

Dec 10, 2020

# 🌐 (Non-UDG treated) double-stranded modern dental calculus DNA library preparation for Illumina sequencing

Forked from [Non-UDG treated double-stranded DNA library preparation for Illumina sequencing of ancient dental calculus](#)

Franziska Aron<sup>1</sup>, Irina Velsko<sup>2</sup>, Gunnar U Neumann<sup>1</sup>, Christina Warinner<sup>2</sup>, Guido Brandt<sup>1</sup>

<sup>1</sup>Department of Archaeogenetics, Max Planck Institute for the Science of Human History;

<sup>2</sup>Max Planck Institute for the Science of Human History

**1** Works for me [dx.doi.org/10.17504/protocols.io.bqd7ms9n](https://dx.doi.org/10.17504/protocols.io.bqd7ms9n)

WarinnerGroup



James Fellows Yates  
Max Planck Institute for the Science of Human History

## ABSTRACT

Protocol for the preparation of double-stranded genomic libraries for Illumina sequencing. This produces adapter ligated DNA fragments that can be used in conjunction with downstream Indexing protocols.

## DOI

[dx.doi.org/10.17504/protocols.io.bqd7ms9n](https://dx.doi.org/10.17504/protocols.io.bqd7ms9n)

## PROTOCOL CITATION

Franziska Aron, Irina Velsko, Gunnar U Neumann, Christina Warinner, Guido Brandt 2020. (Non-UDG treated) double-stranded modern dental calculus DNA library preparation for Illumina sequencing.

**protocols.io**

<https://dx.doi.org/10.17504/protocols.io.bqd7ms9n>



## FORK NOTE

## FORK FROM

Forked from [Non-UDG treated double-stranded DNA library preparation for Illumina sequencing of ancient dental calculus](#), James Fellows Yates

## KEYWORDS

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

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## IMAGE ATTRIBUTION

Matthäus Rest

## CREATED

Dec 04, 2020

## LAST MODIFIED

Dec 10, 2020

## 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 separate dedicated 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

 microTUBE-50 AFA Fiber Screw-

Cap Covaris Catalog #520166

 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

[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 Polynucleotide 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	ACACTCTTCCCTACACGACGC	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<sup>9</sup> to 10<sup>3</sup> 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)  
Ultrasonicator (e.g. Covaris M220 Focused-ultrasonicator)

### 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 decontamination. 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.



#### ABSTRACT

Protocol for the preparation of double-stranded genomic libraries for Illumina sequencing. This produces adapter ligated DNA fragments that can be used in conjunction with downstream Indexing protocols.

#### 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 according 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](https://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.

## DNA shearing

- 1 Prepare for mechanical shearing of extracted DNA. This protocol describes shearing to 150bp for Illumina sequencing using an ultrasonicator.

If the library is to be sequenced with a 75bp kit, shear it to 150bp. If it will be sequenced with a 150bp kit, shear it to 300bp, or longer if desired.

- 2 Place up to **250 ng** of extracted DNA in **50 µl** ultrapure water into a microTUBE-50 AFA Fiber Screw-Cap tube.

**250 ng** is the recommended amount of DNA to be added for this protocol. More or less DNA can be used, but it will not necessarily be the desired length of 150bp. If more is used, the length will be longer, and if less is used, the length will be shorter.

Any remaining sample that will not be sonicated should be stored at **-20 °C**.



Make sure there are no air bubbles in the tube, as this will interfere with the sonication and inhibit shearing.

- 3 Place the Screw-Cap tube into the sonicator, and select the appropriate sonication protocol based on the tube you are using and the desired length of the DNA fragments.

To shear to 150bp with a Covaris M220 focused ultrasonicator using a 50 µL Screw Cap microTUBE, select the protocol **DNA\_0150\_bp\_050\_ul\_Screw\_Cap\_microTUBE** in the sonication software. Run the program until it completes.



Be sure to label the tubes on both the tube side and cap, as the sonication can partially rub off labels.

- 4 Remove the sonicated sample from the Screw Cap tube. Unscrew the cap, and use a 10µL pipette to suck up any droplets on the top of the cap. Then go around the rim of the tube and suck up any droplets on the rim. Then remove the liquid from the tube in 10µL intervals and place in a 1.5 ml LoBind tube. Be sure to move the fiber in the tube with the pipette tip to get all liquid. Discard the Screw Cap tube once all liquid is removed.
- 5 At this point, the sonicated DNA can be either immediately taken into the library preparation protocol (step 6), or stored at **-20 °C** until ready for use.

## Blunt End Repair (aDNA library preparation room)

- 6 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	0.25 mM	0.5
T4 PNK	10 U	0.4 U	2
T4 Polymerase	3 U	0.024 U	0.4
UV HPLC-water			15.1
DNA or UV HPLC-water			20
<b>Total</b>			<b>50</b>

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

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

- 6.1 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.

- 7 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

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

- 8.1 Pre-heat elution buffer EBT to **50 °C**.

## 8.2

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.

## 8.3 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.

## 8.4 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.

## 8.5

Add  **700 µl** PE (wash) buffer to the MinElute column.

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




## 8.7 Dry spin for **15800 x g, 00:01:00** .

## 8.8

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


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



- 8.10 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

- 9 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
<b>Total</b>			<b>40</b>

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

- 9.1 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.

#### 10

Then add  **1 µl** of Quick ligase (  **5 U** stock,  **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.

- 11 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

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

12.1 Pre-heat EBT to  **50 °C** .

12.2 

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.

12.3 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

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


12.5 

Add  **700 µl** PE buffer to the MinElute column.




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

12.7 Dry spin at  **15800 x g, 00:01:00** .

12.8 


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

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

12.10 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

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



A	B	C	D
Reagent	Stock concentration	Final concentration	1x Volume [µl]
Isothermal Buffer	10 x	1 x	4
dNTPs	25 mM each	0.25 mM each	0.4
Bst Polymerase	8 U	0.4 U	2
UV HPLC-water			13.6
Eluate from step 7.10			20
<b>Assay total</b>			<b>40</b>

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

13.1 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.

14 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.

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

16 Transfer the remaining ~  **36 µl** of final library to a fresh 1.5 ml LoBind tube.

17 Freeze the library at  $-20^{\circ}\text{C}$  without purification until further processing.

qPCR\_quality check (modern DNA facility)

18 Prepare a qPCR assay calculating  $20\ \mu\text{l}$  / 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 [ $\mu\text{l}$ ]
DyNAmo MasterMix	2 x	1 x	10
IS7 primer	10 $\mu\text{M}$	1 $\mu\text{M}$	1
IS8 primer	10 $\mu\text{M}$	1 $\mu\text{M}$	1
HPLC-Water (non UVed)			7
DNA or HPLC-Water (1:10 dilution)			1
<b>Total</b>			<b>20</b>

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.

18.1 Add  $19\ \mu\text{l}$  mastermix and  $1\ \mu\text{l}$  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.

19 Amplify the qPCR reactions with the following program:

Temperature	Time	
95°C	10 min	Initial 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.