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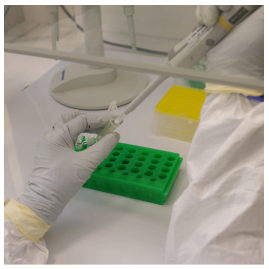
Non-UDG treated double-stranded ancient DNA library preparation for Illumina sequencing



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We use this protocol and it's working

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Abstract

Protocol for the preparation of double-stranded genomic libraries for Illumina sequencing, optimised for ancient DNA (aDNA). This protocol generates adapter ligated DNA fragments that can be used in conjunction with downstream Indexing protocols.

This protocol does **not** include UDG (USER) treatment, in order to retain molecular 'damage' in the form of deaminated cytosines characteristic of aDNA. This protocol is modified after Meyer & Kircher (2010) Cold Spring Harb. Protoc. (doi: **10.1101/pdb.prot5448**).

Image Attribution

Matthäus Rest

Guidelines

Working in an Ancient DNA Laboratory

- All steps of the protocol (except the qPCR at the end) should take place in a clean room facility specifically designed for ancient DNA.
- The researcher performing lab work should be dressed in correspondingly suitable lab-wear, such as:
  - full-body suit with hood (e.g., Tyvek)
  - hairnet
  - face mask
  - two pairs of clean gloves
  - clean shoes
  - protective glasses
- Sample processing should be carried out in separated work benches with integrated UV irradiation (e.g. Dead Air PCR work bench)
- Surfaces and equipment should be regularly decontaminated with e.g. bleach solution or Thermofisher's DNA AWAY (or similar) and irradiated with UV.
- All home-made buffers should be prepared in a seperate decidated PCR-free ultra-clean room and UV-irradiated for 30 min.

Please see the following for more detailed guidance:

Llamas, B. et al., 2017. From the field to the laboratory: Controlling DNA contamination in human ancient DNA research in the high-throughput sequencing era. *STAR: Science & Technology of Archaeological Research*, 3(1), pp.1–14. Available at: https://doi.org/10.1080/20548923.2016.1258824.

Working in an Molecular Biology Laboratory

The qPCR reaction takes place in a standard DNA-based molecular biology lab.
Please keep in mind the safety guidelines of your specific country and institution.
Recommendations include wearing of:

- lab coats
- closed shoes and trousers
- safety glasses
- nitrile or latex gloves

Protocol Specific Guidelines

This protocol requires the use of two rooms - a dedicated PCR-free ultra-clean library building room and a standard molecular biology lab for qPCR.

Materials

MATERIALS

- 1.5 mL Biopur Safe-Lock Tubes **Eppendorf** **Catalog #**0030121589
- Adhesive clear qPCR sheets **Biozym** **Catalog #**600238
- DNA LoBind Tubes 1.5 mL **Eppendorf** **Catalog #**0030108051
- FrameStar® 96 Well Semi-Skirted PCR Plate Roche Style **Catalog #**4ti-0951
- 0.2 mL PCR Tube strips **Eppendorf** **Catalog #**0030124359
- Adenosine-5 Triphosphate (ATP) 1 mL **New England Biolabs** **Catalog #**P0756S
- BSA molecular biology grade 20 mg/ml **New England Biolabs** **Catalog #**B9000S
- Bst 2.0 DNA Polymerase - 1600 units **New England Biolabs** **Catalog #**M0537S
- dNTP Mix - 25 mM each **Thermo Scientific** **Catalog #**R1121
- Ethanol **Merck Millipore (EMD Millipore)** **Catalog #**100983
- NEB Buffer 2 **New England Biolabs** **Catalog #**B7002S
- T4 DNA polymerase - 750 units **New England Biolabs** **Catalog #**M0203L
- T4 Polynucelotide Kinase - 2500 units **New England Biolabs** **Catalog #**M0201L
- TWEEN® 20 **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**P9416-50ML
- Water Chromasolv Plus for HPLC 2.5L **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**34877-2.5L
- DyNAmo Flash sYBR Green qPCR Kit **Thermo Fisher Scientific** **Catalog #**F415L
- MinElute PCR Purification Kit **Qiagen** **Catalog #**28004
- Quick Ligation Kit - 150 reactions **New England Biolabs** **Catalog #**M2200L

Additional Reagents

Adapter-Mix (10 µM), for preparation see the following protocol: **Library Adapter Preparation for Dual-Index Double Stranded DNA Illumina Sequencing**

Primers

	Primer ID	Sequence (5'-3')	Concentration
	IS7	ACACTCTTTCCCTACACGACGC	10 µM
	IS8	GTGACTGGAGTTCAGACGTGTGC	10 µM

qPCR-Standard

- Custom-ordered standard based on a synthetic 224 bp oligo including primer binding sites for the primer pairs IS7/8 (and IS5/IS6 - this standard is also used for the indexing protocol Illumina double-stranded DNA dual-indexing for ancient DNA) ranging from 10^9 to 10^3 DNA copies/µl in serial 1:10 dilutions.

Lab equipment

PCR Thermocycler (e.g. Eppendorf Thermomaster Nexus)
Thermomixer or heatblock (to pre-heat buffers)
Centrifuge 1.5/2.0 ml (e.g. Eppendorf 5424)
Rotor 1.5/2.0ml (e.g. Eppendorf F-45-24-11)
Mini table centrifuge
qPCR machine (e.g. Roche LightCycler® 96 System)
PCR work bench (e.g. AirClean Dead Air PCR Werkbank, 48´´)
UV irradiation box or cross linker (e.g. Vilber Lourmat Bio-Link BLX-254)
Vortex mixer (e.g. Scientific Industries Vortex-Genie® 2)

Generic Reagents

Solution of household bleach (2-6% NaClO, then diluted to a working solution concentration of 0.2-0.5% NaClO)
Thermofisher DNA AWAY
Paper towels or tissues

Troubleshooting

Safety warnings

Reagents

Household bleach solution (0.2-0.5 % NaClO in total)
- 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 AWAY
- H314 Causes severe skin burns and eye damage.

Note: Both bleach solutions and DNA AWAY are used for decontamintation. DNA AWAY is less corrosive than bleach and should be preferred for decontamination of sensitive equipments such as surfaces of electric devices.

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 vapour.
- H319 Causes serious eye irritation.

Kits
Check manufacturer's safety information for the High Pure Viral Nucleic Acid Large Volume Kit used in this protocol.

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

Planning
This protocol takes approximately 6 hours.

While all steps of the protocol are performed in an Ancient DNA facility, the qPCR will be performed in a modern DNA facility with a 1:10 dilution of the samples, which were prepared in the cleanroom facilities. The library remains in the cleanroom for further processing.

Check waste disposal guidance for all reagents in this protocol against your corresponding laboratory regulations.

Preparation of reagents
All home-made buffers should be prepared in a separate dedicated PCR-free ultra-clean room and UV-irradiated for 30 min. Purchased kits should be DNA-free.

Qiagen MinElute kit
- Add ethanol to PE wash buffer acccording to manufacturer's instructions.
- Add Tween-20 to EB elution buffer to a final concentration of 0.05% (Tween-20 in EB). This solution is referred to as **EBT** throughout the protocol.
- All reagents of MinElute PCR Purification Kit should be decontaminated with a **30 min UV irradiation** before use.

HPLC-Water should be decontaminated with a **30 min UV irradiation** before use.

Equipment
Make sure all necessary equipment is available (see Materials).

Abbreviations
EBT = EB elution buffer with 0.05% Tween-20
GuHCl = Guanidinium chloride or Guanidine hydrochloride
HPLC = High Performance Liquid Chromatography (-Grade Water)
NaClO = Sodium hypochlorite
UV = Ultraviolet (radiation)

Controls
Take along extraction positive and negative (blank) controls generated during your extraction (see example Extraction Protocol dx.doi.org/10.17504/protocols.io.baksicwe)

For this protocol you should also add another negative control (HPLC water) to monitor the library preparation experiment. Consider these three extra samples in your calculations for mastermixes and buffer preparations.

Additional Tips

It is recommended to prepare 10% more of the calculated volume of all mastermixes to compensate for possible pipetting error.

Blunt End Repair (aDNA library preparation room)

1 Prepare a mastermix for the blunt end repair calculating 50 µL / reaction . Use a new 1.5 ml LoBind tube to set up the mastermix.

	Reagent	Stock concentration	Final concentration	1x Volume [µl]
	NEB Buffer 2	10 x	1 x	5
	ATP	10 mM	1 mM	5
	BSA	20 mg/ml	0.8 mg/ml	2
	dNTPs	25 mM each	0.1 mM	0.2
	T4 PNK	10 U	0.4 U	2
	T4 Polymerase	3 U	0.024 U	0.4
	UV HPLC-water			25.4
	DNA or UV HPLC-water			10
	Total			**50**

Do not add the DNA to the mastermix if you set it up for 2 or more reactions!

1.1 Add 40 µL mastermix to each reaction tube and then add 10 µL sample DNA to each tube (use 0.2 ml PCR tube strips). Mix by flicking the tubes and spin down briefly.

2 Incubate at 25 °C for 00:20:00 , then at 12 °C for 00:10:00 in the thermocycler.

Note

During this incubation take MinElute tubes out of the fridge so that they warm to room temperature before use in the next step. Also label two sets of 1.5 ml LoBind tubes per sample and one set of 0.2 ml PCR tubes for the next steps. At this time you can also add the PB-Buffer (650µl) to one set of 1.5 ml LoBind tubes for Step 3.2.

MinElute Purification

3 Purify the blunt-end repaired DNA with a MinElute kit, with the following modifications to the manufacturer's protocol.

3.1 Pre-heat elution buffer EBT to 50 °C .

3.2 For each reaction, add 650 µL PB (binding) buffer to a new 1.5 ml LoBind tube and add the blunt end repaired sample, then vortex briefly to mix.

3.3 Load each reaction (PB buffer + blunt-end repaired sample) onto a MinElute column and incubate at RT for 00:02:00 .

Note

This allows sufficient time for the DNA to bind to the silica membrane.

3.4 Spin 15800 x g, 00:01:00 and discard flow-through.

Note

Discard flow-through in one of two following ways:
- Remove all liquid in the collection tube with a pipette, or
- Pour off the liquid into a fresh waste tube, and pat the rim of the collection tube dry on a paper tissue or towel. Use just one spot on the paper tissue per sample. Be careful not to touch the rim of the tube on the waste container. After you are finished with all samples, discard the paper and wipe clean the surface underneath with water and then sterilize the surface with bleach (or DNA Away).

Safety information

During cleaning of surfaces after flow-through discard, do NOT apply bleach before first cleaning with water. Residual GuHCl will react with bleach to form toxic gases.

3.5 Add 700 µL PE (wash) buffer to the MinElute column.

3.6 Spin at 15800 x g, 00:01:00 and discard flow-through *as in step 3.4*.

3.7 Dry spin for 15800 x g, 00:01:00 .

3.8 Flip columns 180° and dry spin again for 15800 x g, 00:01:00 .

3.9 Remove columns from their collection tubes and place them in new 1.5 ml LoBind tubes.

3.10 Add 20 µL pre-heated EBT to the column, let stand for 00:01:00 then spin 15800 x g, 00:01:00 to elute.

Note

Carefully pipette EBT directly onto the center of the membrane without touching the membrane.

Adapter Ligation

4 Prepare a mastermix for adapter ligation calculating 40 µL / reaction . Use a 1.5 ml LoBind tube to set up the ligation mastermix.

Note

The mastermix is calculated to accommodate addition of Quick Ligase to each reaction individually after aliquotting to individual reaction tubes.

	Reagent	Stock concentration	Final concentration	1 X Volume [µl]
	Quick Ligase Buffer	2 x	1 x	20
	Adapter Mix	10 µM	0.25 µM	1
	Eluate from Step 3.10			18
	Total			**40**

Do not add the eluate to the mastermix if you set it up for 2 or more reactions!

4.1 Aliquot 21 µL of mastermix to each tube (use 0.2 ml PCR strips). Transfer the complete eluate (step 3.10) (~ 18 µL) per sample to each new tube.

5 Then add 1 µL of Quick ligase ([M] 5 U stock, [M] 0.125 U final concentration) to each library sample. Mix by flicking the tubes and spin down.

Note

Make sure to add the full volume of ligase by pipetting up and down several times to get all of it off the walls of the pipette tip.

6 Incubate at 22 °C for 00:20:00 in the thermocycler.

Note

During this incubation take MinElute tubes out of the fridge so that they warm to room temperature before use in the next step. Also label two sets of 1.5 ml LoBind tubes and 0.2 ml PCR tubes per sample for the next steps. At this time you can also add the PB-Buffer (650µl) to one set of 1.5 ml LoBind tubes for Step 7.2.

MinElute Purification

7 Purify the adapter-ligated libraries with a MinElute kit, with the following modifications to the manufacturer's instructions

7.1 Pre-heat EBT to 50 °C .

7.2 For each sample, add 650 µL PB buffer to a new 1.5 ml LoBind tube and add the entire volume of the adapter ligation mix, then vortex briefly.

7.3 Load each reaction (PB buffer + adapter-ligated library) onto a MinElute column and incubate for 00:02:00 .

Note

This allows the DNA sufficient time to bind to the silica membrane

7.4 Spin at 15800 x g, 00:01:00 and discard flow-through *as in step 3.4*.

7.5 Add 700 µL PE buffer to the MinElute column.

7.6 Spin at 15800 x g, 00:01:00 and discard flow-through *as in step 3.4*.

7.7 Dry spin at 15800 x g, 00:01:00 .

7.8 Flip columns by 180° and dry spin again at 15800 x g, 00:01:00 .

7.9 Remove columns from their collection tubes and place them in new 1.5 ml LoBind tubes.

7.10 Add 22 µL of pre-heated EBT to column, let stand for 00:01:00 , then spin 15800 x g, 00:01:00 to elute.

Note

Carefully pipette EBT directly onto the center of the membrane without touching it.

Adapter Fill-in

8 Prepare adapter fill-in reaction calculating 40 µL / reaction . Use a 1.5 ml LoBind tube to set up the adapter fill-in mastermix.

	Reagent	Stock concentration	Final concentration	1x Volume [µl]
	Isothermal Buffer	10 x	1 x	4
	dNTPs	25 mM each	0.125 mM each	0.2
	Bst Polymerase	8 U	0.4 U	2
	UV HPLC-water			13.8
	Eluate from step 7.10			20
	Assay total			**40**

Do not add the eluate to the mastermix if you set it up for 2 or more reactions!

8.1 Add 20 µL of mastermix and the complete eluate (step 7.10) (~ 20 µL) to each tube (use 0.2 ml PCR strips). Mix by flicking the tubes and spin down briefly.

9 Incubate at 37 °C for 00:30:00 then 80 °C for 00:10:00 in the thermocycler.

Note

During this incubation label new 0.2 ml tubes for step 10 and new 1.5 ml LoBind tubes for step 11.

10 Aliquot 18 µL water to the 0.2 ml PCR tubes. Aliquot 2 µL per library to 0.2 ml PCR tubes with 18 uL water (making a 1:10 dilution of the library) for the qPCR quality check (steps 13-14).

11 Transfer the remaining ~ 36 µL of final library to a fresh 1.5 ml LoBind tube.

12 Freeze the library at -20 °C without purification until further processing.

qPCR_quality check (modern DNA facility)

13 Prepare a qPCR assay calculating 20 µL / reaction. Prepare 2 reactions per sample, plus 16 additional reactions for 7 qPCR standards in duplicates and 2 qPCR blanks. This qPCR uses a 1:10 dilution of the samples.

Note

The 1:10 dilutions of the samples are prepared in the cleanroom, but the qPCR is performed in the modern lab.

	Reagent	Stock concentration	Final concentration	1x Volume [µl]

	DyNAmo MasterMix	2 x	1 x	10
	IS7 primer	10 µM	1 µM	1
	IS8 primer	10 µM	1 µM	1
	HPLC-Water (non UVed)			7
	DNA or HPLC-Water (1:10 dilution)			1
	Total			**20**

Do not add the DNA dilutions to the mastermix.

Note

Important Do NOT vortex the DyNAmo MasterMix, it will create bubbles that are nearly impossible to remove. The bubbles will interfere with the qPCR measurements. Gently pipette the DyNAmo MasterMix to equally distribute the fluorescent dye.

Gently pipette the qPCR master mix, or gently invert the tube several times to mix. Again avoid creating any bubbles.

13.1 Add 19 µL mastermix and 1 µL 1:10 diluted libraries, standard, or water to a fresh 96-well qPCR plate. Seal with adhesive clear qPCR seal sheets and briefly spin down.

Note

Be sure to check that the reactions do not contain bubbles, because this will affect the qPCR readings and subsequent downstream calculations. If bubbles are present, briefly centrifuge the plate at maximum speed to remove them.

14 Amplify the qPCR reactions with the following program:

	Temperature	**Time**	
	95°C	10 min	Inital denaturation
	95°C	30 sec	40 cycl

	60°C	1 min	es
	72°C	30 sec	
	60-95˚C		Melting curve
	Finally hold the reactions at 37°C.		

Note

The number of DNA copies determined with this qPCR is used to determine the amount of sample used as input for indexing protocols.