Mix	2.5	0.25	µM	5	vortex

	A	B	C	D	E	F
	End Repair Enzyme Mix	1.0	0.10	U	5	on ice
	Master Mix total				20.00	
	Template DNA or water				30.00	
	REACTION TOTAL				50.00	

Calculate +20% for all Master Mix components.

34 Add 20 µl of Master Mix to each tube of the AL strip.

35 Add 30 µl of end-repaired template DNA or water to each tube.

36 Incubate at 20 °C for 00:15:00 . 15m

MinElute purification 2

37 Take MinElute columns out of the fridge.

38 Turn on heat block 37 °C for elution if not turned on already.

39 Label columns and tubes with sample ID numbers.

40 Add 500 µl PB buffer (binding buffer) to MinElute columns. You can use the same tip.

41 Add adapter ligation reaction mix to the PB buffer inside the MinElute columns and mix by resuspending.

42 Spin at 13000 rpm, 00:01:00, discard supernatant, change gloves.

43 Add 690 µl PE buffer (wash buffer), change tip for every sample.

44 Spin at 13000 rpm, 00:01:00, discard supernatant, change gloves.

45 Spin at 13000 rpm, 00:01:00 (dry spin).

46 Put column in new tube, change gloves.

47 Elute in 30 µl EB buffer (elution buffer), change tip for every sample.

48 Incubate at 37 °C for 00:10:00. 10m

49 Spin at 13000 rpm, 00:02:00.

50 Discard the silica column, close the lid.

51

Note

Potential stopping point, place adapter-ligated DNA in freezer until ready for next step.

Fill-in reaction

52 Use 1.5 ml FI tube to set up the Adapter Ligation Master Mix On ice.

	A	B	C	D	E	F
	REAGENT	STOCK	FINAL	UNIT	1× VOL (µl)	NOTE
	Water [already added]				12.2	
	Thermopol Buffer	10	1.00	×	5.0	vortex
	dNTPs	25	0.40	mM	0.8	vortex
	Bst polymerase	8	0.32	U/µl	2.0	on ice
	Master Mix total				20	
	Template DNA or water				30	
	REACTION TOTAL				50	

Calculate +20% for all Master Mix components.

53 Add 20 µl Master Mix to each tube of the FI strip.

54 Add 30 µl of adapter-ligated template DNA or water to each tube.

55 Incubate at 37 °C for 00:30:00, then at 80 °C for 00:20:00. 50m

Note

Use free time for preparing the Master Mix for the following step, Master Mixes should be stored in the fridge

Library amplification (PCR)

56 Use 5 ml PCR tube to set up PCR Master Mix On ice.

	A	B	C	D	E	F
	REAGENTS	STOCK	FINAL	UNIT	1× VOL (µl)	NOTE
	Water [already added]				17	
	10x PCR buffer	10	1.00	X	10	vortex
	MgCl2	25	2.50	mM	10	vortex
	BSA	20	1.00	mg/ml	5	vortex
	dNTPs 10 mM	40	0.80	mM	2	vortex
	HGS Taq Diamond	1	0.02	U	2	on ice
	Universal primer 1.0	10	0.2	µM	2	vortex

	A	B	C	D	E	F
	Master Mix total				48	
	Indexing primer	10	0.2	µM	2	vortex
	Template DNA or water				50	
	REACTION TOTAL				100	

Calculate +10% for all Master Mix components.

57 Aliquot 48 µl of Master Mix to the tubes of the PCR strip.

58 Vortex indexes slightly and spin them down with a table centrifuge.

59 Add 2 µl of the indexing primer (10 µM) to the respective tube with Master Mix.

60 Add 50 µl of adapter fill-in reaction mix or water to the respective tube with Master Mix. Mix by resuspending with the pipet.

61 Check that the lids are tightly sealed and take the strips to the modern lab for PCR.