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Hamilton NGS Star workstation: library indexing using AccuPrime Pfx DNA polymerase, purification and quantification V.1

 Forked from [Bravo workstation: automated indexing, purification and quantification of DNA libraries](#)

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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 up to 384 DNA libraries through automated liquid handling using the Hamilton NGS Star workstation. 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 μ l (the final library volume in library preparation with the ssDNA2.0 method), for a total reaction volume of 100 μ l. However, lower input volumes are possible.

Input files are provided containing unique index combinations for column-wise (or half column-wise) pipetting of 576 indexing PCR plates, each with 96 reactions, making for a total of 55,296 reactions. 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.

In addition to the purified, indexed libraries, the protocol produces plates with 2.5-fold and 10-fold dilutions intended for Nanodrop measurements and templates for reamplification, as well as a backup of unpurified library.

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.

Gansauge, M.-T., Aximu-Petri, A., Nagel, S., & Meyer, M. (2020). Manual and automated preparation of single-stranded DNA libraries for the sequencing of DNA from ancient biological remains and other sources of highly degraded DNA. *Nature Protocols*, 15, 2279–2300.

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Meyer, M., & Kircher, M. (2010). Illumina sequencing library preparation for highly multiplexed target capture and sequencing. *Cold Spring Harbor Protocols*, 2010(6): prot5448.

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.
<i>Large-volume buffers/reagents</i>		
80% EtOH (v/v) ‡	self	-
33 % SPRI beads †	self	-
TE buffer †	self	-
<i>Reagents</i>		
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	-
<i>Consumables</i>		
Hamilton conductive filter tips, 50 µl	VWR	736-0767
Hamilton conductive filter tips, 300 µl	VWR	736-0787
Hamilton conductive filter tips, 1000 µl	VWR	736-0794
15 ml Falcon tube	Greiner Bio-one	188261
5 ml screw cap tubes (rack 2d Lp W/barcode)	Thermo Fisher Scientific	SCT-5ML-S
Peelable heatseal foil	Bio-Rad	1814045
Twin.tec PCR Plate 96, semi-skirted	neoLab Migge	VB-0398
Twintec PCR Plate 96, skirted	neoLab Migge	VB_0403
Filter tip, 1000 µl, Diamond tips	neoLab Migge	CF-0425

	Reagents/consumables	Supplier	Cat. no.
	Filter tip, 200 µl, Diamond tips	neoLab Migge	CF-0438
	Filter tip, 10 µl, Diamond tips	neoLab Migge	CF-0417
	reagent container with lid, liquid reservoir 60 ml	VWR	736-0831
	Depp-well MIDI plate, 96 well storage plate	Thermo Fisher Scientific	12194162

† 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

- Hamilton work station - NGS STAR MOA (basic configuration: ML STAR with 8 independent channels, MPH96, 5 HHS, 1 CPAC). Required add-ons are: iSWAP Plate Handler (for full-deck plate transport), MPH Extension Left Standard (for usage of MPH96) and the ODTG (for on-deck thermal cycling of 96 well plates). See document in the Appendix for custom-specific modifications of the deck layout. Compatible robots at the MPI-EVA: Elfi, Sherlock, Richard and Watson.
- Centrifuge for PCR plates (e.g., Eppendorf, cat. no. 5948000913)
- Centrifuge for 15 ml Falcon tubes (e.g., Carl Roth table centrifuge, cat. no. KEC8.1)
- 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)
- Label printer and corresponding labels (e.g., Brady, cat. no. BRDY317844)
- NanoDrop system (NanoDrop™ 8000 Spektralphotometer)

Electronic protocol files

Available on Github : <https://github.com/adnacore/Hamilton-NGS-Star-workstation-automated-indexing-setup-and-purification/tree/v1.1>

Appendix

Document for custom-specific modifications of the deck layout

Document

NAME

DeckLayout: automated indexing, purification and quantification of DNA libraries

CREATED BY

Julia Zorn

Preview

Indexing primer sequences

 p5_indices_384set.xlsx 23KB

 p7_indices_384set.xlsx 23KB

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

384 Indexing primer plate layout



8bp_iPCR_P5_P7_plateLayout.xlsx 10KB

Troubleshooting



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.

- 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]

If you do not know the required reagents and their volumes, thaw reagents only after reaching  13 .

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

- 3 Label the indexing PCR plates (between one and four fresh semi-skirted 96-well PCR plates) with Plate ID, 'indexing PCR', date and your Initials.
- 4 Briefly centrifuge the library plates containing the unindexed libraries (skirted 96-well PCR plates).
- 5 Turn on the Hamilton robot and the connected components beneath (three components).
- 6 Log into the computer as user 'Hamilton' (password: 'hamilton').



- 7 Open the software Method Manager and log into the 'labuser' account (password: 'labuser'). Make sure the software is set to running mode (status bar is green), not simulation mode (status bar is yellow). Open the protocol 'Indexing Setup' which can be found in the section 'MPI Library preparation'. A dialog will ask to record the run. Click 'Ok' to save the run data for possible later run evaluation.

Note

[Note]

A weekly maintenance has to be performed on the Hamilton system. In case you are the first person using the robot in the week, open the software 'Method Manager' and the protocol 'Maintenance' located in the 'Maintenance' section to perform the weekly checkup.

Once the protocol is opened in the software, it will guide you through the procedure step-by-step until the run is ready to be started. Don't leave out any steps!

- 8 Enter your email address to receive notifications from the robot.
- 9 Choose "**AccuPrime Setup**" and specify the number of plates to be indexed. Click "Ok" to continue.

Note

[Note]

The most efficient workflow involves the indexing of four library plates at once, but between one and three plates are also possible. Indexing of individual plate columns is also possible if the input file is adjusted accordingly. Do not choose the "Equinox" option, which is intended for indexing with an alternative polymerase.

- 10 Specify the library volume that should be used as input for indexing PCR.

Note

[Note]

Default library input volume is 49 μ l. If smaller volumes of library are indexed, the robot will use water to substitute the reduced input volume. If you chose to index more than one library plate, you can specify the library input volume for each plate separately.

- 11 Load an input file (.txt) containing the desired index combinations for each reaction. If indexing multiple plates, provide the corresponding input files sequentially, one for each plate.

Note

[Note]

Input files are available at "P:\AncientDNA\indices\8bp_indices_Hamilton_files", 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_Hamilton_PL_397.txt), and transfer it to the following folder: "P:\AncientDNA\indices\8bp_indices_Hamilton_files\used_indices". From there, load it into the protocol. Repeat this step for each library plate.

- 12 Set up the Hamilton deck as indicated by the robot dialog and confirm by clicking "Ok". This includes the following steps:
 - Load the required pipette tips and mark the filled positions in the pop-up window. When loading the pipette tips, make sure the colored barcode is facing to the right side so that the barcode scanner can read them. The Hamilton deck has to be filled as indicated in the table below.

	Tip volume (μ l)	Color	Hamilton track
	50	purple	6-11
	300	yellow	12-17
	1000	white	18-23

- Track 24-29: Place the fresh semi-skirted 96-well PCR plate(s) from step 3 at the positions 2-5 as indicated by the dialogue.

Note

[Note]

Make sure the 4-plate carrier is placed in track 24-29. If another carrier is present, exchange it.

- Track 30-35: Place the library plates containing the unindexed library in skirted 96-well PCR plates at position 2-5 as indicated by the dialogue.

Note

[Note]

Make sure the 4-plate carrier is placed in track 30-35. If another carrier is present, exchange it.

- 13 A dialog will open displaying the composition of the PCR master mix required for the run. The volumes will be calculated individually for each experiment. The corresponding file 'MastermixSummary.csv' can be found in: C:\Program Files (x86)\Hamilton\Logfiles. Click "Ok" to continue.

Reagent	Volume (μl)	Volume per reaction (μl)	Final concentration
Water	8359.7	20	
10x AccuPrime Pfx buffer	4179.8	10	1x
AccuPrime Pfx DNA polymerase	418	1	

Note

[Note]

The table above exemplifies the required volumes for indexing four library plates at once (the most efficient workflow). Excess is included in the calculation of the required volumes.

[Documentation]

Attach the master mix file to your Labfolder entry if working with less than four full library plates.



- 14
- Briefly vortex and centrifuge the 10x AccuPrime *Pfx* buffer. Prepare the PCR master mix in a 15 ml Falcon tube as indicated in the master mix file (a smaller tube may be used if fewer than four plates are being indexed simultaneously). Mix the master mix by flicking the tube with your finger, then briefly spin it in a centrifuge. Aliquot the master mix into four 5 ml screw cap tubes, as indicated in the dialog and the MastermixSummary file.

Note**[Documentation]**

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

- 15
- Briefly centrifuge the P5 and P7 primer plates in a plate centrifuge to ensure that the plate seal is free of liquid, then carefully remove the foil.

Note**[Note]**

To reduce the risk of cross-contamination between primers do not vortex the 384 well primer plates and handle them gently to avoid spilling of liquid.

- 16
- Continue to set up the Hamilton deck as indicated by the robot dialog and confirm by clicking "Ok":
- Track 36-42: Add the silver 384 adapter to position 4 and put the P7 primer plate on top of the adapter.
 - Track 43-49: Add the silver 384 adapter to position 4 and put the P5 primer plate on top of the adapter. Remove the caps from the Master mix tubes and put them into wells A1, B1, C1, etc. of the CPAC adapter (the black 24-well aluminum adapter for big and small tubes) on position 3.
 - OPTIONAL: Track 53: If less than 49 μ l library are indexed, the robot will use water to substitute the reduced input volume. In this case, label a liquid reservoir with "water" on the upper part (so that the label is visible when placed in the robot). Remove the plastic lid and fill the reservoir with water as indicated by the dialog. Place the reservoir at position 1.
- 17
- Start run by clicking "Ok" and follow the instructions.

**Note****[Note]**

Run time is ca. 20 min per library plate.

Completing indexing PCR setup and cycling

- 18 The robot will send a notification via E-Mail once the run is complete. Press "Ok" to move on to the robot cleanup.
- 19 Remove the indexing PCR plates from position 2-5 on track 24-29 and seal the plates carefully with peelable foil in the Bio-Rad plate sealer (185 °C, 3 s, use sealing frame). Store the indexing PCR plates in the fridge until they are transferred to the post-PCR lab for cycling. If less than 49 µl library were used as input for PCR, seal the library plate containing the leftover libraries (180 °C, 3 s, use sealing frame) and put it back to the freezer.
- 20 Press "Ok" to finish the run and clean up the robot as indicated by the protocol. Empty the plastic waste and wipe the metal part of the tip trash with Ethanol.

Note**[Note]**

Before discarding the plasticware, carefully inspect the skirted 96-well PCR plates that previously contained the unindexed libraries. If the standard procedure of indexing 49 µl library volume was followed, the wells should now be empty.

- 21 Restart the computer and turn off the connected devices beneath. The robot itself should only be switched off by the last person using it at the end of a work week.

Note**[Note]**

Discard the used indexing primer plates even if fewer than four indexing PCR plates were prepared. Sealing and re-using primer plates is strongly discouraged due to the increased risk of cross-contaminating primers.

- 22 Transfer the indexing PCR plates 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.

- 23 When cycling has completed, store the PCR plates in the fridge until indexing library purification is performed. Perform purification within one week.

SPRI purification set-up

- 24 Turn on the Hamilton robot and the connected devices beneath (three devices).
- 25 Log into the computer as user 'Hamilton' (password: 'hamilton').
- 26 Open the software Method Manager and log into the 'labuser' account (password: 'labuser'). Make sure the software is set to running mode (status bar is green), not



simulation mode (status bar is yellow). Open the protocol 'SPRI purification' which can be found in the section 'SPRI purification'. A dialog will ask to record the run. Click 'Ok' to save the run data for possible later run evaluation.

27 Enter your email address to receive notifications from the robot.

28 Choose the plate type "indexing PCR (regular pipeline)" and click "Ok".

Note

[Note]

The Hamilton purification protocol can also be used for different types of purification (e.g., indexing backup purification, reamp purification). Therefore, different plate types can be selected.

29 A dialog will open displaying the parameters relevant for purification. Click "Continue" to proceed the run.

Note

[Note]

The default values for post-indexing purification are as follows:

- Number of samples: 96
- SPRI bead PEG concentration: 33% PEG SPRI beads (0.75x beads suspension/sample ratio)
- Labware input plate: Eppendorf twin.tec PCR plate 96, semi-skirted

Output plate selection:

- 5 µl backup plate (unpurified library input): yes
- NanoDrop dilution plate (2.5x dilution): yes
- purified library backup plate (10x dilution): yes
- PCR product input volume (µl): 90
- Eluate volume (µl): 40
- EBT volume (µl) to add before purification: no

For experimental setups, these parameters can be adjusted if necessary.

30 Take the indexing PCR plate out of the fridge and briefly centrifuge it in a plate centrifuge to ensure that no droplets remain on the foil. Do not vortex the plate.

31 Set up the Hamilton deck as indicated by the robot dialog and confirm by clicking "Ok".

This includes the following steps:

- Track 1-5: Place a MIDI plate to position 1 and four Eppendorf full skirted 96 well plates (HSP plates) to position 4 (stacked).

- Load the required pipette tips and mark the filled positions in the pop-up window. When loading the pipette tips, make sure the colored barcode is facing to the right side so that the barcode scanner can read them. The Hamilton deck has to be filled as indicated in the table below.

Tip volume (µl)	Color	Hamilton track
50	purple	6-11
300	yellow	12-23
1000	white	24-29

Note

[Note]

Hamilton Richard II requires Core II tips (C2 on the barcode).

- Track 24-29: Add the black 96 well adapter to position 1.
- Track 30-35: Place the semi-skirted Eppendorf 96 well plate containing the indexed library to position 3.
- Track 53: Label the liquid reservoirs with name of reagent on the upper part (so that the label is visible when placed in the robot). Remove the plastic lids and fill the reservoirs according to the table below. Each reservoir can hold ca. 60 ml. Add slightly more of the reagents than the minimum volumes listed below to ensure there is enough reagent available. Use the metal lids to cover the reservoirs on positions 1-3.

Track	Position	Reagent	Volume (ml)
53	1	EtOH 80%	45.2
53	2	SPRI beads (33% PEG)	9.7
53	3	TE buffer	7.2
53	4	empty	N/A
53	5	placeholder reservoir	N/A

**Note****[Note]**

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

The spacer reservoir in track 53 (position 5) serves as a lid holder during the run. Place it into the correct position and remove the plastic lid.

[Documentation]

Note down the reagent information in the respective fields in Labfolder.

- 32 Start run by clicking "Ok" and follow the instructions.

Note**[Note]**

The total run time is ca. 2 h. 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.

- 33 When prompted by the software, remove the plate containing the remaining unpurified library. Seal the plate with peelable foil using 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

- 34
- The robot will send a notification via E-Mail once the run is complete. Remove the final indexed and purified library plate from position 3, the 2.5-fold dilution plate for Nanodrop measurement from position 2 on track 30-35, and the 10-fold dilution backup plate from position 4 (CPAC 3) on track 36-42. Seal the plates with peelable foil using the Bio-Rad plate sealer (180 °C, 3 s, use sealing frame).

**Note****[Note]**

There are three plates to store after library purification. Label them properly using the Brady label printer:

- The final indexed and purified library plate (40 μ l). 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 μ l 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 μ l 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!

- 35 Press "Ok" to finish the run. If additional PCR plate require purification,  26 to repeat the purification process. Once all purifications are finished, clean the robot as indicated by the protocol: Empty the plastic waste and the liquid waste container and wipe the metal part of the tip trash with ethanol.
- 36 Restart the computer and turn off the connected devices beneath. The robot itself should only be switched off by the last person using it at the end of a work week.

Indexed library quantification using the Nanodrop spectrophotometer

- 37 Remove the 2.5-fold dilution plate for NanoDrop measurement from the fridge and briefly centrifuge.

Note**[Note]**

To avoid freezing-induced DNA concentration gradients, the indexed library plate should **always be stored in the fridge** until the NanoDrop measurements are performed.

- 38 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 provided by the software.
- 39 Clean the NanoDrop pedestal by adding 1 μ l of water using a multi-channel pipette. Close the pedestal and click "Ok" to initialize the instrument. Open it and remove the water with



a soft tissue.

- 40 Blank the device by adding 1 μl of TE buffer to the pedestal. Close the pedestal and click "Ok". Open the pedestal and remove the liquid with a soft tissue.

Note

[Documentation]

Note down the reagent information in the respective fields in Labfolder.

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

Expected result

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

- 42 Save the measurements in MS Excel format 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.

- 43 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.