

Apr 05, 2023

Library preparation (dsDNA double indexing, non-UDG, 2x split)

 In 1 collection

DOI

<https://dx.doi.org/10.17504/protocols.io.n2bvj6xqxik5/v1>

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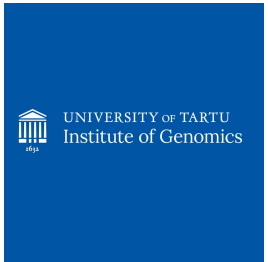
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Protocol Citation: Marcel Keller, Christiana L Scheib, Biancamaria Bonucci 2023. Library preparation (dsDNA double indexing, non-UDG, 2x split). **protocols.io** <https://dx.doi.org/10.17504/protocols.io.n2bvj6xqxik5/v1>

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Protocol status: Working

We use this protocol and it's working

Created: January 24, 2022

Last Modified: April 05, 2023

Protocol Integer ID: 57339

Keywords: ancient DNA, aDNA, archeogenetics, archaeogenetics, paleogenetics, palaeogenetics, library preparation, dsdna double indexing, dna libraries for illumina, stranded dna library, dsdna, dna, dna damage, treatment with udg, dna damage in form, double indexing, udg, protocol for the preparation, deaminated cytosine, library preparation

Abstract

Protocol for the preparation of double 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](https://doi.org/10.1101/pdb.prot5448)). This protocol does not 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	Con c.	Unit	Manufacturer	Kit/full description	Product number
	End repair	Water			Fisher chemicals	Water for HPLC 1L	10367171
	End repair	End Repair Reaction Buffer	10	X	New England Biolabs	NEBNext End Repair Module	E6050L
	End repair	End Repair Enzyme Mix	1	X	New England Biolabs	NEBNext End Repair Module	E6050L
	Adapter Ligation	Quick Ligation Buffer	5.0	X	New England Biolabs	NEBNext Quick Ligation Module	E6056L
	Adapter Ligation	Adapter Mix (2.5 μ M)	2.5	μ M		*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/ μ l	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	MgCl ₂	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	Dual Index Primers Set 1	10	μ M	New England Biolabs	NEBNext® Multiplex Oligos for Illumina® (Dual Index Primers Set 1)	E7600S



	A	B	C	D	E	F	G
	PCR	Dual Index Primers Set 2	10	μM	New England Biolabs	NEBNext® Multiplex Oligos for Illumina® (Dual Index Primers Set 2)	E7780S
	Purification	PB Buffer			Qiagen	MinElute PCR Purification Kit	19066
	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]×5 (tubes)	0.2 μl PCR strips (8 tubes)
[# of samples]×2+5	1.5 ml tubes
1	5 ml tube
[# of samples]×2	MinElute columns
3	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

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]×5 (tubes)	0.2 µl PCR strips (8 tubes)
	[# of samples]×2+5	1.5 ml tubes
	1	5 ml tube
	[# of samples]×2	MinElute columns
	1	50 ml tube



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-A, PCR-B
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]×30 µl plus 10%
- 6 Aliquot water for Master Mixes:
ER: [# of samples]×12.50 µl plus 10%
FI: [# of samples]×12.20 µl plus 10%
PCR-A: [# of samples]×84.00 µl plus 10%
- 7 Aliquot PB buffer to a 50 ml tube: [# of samples]×1000 µl plus 10%
- 8 Prepare PE (wash) buffer by adding ethanol. 
- 9 Aliquot PE buffer to a 50 ml tube: [# of samples]×1380 µl plus 10%
- 10 Take DNA extracts out of the freezer to thaw at room temperature. Change gloves. 

Blunt End Repair

- 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]				12.50	
	End Repair Reaction Buffer	10	1.000	×	5.00	vortex
	End Repair Enzyme Mix	1	0.050	×	2.50	on ice
	Master Mix total				20.00	
	Template DNA or water				30.00	
	REACTION TOTAL				50.00	

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 30 μl of template DNA or water to each tube. 
- 14 Mix carefully by resuspending, remove bubbles and spin down. 
- 15 Incubate at  20 °C for  00:30:00 . 

Note

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

MinElute Purification 1

- 16 Take MinElute columns out of the fridge.



17 Turn on heat block  37 °C for elution.

18 Label columns and tubes with sample ID numbers.

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

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

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

1m



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

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

1m



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

1m



25 Put column in new tube, change gloves.

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

27 Incubate at  37 °C for  00:10:00 .

10m



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

2m



29 Discard the silica column and close the lid.

30

Note

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

Adapter ligation

31 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
	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.

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

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

34 Incubate at  20 °C for  00:15:00 .

15m



MinElute purification 2

- 35 Take MinElute columns out of the fridge.
- 36 Turn on heat block  37 °C for elution if not turned on already.
- 37 Label columns and tubes with sample ID numbers.
- 38 Add 500 µl PB buffer (binding buffer) to MinElute columns. You can use the same tip. 
- 39 Add adapter ligation reaction mix to the PB buffer inside the MinElute columns and mix by resuspending. 
- 40 Spin at  13000 rpm, 00:01:00 , discard supernatant, change gloves. 
- 41 Add 690 µl PE buffer (wash buffer), change tip for every sample. 
- 42 Spin at  13000 rpm, 00:01:00 , discard supernatant, change gloves. 
- 43 Spin at  13000 rpm, 00:01:00 (dry spin). 
- 44 Put column in new tube, change gloves.
- 45 Elute in 30 µl EB buffer (elution buffer), change tip for every sample. 
- 46 Incubate at  37 °C for  00:10:00 . 



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



48 Discard the silica column, close the lid.

49



Note

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

Fill-in reaction

50 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.

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



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



53 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)

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



Note

The PCR reaction will be set up with 2x volume and split into two tubes after adding the fill-in reaction mix. Add indices after aliquoting the Master Mix to the PCR-A strip tubes.

	A	B	C	D	E	F
	REAGENTS	STOCK	FINAL	UNIT	1× VOL (µl)	NOTE
	Water [already added]				84	
	10x PCR buffer	10	1.00	X	20	vortex
	MgCl ₂	25	2.50	mM	20	vortex
	BSA	20	1.00	mg/ml	10	vortex
	dNTPs 10 mM	40	0.80	mM	4	vortex

	A	B	C	D	E	F
	HGS Taq Diamond	1	0.02	U	4	on ice
	Master Mix total				142	
	i7 Index	10	0.2	μM	4	vortex
	i5 Index	10	0.2	μM	4	vortex
	Template DNA or water				50	
	REACTION TOTAL				100	

Calculate +10% for all Master Mix components.

- 55 Aliquot 142 μl of Master Mix to the tubes of the PCR-A strip. 
- 56 Vortex indexes slightly and spin them down with a table centrifuge. 
- 57 Add 4 μl of i7 indexing primer (10 μM) to the respective tube with Master Mix. 
- 58 Add 4 μl of i5 indexing primer (10 μM) to the respective tube with Master Mix. 
- 59 Add 50 μl of adapter fill-in reaction mix or water to the respective tube with Master Mix. 
- 60 Mix by resuspending and pipet 100 μl of the reaction to the PCR-B strip tubes. 
- 61 Check that the lids are tightly sealed and take the strips to the modern lab for PCR. 