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Illumina double-stranded DNA dual indexing for ancient DNA V.3

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ABSTRACT

This protocol converts partially completed double-stranded DNA libraries e.g. from:

Non-UDG treated double-stranded ancient DNA library preparation for Illumina sequencing (dx.doi.org/10.17504/protocols.io.bakricv6)

into dual-indexed Illumina libraries ready for sequencing on an Illumina platform. Other variants of the above protocol can also be used.

This protocol includes steps for indexing PCR, clean-up, and post-indexing qPCR. This protocol is modified after Kircher, M., Sawyer, S. & Meyer, M., 2012. Double indexing overcomes inaccuracies in multiplex sequencing on the Illumina platform. Nucleic acids research, 40(1), p.e3. http://dx.doi.org/10.1093/nar/gkr771.

IMAGE ATTRIBUTION

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We use this protocol and it's working

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GUIDELINES

Working in an Ancient DNA Laboratory

- All steps of this protocol **prior to PCR amplification** 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 and buffer/master mix preparation 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.

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

- From PCR amplification onwards, all steps 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

Definitions

Stock-aliquot refers to a personal 'stock' (e.g. in a 50ml Falcon Tube) of reagents you can use across multiple sessions of this protocol. An 'aliquot' refers to a sub-aliquot of the stock, that is used for a single session of this specific protocol.

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

- 0.2 ml PCR strips **Eppendorf Catalog #0030124839**
- Safe-Lock Tubes 1.5 ml PCR clean DNA LoBind **Eppendorf Catalog #0030108051**
- FrameStar® 480 PCR plate 96 semi-skirted **4titude Catalog #4ti-0951**
- Adhesive clear qPCR seal sheets **Biozym Catalog #600238**
- Ethanol Absolute **Merck Millipore Catalog #1009831011**
- Water Chromasolv Plus **Sigma Aldrich Catalog #34877-2.5L**
- Tween-20 **Sigma Aldrich Catalog #P9416-50ML**
- dNTPs 25mM **Thermo Scientific Catalog #R1121**
- Adenosine-5 Triphosphate (ATP) - 1 ml **New England Biolabs Catalog #P0756S**
- BSA Molecular Biology Grade (20 mg/ml) **New England Biolabs Catalog #B9000 S**
- PfuTurbo Cx Hotstart DNA Polymerase **Agilent Technologies Catalog #600412**
- DyNAmo Flash SYBR Green qPCR Kit **Thermofisher Catalog #F415L**
- MinElute PCR Purification Kit **Qiagen Catalog #28006**
- Eppendorf Tubes 5 ml PCR clean **Eppendorf Catalog #0030119460**

Primers

	Oligo_ID	Sequence (5'-3')	Coce ntrati on
	P5_Jen_8nt (µM)	AATGATACGGCGACCACCGAGATCTACAC???????? ACACTCTTTCCCTACACGACGC	10 µM
	P7_Jen_8nt (µM)	CAAGCAGAAGACGGCATACGAGAT???????? GTGACTGGAGTTCAGACGTGTGC	10 µM
	IS5	AATGATACGGCGACCACCGA	10 µM
	IS6	CAAGCAGAAGACGGCATACGA	10 µM

???????? indicates a 8 bp long unique index sequence. Primers can be ordered from any company.

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

SAFETY WARNINGS

Reagents

Household bleach solution (2-6%) diluted to a working concentration of 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 Qiagen MinElute PCR Purification 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 INSTRUCTIONS

Planning
This protocol takes 1 day.

During the protocol samples move from the ancient DNA laboratory to a modern molecular biology lab:
Indexing reactions are prepared in the ancient DNA lab, and then closed tubes are transferred to a standard modern molecular biology lab. All PCR amplification must take place in the modern molecular biology lab and NOT in the ancient DNA lab.

Safety information

Do not amplify the index reaction in a cleanroom! This will produce small PCR fragments that are easily aerosolized, and can contaminate all other libraries produced in that room.

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

Preparation of buffers (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.

Equipment

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

Abbreviations

EBT = modified EB-Buffer (MinElute Kit), see Preparation of buffers
HPLC = High Performance Liquid Chromatography (-Grade Water)
PE = PE-Buffer from Qiagen MinElute Kit
PB = PB-Buffer from Qiagen MinElute Kit
UV = Ultraviolet (radiation)

Samples

This protocol uses around 36 µL of the remaining eluate after the final qPCR from library preprartion (e.g. dx.doi.org/10.17504/protocols.io.bakricv6), that is stored at -20 °C in the ancient DNA lab.

Controls

Take along a positive control and 2 negative controls (e.g. all three from the extraction blank control from Ancient DNA Extraction from Skeletal Material - dx.doi.org/10.17504/protocols.io.baksicwe) and the library build control (e.g. from non-UDG treated double-stranded ancient DNA library preparation for Illumina sequencing dx.doi.org/10.17504/protocols.io.bakricv6) to assess the performance of the protocol and the level of background contamination. Consider these three extra samples in your calculations for buffer preparations. To take along the positive controls is optional after you know that the library preparation worked out.

Additional Tips

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

Indexing preparation (aDNA library preparation room)

1 Calculate the total number of DNA molecules (total copy number) DNA concentration in each library based on qPCR performed at the end of library preparation (see Before Start). Do not use more than 1.5x10^10 copies per indexing reaction. Adjust the amount of DNA used per reaction based on the initial library quantification. Depending on the total amount of DNA, split the indexing PCR into 2, 4, or 6 reactions per library.

Note

The number of indexing reactions per library depends on the DNA concentration of the library, which comes from the qPCR run at the end of library preparation (see Before Start).

Note

If the total copy number is high enough that six splits are not sufficient to bring the number of DNA copies per reaction to 1.5x10^10, then calculate the amount of splits nessesary to stay below this threshold. Divide the total volume of the library (36 µL) by the number of splits to know the maximum input volume of library per index reaction. Set up a maximum of six indexing reactions. Keep the remaining DNA-Library as a backup at -20 °C .

Store all remaining libraries eluates in the freezer at -20 °C for short-term storage (1-2 months) or the -80 °C freezer for long-term storage.

2 Assign unique dual index combinations of index primer pairs to each library.

Splits: 2 (aDNA library preparation room)

3 Calculate the amount of master mix needed for the number of samples being processed. Prepare the master mix according to the table below within a 1.5 mL LoBind tube.

Add 78 µL mastermix, 2 µL of each index primer and 18 µL sample to each tube (use 0.2 mL PCR strips).

	A	B	C	D

	A	B	C	D
	Reagent	Stock Concentration	Final concentration	1× Volume [µl]
	Pfu Turbo Cx Buffer	10 ×	1 ×	10.0
	BSA	20 mg/mL	0.3 mg/mL	1.5
	dNTPs	25 mM each	0.25 mM each	1.0
	Pfu Turbo Cx Polymerase	2.5 U	0.025 U	1.0
	Index P5_Jen_8nt	10 µM	0.2	2.0
	Index P7_Jen_8nt	10 µM	0.2 µM	2.0
	UV HPLC-water	-	-	64.5
	DNA or UV HPLC-water	-	-	18.0
	Total			100.0

Note

Each library will have 2 reactions of 100 µL each.

Splits: 4 (aDNA library preparation room)

4 Calculate the amount of master mix needed for the number of samples being processed. Prepare the master mix according to the table below within a 1.5 mL LoBind tube.

Add 87 µL mastermix, 2 µL of each index primer and 9 µL sample to each tube (use 0.2 mL PCR strips).

	A	B	C	D
	Reagent	Stock conconcentration	Final concentration	1× Volume [µL]
	Pfu Turbo Cx Buffer	10 ×	1 ×	10.0
	BSA	20 mg/mL	0.3 mg/mL	1.5
	dNTPs	25 mM	0.25 mM	1.0

	A	B	C	D
	Pfu Turbo Cx Polymerase	2.5 U	0.025 U	1.0
	Index P5_Jen_8nt	10 µM	0.2 µM	2.0
	Index P7_Jen_8nt	10 µM	0.2 µM	2.0
	UV HPLC-water	-	-	73.5
	DNA or UV HPLC-water	-	-	9.0
	Total			100.0

Note

Each library will have 4 reactions of 100 µL each.

Splits: 6 (aDNA library preparation room)

5 Calculate the amount of master mix needed for the number of samples being processed. Mix the master mix from the table below in a 1.5 mL LoBind tube.

Add 90 µL mastermix, 2 µL of each index primer and 6 µL sample to each tube (use 0.2 ml PCR strips).

	A	B	C	D
	Reagent	Stock concentration	Final concentration	1× Volume [µl]
	Pfu Turbo Cx Buffer	10 ×	1 ×	10.0
	BSA	20 mg/mL	0.3 mg/mL	1.5
	dNTPs	25 mM each	0.25 mM each	1.0
	Pfu Turbo Cx Polymerase	2.5 U	0.025 U	1.0
	Index P5_Jen_8nt	10 µM	0.2 µM	2.0
	Index P7_Jen_8nt	10 µM	0.2 µM	2.0

	A	B	C	D
	UV HPLC-water	-	-	76.5
	DNA or UV HPLC-water	-	-	6.0
	Total			100.0

Note

Each library will have 6 reactions of 100 µL each.

Move to modern DNA lab

6 Securely close the reactions and transfer to modern DNA laboratory. If possible, keep the reactions on ice during the transfer.

Indexed adapter ligation with PCR (modern DNA lab)

7 In a modern DNA lab, use a thermocycler to amplify the reactions with the following program:

	Temperature	Time	
	95°C	2 min	Inital denaturation
	95°C	30 sec	10 cycles
	58°C	30 sec	
	72°C	1 min	
	72˚C	10 min	Final elongation
	10˚C	until further processing	

Note

During this incubation, label new 1.5mL LoBind tubes for post-purification elution (step 10).

MinElute Purification (modern DNA lab)

8 Purify the indexed libraries with a MinElute kit, with the following modifications to the manufacturer's protocol.

8.1 For each reaction, add 650 µL PB (binding) buffer to a new 5 mL LoBind tube. Add the library, then vortex briefly to mix. A single column can be used for up to 4 index reactions of one library. Therefore, each PB and library mix of a single library that was split in 2 to 4 reactions will be loaded onto the same column.

Note

If 6 or more splits were performed for the indexing reactions, the PB and library mix can be split over 2 columns for purification. In this case, elute the DNA from each column in step 8.8 with 25 µL EBT and combine the eluate to get 50 µL.

8.2 Load each reaction (PB buffer + library) 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.

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

Note

Pour off the liquid into a 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 soap.

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

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

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

8.7 Remove columns from their collection tubes and place them in new 1.5 mL LoBind tubes.

8.8 Add 50 µL EBT to the column of the filter, let stand for 00:01:00, then spin at 15800 x g, 00:01:00 to elute.

Note

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

Note

If two columns were used for the purification of one library, elute each in 25 µL and pool both reactions to produce a total elution volume of 50 µL.

qPCR Quality Check (modern DNA facility)

9 Dilute 2 µL of the indexed library 1:1000 for qPCR. Do this in 2 steps: Make a 1:10 dilution, and then make a 1:100 dilution of the 1:10 dilution, for a final dilution of 1:1000.

10 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.

	Reagent	Stock concentration	Final concentration	1× Volume [µl]
	DyNAmo Master Mix	2 ×	1 ×	10
	IS5 primer	10 µM	0.5 µM	1
	IS6 primer	10 µM	0.5 µM	1
	HPLC-Water (non UVed)	-	-	7
	DNA or HPLC-Water (1:1000 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 very difficult 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 vortexing so you do not create any bubbles.

10.1 Add 19 µL mastermix and 1 µL diluted libraries, standard, or water for each reaction to a fresh 96-well plate.

> **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.

11 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.		

> **Note**
>
> The number of DNA copies determined with this qPCR is used to determine the amount of sample used as input for final amplification and pooling for sequencing (separate protocol).