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Bravo workstation: automated indexing, purification and quantification of DNA libraries V.1

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MPI EVA Ancient DNA C...

Ancient DNA Core Unit
MPI EVA

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We use this protocol and it's working

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Abstract

We here provide a protocol for the simultaneous amplification and dual-indexing of 96 DNA libraries through automated liquid handling using the Bravo NGS workstation (Agilent Technologies). The benefits of dual-indexing for preventing and detecting cross-contamination have been documented elsewhere (Kircher et al. 2012, Zavala et al. 2022). This protocol is optimized specifically for ancient and degraded DNA (i.e., libraries with short inserts) and is compatible with both double- and single-stranded methods for preparing Illumina-type libraries (e.g., Meyer and Kircher 2010, Gansauge et al. 2020). Amplification is carried out to PCR plateau, ensuring approximately equal yields across all samples. The use of AccuPrime *Pfx* polymerase minimizes length and GC biases during amplification (Dabney et al. 2012). Additionally, we provide sequences for 384 unique 8-bp indices, incorporated into the interior of both the P5 and P7 indexing primers.

The protocol also includes steps for purifying indexed libraries using Solid Phase Reversible Immobilization (SPRI) technology (deAngelis et al. 1995) and for determining the concentration of purified indexed libraries using a NanoDrop photospectrometer.

Some of the instructions, for example regarding the documentation and location of files, are specific to the environment and workflows of the Ancient DNA Core Unit of the MPI-EVA and have to be amended in other environments.

Notes

Default input volume of library into indexing PCR is 49 µl (the final library volume in library preparation with the ssDNA2.0 method), for a total reaction volume of 100 µl. However, lower input volumes are possible.

Input files are provided containing index combinations and Bravo pipetting commands for 384-well PCR plates, making for a total of 36,864 libraries that can be indexed with unique combinations of P7 and P5 indices with fast, column-wise pipetting. There is no overlap in P5 or P7 indices among four consecutive plates (labeled A, B, C and D), which together form one set of plates. Thus, four PCR plates can be set up consecutively using only a single P5 and P7 index primer plate each. Note that for half of the index combinations provided, the P5 index primer plate has to be rotated by 180° before insertion (indicated in the file name).

References

Dabney, J., & Meyer, M. (2012). Length and GC-biases during sequencing library amplification: A comparison of various polymerase-buffer systems with ancient and modern DNA sequencing libraries. BioTechniques, 52(2), 87-94.

DeAngelis, M. M., Wang, D. G., Hawkins, T. L. (1995) Solid-phase reversible immobilization for the isolation of PCR products. Nucleic Acids Res. 23(22):4742-3. doi: 10.1093/nar/23.22.4742.

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Zavala, E. I., Ayinuer-Petri, A., Richter, J., Nickel, B., Vernot, B., & Meyer, M. (2022). Quantifying and reducing cross-contamination in single- and multiplex hybridization capture of ancient DNA. Molecular Ecology Resources, 22(6), 2196-2207.

Materials

Materials

	Reagents/consumables	Supplier	Cat. no.
	Large-volume buffers/reagents		
	80% EtOH (v/v) ǂ	self	-
	33 % SPRI beads †	self	-
	TE buffer †	self	-
	Reagents		
	water	Sigma/Merck	1153332500
	10x AccuPrime Pfx buffer	ThermoFisher Scientific	12344032
	AccuPrime Pfx DNA Polymerase	ThermoFisher Scientific	12344032
	P5 primer plate 384 *	self	-
	P7 primer plate 384 *	self	-
	Consumables		
	Bravo 96LT 250 µl sterile, filtered tips	Agilent	19477-022
	Bravo 96LT 250 µl sterile, filtered tips, empty boxes	Agilent	19477-022
	5 ml Eppendorf tubes	Kisker	G05-ML
	NUNC 96 deepwell plate	NUNC	736-0600
	Peelable heatseal foil	Bio-Rad	1814045
	Twin.tec PCR Plate 96, semiskirted	neoLab Migge	VB-0398
	Filter tip, 1000 µl, Diamond tips	neoLab Migge	CF-0425
	Filter tip, 200 µl, Diamond tips	neoLab Migge	CF-0438
	Filter tip, 10 µl, Diamond tips	neoLab Migge	CF-0417

	Reagents/consumables	Supplier	Cat. no.
	HTS deep well reservoirs	Kisker	97813
	HTS low well reservoirs	Kisker	97817
	96 Deep well PP Sqr well plate	Axygen	P-2ML-SQ-C-S
	Twin.tec PCR Plates 96, skirted	neoLab Migge	VB-0403

† See documents in the Appendix for preparation of reagents, buffers and SPRI beads.
‡ Dilute 100% EtOH in water to obtain a 80 % solution.
* Order P5 and P7 indexing primers at 40 nmol synthesis scale (Eurogentec, RP-cartridge purification). See Appendix for the document describing the preparation of 384-well primer aliquot plates.

Equipment

- Bravo-B NGS workstation G5522A with 96-channel LT pipette head and associated equipment (red thermal plate insert for 96-well PCR plates, silver thermal plate insert for 96-well Nunc deep-well plates, two silver thermal plate insert for 384-well PCR plates, 96-well magnetic rack) for indexing library preparation
- Bravo-B NGS workstation G5522A with 96-channel LT pipette head and associated equipment (two red thermal plate insert for 96-well PCR plates, 96-well magnetic rack) for indexing library purification
- Hood for NGS workstation B with HEPA-filtered ventilation system and strip curtain (custom-built, optional)
- Centrifuge for PCR plates (e.g., Eppendorf, cat. no. 5948000913)
- Table-top micro-centrifuge for 1.5 ml tubes (e.g., Carl Roth Mini-Zentrifuge ROTILABO‰, cat. no. T464.1)
- Table-top micro-centrifuge for 5 ml tubes (e.g., myFuge 5D Digital 5mL Centrifuge, cat. no. C2595-E)
- Thermal PCR cycler (e.g., MJ Research, DNA Engine Thermal Cycler PTC-200, cat. no. 8252-30-0001)
- Plate sealer (e.g., Bio-Rad Px1 PCR plate sealer, cat. no. 1814000)

Bravo electronic protocol files

Available on Github: https://github.com/adnacore/Bravo-workstation-single-stranded-DNA-library-preparation-ssDNA2.0/tree/v1.0

Appendix

Indexing primer sequences

8bp_iPCR_P5_P7_plateLayout_2021... 27.9KB

Documents for buffers and reagents

Document

NAME

TE buffer

CREATED BY

Anna Schmidt

Preview

Document

NAME

SPRI beads, variable PEG concentration

CREATED BY

Ancient DNA Core Unit

Preview

Documents for primer plates

Document

NAME

Bravo workstation: Preparation of indexing primer aliquots in 384-well plates

CREATED BY

Ancient DNA Core Unit

Preview

Indexing PCR setup

1 Before each run, follow the instructions in Labfolder and document the experiment.

Note

[Documentation]
Document the experiment in Labfolder.

[Note]
The Labfolder entry name consists of the name of the Labfolder template and the library plate ID. To document your experiment, fill the data element fields in Labfolder. They serve as a template for creating CoreDB entries later on. Providing the plate ID and performing the database work is usually done by the main person responsible for library preparation in the Core Unit, not by the person performing the experiment.

2 Thaw the following components at room temperature:

- Library plate(s) (up to four plates to be indexed with one set of indexing primers)
- 10x AccuPrime *Pfx* buffer
- P5 primer plate 384 well
- P7 primer plate 384 well

Note

[Note]
See MATERIALS for the preparation of the 384-well P5 and P7 primer plates. Do NOT vortex the plates after thawing.

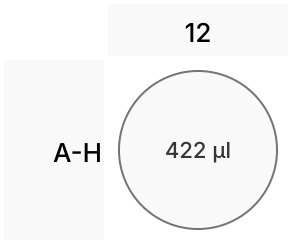
3 Prepare PCR master mix in a 5 ml Eppendorf tube by combining the reagents below. Mix by flicking the tube with a finger and briefly centrifuge in a 5 ml table centrifuge.

	Reagent	Volume (µl)	Volume per reaction (µl)	Final concentration
	Water	2200	20	
	10x AccuPrime Pfx buffer	1100	10	1x
	AccuPrime Pfx DNA Polymerase	110	1	

Note

[Documentation]
Note down the lot/batch information of the reagents in the respective fields in Labfolder.

4 Pipette 422 µl of the master mix to each well of column 12 of a NUNC Plate. Pipette very slowly to prevent foaming. Leave the plate on the bench until used.



5 Switch on all components of the Bravo system, including the external cooling device (set to 4 °C). Switch on light and ventilation ("Betrieb") inside the robot hood.

6 Log into the VWworks software using the administrator account (password "a") and open the protocol under "S:\Bravo_protocols\MPI-EVAN-homebrew\forms\ssDNA_Library_preparation\96_ssDNAprep_indexingPCR_8bp_indices.VWForm". Initialize the system.

7 Briefly centrifuge the library plate as well as P5 and P7 primer plates in a plate centrifuge to ensure that the foil is clean and free of liquid before you carefully remove the foil.

8 Set up the Bravo deck as indicated by the form file and specify the volume of library that will be indexed.

Note

[Note]
Default library input volume is 49 µl. If smaller volumes of library should be indexed, put an HTS deep well reservoir filled approximately half with water on position 1 of the Bravo deck. This reservoir will be used to substitute the reduced input volume.

9 Load a text file containing the desired combinations of indices for each reaction and associated Bravo pipetting instructions.

Note

[Note]
Input files are available at "P:\AncientDNA\indices\8bp_indices_correct", sorted alphabetically in the order in which they should be used. Pick the first file in the folder, rename it by adding the library plate ID (e.g., 01A_Pl_397.txt), and transfer it to the following folder: "P:\AncientDNA\indices\8bp_indices_correct\used_indices". From there, load it into the protocol.

CAUTION: In case the input file names contains "**revP5**" (e.g., "49A.revP5.txt"), the P5 primer plate needs to be rotated 180 ° on the deck, so that the numbers and letters are upside down.

10 Start the run by clicking the "start" button and follow the instructions by VWorks.

Note

[Note]
Run time is ca. 30 min. The first 15 min are used for aliquoting the P5 and P7 primers to the indexing PCR plate. After this, a pause point is reached, at which the user will be prompted to add the master mix if it has not been added already. Inexperienced users are advised to start the run only after all components have been added to the Bravo deck, as indicated in the form file.

Finishing indexing PCR setup and cycling

11 After the run has finished, seal the PCR plate with peelable foil in the Bio-Rad plate sealer (185 °C, 3 s, use sealing frame). Store PCR plate in the fridge until it is transferred to the post-PCR lab for cycling. If less than 49 µl library were used for PCR, seal the library plate containing the leftover libraries (180 °C, 3 s, use sealing frame) and put it back to

the freezer. If needed, go to step #1 to prepare additional indexing PCR plates using P5 and P7 indexing primers from the same set.

12 After all PCR plates have been prepared, discard all plastic ware that is still in the robot and clean the robot deck using ethanol and water. Switch off the robot and restart the computer.

Note

[Note]
If less than four indexing PCR plates were prepared, also discard the remaining indexing primer aliquots. Sealing and re-using primer aliquots is strongly discouraged due to the risk of cross-contamination between primers.

13 Take the indexing PCR plate(s) to a post-PCR lab equipped with thermo cyclers and start PCR cycling using the profile below.

Note

Cycling is performed using the following parameters:

	Step	Temperature °C	Time
	Initial denaturation	95	2 min
	Per cycle denaturation	95	20 s
	Per cycle annealing	60	30 s
	Per cycle extension	68	1 min
	Goto 2, 34 times		
	Final extension	68	5 min
	Hold	10	forever

- The cycling takes about 2 h 20 min.

Note

[Documentation]
Add cycler and room number to your entry in Labfolder.

14 When cycling has completed, store the PCR plate(s) in the fridge until indexing library purification is performed. Perform purification within one week.

SPRI purification set-up

15 Switch on all components of the Bravo system. The external cooling device is not needed for this protocol.

16 Log into the VWworks software using the administrator account (password "a") and open the automated purification protocol under "S:\Bravo_protocols\MPI-EVAN-homebrew\forms\SPRI_purification\V2_2_1_96_SPRI_33_Purification_Dilutions.VWForm". Initialize the system.

17 Take the indexing PCR plate out of the fridge and briefly centrifuge it in a plate centrifuge to make sure that there are no droplets remaining at the foil. Do not vortex the plate.

18 Specify the parameters in the menu of the form file.

Note

[Note]

The default values for post-indexing purification are as follows:
- Sample volume: 85 µl
- Elution volume: 40 µl
- Number of columns to be purified: 12
- Specify last filled column in tip box: 12
- Specify first filled column in tip box: 1
- Specify sample plate type (cannot be changed): Eppendorf twin. tec semi-skirted PCR plate
- Specify SPRI beads reservoir type: 96 Ay Deep-well HTS reservoir

19 Set up the Bravo deck as indicated by the form file.

20 Fill buffer reservoirs as listed in the table below and load them into the MiniHub as indicated by the form file.

	Reagent	Volume (ml)
	80% EtOH	approx. 50
	TE buffer	approx. 30
	SPRI beads (33% PEG)	approx. 30

Note

[Note]
Mix the SPRI bead suspension thoroughly by shaking. Make sure that no bead pellet or clumps are remaining.

Note

[Documentation]
Note down the reagent batches in the respective fields in Labfolder.

21 Start the purification by clicking the "Run" button.

Note

[Note]
- Run time is approximately 1 h 20 min. The run starts with aliquoting 5 µl backup of the unpurified libraries into a fresh empty Eppendorf full skirted plate. This takes ca. 5 min. After this step the run will be paused automatically.

22 When prompted by the software, seal the plate with peelable foil in the Bio-Rad plate sealer (180 °C, 3 s, use sealing frame). Label the plate "5ul backup indexed & UNpurified" and store it in the freezer. Continue the run by clicking "Continue".

Finishing SPRI purification

23 After the run has finished, seal the plates with peelable foil using the Bio-Rad sealer (180 °C, 3 s, use sealing frame).

Note

[Note]
There are three plates to store after library purification:

- The final indexed and purified library plate (40 µl). Label the plate with plate ID, "indexed and purified", the date and your initials. Store the plate in the fridge for subsequent generation of library pools. After pooling, the plate should be stored in the respective library box in the freezer.
- A 10-fold dilution plate (20 µl total volume), to be used as a template for reamplification if necessary. Label the plate with plate ID, "1:10 reamp backup", the date and your initials. Freeze it for later use.
- A 2.5-fold dilution plate (5 µl total volume), to be used for NanoDrop measurement. Label the plate with plate ID, "NanoDrop dilution indexed and purified', the date and your initials. Store the plate in the fridge until NanoDrop measurement is performed. **Important:** NanoDrop measurements should never be performed when the plate was already stored in the freezer!

24 Remove and discard all the plastic ware that is still in the robot. Discard any leftover reagents in liquid waste container and clean the robot using ethanol and water. Switch off the robot and restart the computer.

Indexing library quantification using Nanodrop device

25 Take the 2.5-fold dilution plate for NanoDrop measurement from the fridge and briefly centrifuge.

Note

[Note]
To avoid freezing/thawing-induced concentration gradients, the indexed library plate should always be stored in the FRIDGE until the NanoDrop measurements are performed.

26 Start the NanoDrop Software on the computer linked to the NanoDrop device (ND 8000). Choose the 8-channel measurement for Nucleic Acid (DNA-50, dsDNA) and follow the instructions by the software.

27 Clean the NanoDrop device by adding 1 µl of water to the measurement pedestal using a multi-channel pipette. Close the pedestal and click "Ok" to initialize the instrument. Open it and clean it with a soft tissue.

28 Blank the device by adding 1 µl of TE buffer to the pedestal. Close the pedestal and click "Ok" to blank the device. Open the pedestal and clean again with soft tissue.

Note

[Documentation]
Note down the reagent information in the respective fields in Labfolder.

29 Carefully remove the plate seal of the NanoDrop dilution plate and measure the concentrations of the indexing libraries column-wise using the multi-channel pipette. Clean pedestal after every measurement with soft tissue.

Expected result

Purified indexed libraries typically yield DNA concentrations between 100 and 200 ng/µl (after correcting for the 2.5-fold dilution). Repeat measurements for samples with values below 50 ng/µl or samples showing unexpected curves.

30 Save the measurements as an MS Excel file in your personal folder on the public server (P:\user\Name). Save a copy on the Coreunit server in the respective library plate folder under "coreunit(V:)\SummaryTables\Library/PI_XXX".

Note

[Note]
Attach the file to your entry in Labfolder.

31 Seal the 2.5-fold dilution plate with peelable foil (180 °C, 3 s, use sealing frame). This plate can be discarded after one year of storage. Label the plate with "Discard in MMYYYY" and put it into the corresponding drawer in the freezer.