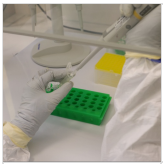

Dec 10, 2020

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

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

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1 Works for me dx.doi.org/10.17504/protocols.io.bqcsmswe

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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).

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FORK NOTE

FORK FROM

Forked from Non-UDG treated double-stranded ancient DNA library preparation for Illumina sequencing , Gunnar Neumann

KEYWORDS

ancient DNA, sequencing, nonUDG, double-stranded, DNA, genomic DNA, genomics, palaeogenetics, archaeogenetics, paleogenetics, archeogenetics, aDNA, Illumina, library preparation, nucleic acids

IMAGE ATTRIBUTION

Matthäus Rest

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45170

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 TEXT

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 **Contributed by users Catalog #4ti-0951**

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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 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 **Sigma Aldrich Catalog #P9416-50ML**

Water Chromasolv Plus for HPLC 2.5L **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)

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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

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

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- 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.

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).

BEFORE STARTING

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.

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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.

A	B	C	D
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			15.4
DNA or UV HPLC-water			20
Total			**50**

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

You can use up to **20 µl** extract, but with a maximum Input of **100 ng** DNA in total.

1.1 Add **30 µl** mastermix to each reaction tube and then add **20 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.

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.

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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** .

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.

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).

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

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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.

> 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.

> 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.

> 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.

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

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** .

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** .

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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.

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.

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).

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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.

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.

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.

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.

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14 Amplify the qPCR reactions with the following program:

Temperature	Time	
95°C	10 min	Inital denaturation
95°C	30 sec	40 cycles
60°C	1 min	
72°C	30 sec	
60-95°C		Melting curve
Finally hold the reactions at 37°C.		

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

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