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Hamilton NGS Star workstation: automated in-solution hybridization capture (MPI-EVA method) V.1

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MPI EVA Ancient DNA C...

Ancient DNA Core Unit
MPI EVA

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Protocol status: Working
We use this protocol and it's working

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Abstract

We here provide a protocol for automated hybridization capture of ancient DNA libraries on the Hamilton NGS Star in 96-well format using a capture method originally developed by Gnirke et al. 2009 and modified at the MPI-EVA for use with ancient DNA by Fu et al. 2013. Modifications to the latter method include the omission of a wash with Wash Buffer 3 and the hydroxide melt step for the elution of the captured library molecules prior to amplification.

In-solution hybridization capture is performed using single-stranded biotinylated DNA probes, which can be generated from oligonucleotide arrays, synthetic oligonucleotide pools or other sources. It has recently been shown that small amounts of cross-contamination can occur in hybridization capture as a result of 'PCR jumping' during post-capture amplification even when working with double-indexed libraries (Zavala et al. 2022). The choice of amplification conditions described here (polymerase, primers and thermal profile) minimizes PCR jumping, despite amplifying libraries into PCR plateau. Yet, we strongly urge users to quantify cross-contamination in the resulting sequence data using the computational strategy described by Zavala et al. 2022.

To use the protocol, a Hamilton NGS Star system with a custom deck layout is required. Calibration of the instrument for this protocol has to be performed by the user and requires significant expertise in using the platform. Electronic protocol files for the NGS Star are provided together with this protocol and a brief description of the steps performed by the liquid handling system. Documents detailing the preparation of the probes and other reagents are also provided. 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. We strongly advise against manual execution of the protocol, as manual handling lacks the precision of automated liquid handling and may lead to cross-contamination of samples or non-optimal results. For a manual protocol, please refer to Zavala et al. 2022.

This protocol consists of several parts: (i) preparation of the sample plate, (ii) hybridization over-night, (iii) post-capture washing, library amplification and clean-up, and (iv) qPCR quantification of capture yields. Capture can be performed in a single or two successive rounds of hybridization capture, with two-round capture being our current default.

References

Fu, Q., Meyer, M., Gao, X., Stenzel, U., Burbano, H. A., Kelso, J., & Pääbo, S. (2013). DNA analysis of an early modern human from Tianyuan Cave, China. Proceedings of the National Academy of Sciences of the United States of America, 110, 2223-2227.

Gnirke, A., Melnikov, A., Maguire, J., Rogov, P., LeProust, E.M., Brockman, W., Fennell, T., Giannoukos, G., Fisher, S., Russ, C., Gabriel, S., Jaffe, D. B., Lander, E. S., Nusbaum, C. (2009) Solution hybrid selection with ultra-long oligonucleotides for massively parallel targeted sequencing. Nat Biotechnol. 27(2), 182-189

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

	Reagent	Supplier	Cat. no.
	Large-volume buffers/reagents		
	1x BWT †	self	-
	Wash buffer 1 †	self	-
	Hot wash buffer (HWT) †	self	-
	EBT and EB buffer †	self	-
	80% EtOH (v/v) ‡	self	-
	SPRI beads (38% PEG) †	self	-
	TE buffer †	self	-
	Other reagents		
	Capture positive controls (CPC)	self	-
	Dynabeads MyOne Streptavidin C1 (10 mg/ml)	Thermo Fisher Scientific	65002
	Water, MilliQ		
	500µM Blocking oligo BO4.P7.part1.R §	IDT	-
	500µM Blocking oligo BO6.P7.part2.R ‖	IDT	-
	500µM Blocking oligo BO8.P5.part1.R ¶	IDT	-
	500µM Blocking oligo BO11.P5.part2.R *	IDT	-
	2x HI-RPM hybridization buffer	Agilent	5188-5380
	Agilent blocking agent	Agilent	5188-5380
	Cot-1 DNA (1µg / µl)	Thermo Fisher Scientific	15279011
	Single-stranded DNA capture probes ‖‖	self	-

	Reagent	Supplier	Cat. no.
	Equinox ready mix 2x ‡‡	Watchmaker Genomics	7K0021-384
	100 µM primer IS105 $	IDT	-
	100 µM primer IS109 ††	IDT	-
	Maxima SYBR Green qPCR Master Mix 2X, aliquotted in X µl volumes to 5 ml Eppendorf tubes)	Thermo Fisher Scientific	K0252
	100 µM primer IS05 **	IDT	-
	100 µM primer IS06 §§	IDT	-
	qPCR standard dilution ¶¶	self	-
	Consumables		
	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
	Eppendorf twin.tec PCR plates 96, skirted	neoLab Migge	VB-0403
	5 ml Eppendorf tubes	Kisker	G05-ML
	1.5 ml micro test tube, safe-lock	neoLab Migge	VB-0306
	Filter tip, 1000 µl, Diamond Tips	neoLab Migge	CF-0425
	Filter tip, 200 µl, Diamond Tips	neoLab Migge	CF-0438
	2 ml screw capped tubes	Sarstedt AG	72.694.005
	25 ml glass pipette	Corning BV	357525
	Peelable aluminium seal (PlateLoc)	Agilent	24214-001
	Optical heat seal foil	Bio-Rad	1814030
	Comfort lid	VWR	HAMI814300
	Deep-well MIDI plate, 96 well storage plate	Thermo Fisher Scientific	12194162

	Reagent	Supplier	Cat. no.
	Reagent container with lid, 60 ml	VWR	736-0831
	Open buffer reservoir, 25 ml	Roth	HT66.1
	Hard Shell PCR plate 384-well, thin-wall, clear shell, white well	Bio-Rad	HSP3805
	Microseal B Adhesive seal, optical	Bio-Rad	MSB1001

† See documents in the Appendix for preparation of buffers and controls.
‡ Dilute 100% EtOH in water to obtain a 80 % solution.
§ Order blocking oligonucleotide BO4.P7.part1.R at 1 µmol synthesis scale (IDT, desalted). Dissolve in water at a concentration of 500 µM. Sequence: 5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-Pho-3'
|| Order blocking oligonucleotide BO6.P7.part2.R at 1 µmol synthesis scale (IDT, desalted). Dissolve in water at a concentration of 500 µM. Sequence: 5'-CAAGCAGAAGACGGCATACGAGAT-Pho-3'
¶ Order blocking oligonuleotide BO8.P5.part1.R at 1 µmol synthesis scale (IDT, desalted). Dissolve in water at a concentration of 500 µM. Sequence: 5'-GTGTAGATCTCGGTGGTCGCCGTATCATT-Pho-3'
* Order blocking oligonucleotide BO11.P5.sslib_part2.R at 1 µmol synthesis scale (IDT, desalted). Dissolve in water at a concentration of 500 µM. Sequence: 5'-GGAAGAGCGTCGTGTAGGGAAAGAGTGT-Pho-3'
‡‡ Mix is pre-aliquoted (Aliquots of 2,151 µl in 5 ml Eppendorf tubes).
$ Order oligonucleotide IS105 at 0.25 µmol synthesis scale (IDT, desalted). Dissolve in TE buffer (see document in the Appendix for preparation of TE buffer) at a concentration of 100 µM.
Sequence: 5'-AATGATACGGCGACCACCGAGATCTACAC-3'
†† Order oligonucleotide IS109 at 0.25 µmol synthesis scale (IDT, desalted). Dissolve in TE buffer (see document in the Appendix for preparation of TE buffer) at a concentration of 100 µM.
Sequence: 5'-CAAGCAGAAGACGGCATACGAGAT-3'
** Order oligonucleotide IS05 at 0.25 µmol synthesis scale (IDT, desalted). Dissolve in TE buffer (see document in the Appendix for preparation of TE buffer) at a concentration of 100 µM. Sequence: 5'-AATGATACGGCGACCACCGA-3'
§§ Order oligonucleotide IS06 at 0.25 µmol synthesis scale (IDT, desalted). Dissolve in TE buffer (see document in the Appendix for preparation of TE buffer) at a concentration of 100 µM. Sequence: 5'-CAAGCAGAAGACGGCATACGA-3'
¶¶ See documents in the Appendix for the preparation of a qPCR standard dilution. The standard should be provided in the first column of an Eppendorf twin.tec PCR plate 96.
||| See documents in the appendix for the preparation of single-stranded DNA capture probes. Probe concentration should be adjusted to 100 ng/µl for capture of nuclear targets (e.g., AA213), 1 ng/µl for capture of mitochondrial genomes (e.g., AA163) and 20 ng/µl for simultaneous capture of many mitochondrial genomes (e.g., AA75). These concentrations result in 500, 5 and 50 ng of probe per capture reaction, respectively.

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 ODTC (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, Holmes and Richard.
- 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)
- Magnetic rack for Eppendorf tubes (e.g., MagJET Separation Rack, ThermoFisher Scientific, cat.no. MR02)
- Plate sealer (G5585B PlateLoc Thermal Microplate Sealer using peelable aluminum seal)
- Plate sealer (e.g., Bio-Rad Px1 PCR plate sealer, cat. no. 1814000)
- Brady Label printer and corresponding labels
- 384-format qPCR system (e.g., C1000 Touch Thermal Cycler CFX 384 Real-Time System, Bio-Rad)
- NanoDrop system (NanoDrop™ 8000 Spektralphotometer)

Electronic protocol files

Available on Github : https://github.com/adnacore/Hamilton-NGS-Star-workstation-in-solution-hybridization-capture/tree/v1.0

Appendix

Document for custom-specific modifications of the deck layout

Document

NAME

DeckLayout: automated in-solution hybridization capture (MPI-EVA method)

CREATED BY

Julia Zorn

Preview

Documents for buffers

Document

NAME

1xBWT buffer

CREATED BY

Ancient DNA Core Unit

Preview

Document

NAME

Wash buffer 1

CREATED BY

Ancient DNA Core Unit

Preview

Document

NAME

HWT buffer

CREATED BY

Ancient DNA Core Unit

Preview

Document

NAME

EBT buffer

CREATED BY

Anna Schmidt

Preview

Document

NAME

EB buffer

CREATED BY

Ancient DNA Core Unit

Preview

Document

NAME

TE buffer

CREATED BY

Anna Schmidt

Preview

Document

NAME

SPRI beads, variable PEG concentration

CREATED BY

Ancient DNA Core Unit

Preview

Documents related to preparation of the qPCR

Document

NAME

qPCR standard for library quantification

CREATED BY

Ancient DNA Core Unit

Preview

Troubleshooting

Reagent and capture sample plate preparation (days/weeks/months before the run)

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

Note

[Documentation]
The Labfolder entry name consists of the name of the Labfolder template and the capture plate ID. Fill in all the required information in the respective fields in Labfolder to track the experiment.
Providing the plate ID and database work is usually performed by the main person responsible for capture preparation in the Core Unit, not by the person performing the experiment.

2 Prepare required buffers and other reagents as described in MATERIALS.

3 Prepare the capture input file and save it in the respective 'in progress' folder on the public server (Z:\AncientDNA\captures\in_progress\in progress_YYYYMMDD_CapPl_XXX_XX).

Note

[Note]
Prepare the capture input file containing libraries and capture negative and positive controls (CNCs and CPCs). Observe the following rules:

- The file requires the following information: the original plate ID (column 'LibPlate'), the position of the library on that plate (column 'LibPlatePos'), the amount of library used (column 'InputVol'), the probe name (column 'CapturePanel'), requirement of human cot-DNA (column 'Blocker'). For CNCs, enter "Control" into the 'LibPlate' field, which will prompt the robot to add water to the respective position of the capture sample plate. CPCs are libraries that were used in previous captures and which provide reference points in qPCR and sequencing for expected capture outcome.
- Add at least 1 CNC per capture experiment. CPCs are optional.
- Calculate the required input volume based on the Nanodrop values of the input libraries: For the first capture round, 1 µg of indexed and purified library is needed. The library input volume is calculated individually for each library (volume = 1000 ng / x, with x being the library concentration in ng/µl). For the second capture round, 500 ng of input is needed. For simplicity, the same input volume is used for all libraries (volume = 500 ng / x, with x being the mean library concentration of the library plate from capture round 1 in ng/µl).
- Make sure the format of the file is "**.xls (Excel 97-2003 Workbook)**". An empty template and an example file are provided below.

[Documentation]
Add the capture input file to the respective entry in Labfolder.

example_file.xls 44KB empty_template.xls 54KB

4 Thaw all required source library plates and controls and briefly centrifuge them. Do not vortex the plates.

Note

[Note]
For capture round 1, the source libraries are indexed and purified libraries found on 96-well plates named Pl_XXX. Visually check if all wells of the source library plates contain at least 10 µl library. If there are wells with lower volume, check if the wells are needed for capture. If so, a reamplification has to be performed (see separate protocol; add plate to the list of libraries for reamplification). Plates containing reamplified libraries are named ReAmpPl_XXX.

For capture round 2, the source libraries are the capture libraries from round 1 that are located on a 96-well plate, which has been stored in the fridge.

5 Label the capture sample plate (an empty full-skirted 96-well PCR plate) with Plate ID, 'sample plate', date and your Initials.

6 Turn on the Hamilton robot and the connected components beneath (three components).

7 Log into the computer as user 'Hamilton' (password: 'hamilton').

8 Open the software Method Manager and log in using 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 'Libraries to 96-well plate setup' which can be found in the section 'preparation protocols'. A dialog will ask to record the run. Click 'Ok' to save the run data for possible later run evaluation. Load the capture input file and click "Ok".

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!

9 Set up the Hamilton deck as indicated by the electronic protocol 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 (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 30-35: Place the capture sample plate at position 2 and the first source library plate at position 3.
- Track 43-49: Fill a 2 ml screw capped tube with 1 ml of water, remove the cap and place the tube into well A1 of the CPCA adapter (the black 24-well aluminum adapter for tubes) on position 4.

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

Note

[Note]
The robot will now transfer the sample libraries from the source library plates to the capture sample plate. Run time is between ca. 30 min and 90 min depending on the number of source plates.

During the preparation of the capture sample plate

11 After dispensing water to the capture sample plate, the robot will ask for the required source plates one by one. When prompted, take out the current plate and seal the it with peelable foil at 175 °C for 1.0 s using the PlateLoc. Insert the next plate when asked. Change gloves every time you handled an open library source plate.

Note

[Note]
Based on volumes provided in the input file, the robot combines sample libraries and water to a final volume of 10 µl for each well.

Finishing the preparation of the capture sample plate

12 When the sample plate preparation has finished, take out the capture sample plate (containing 10 µl sample per well) and seal the plate with peelable foil at 175 °C for 1.0 s

using the PlateLoc. Visually check the plate to verify that all wells contain the same volume. Store the capture sample plate in the fridge until used further.

Note

[Documentation]
In case of unexpected observations, add comments in the 'notes' section of Labfolder as well as in the documentation of the experiment on Mattermost.

13 Discard the 2 ml screw capped tube containing water and clean out the trash bins.

14 If necessary, return the source library plates to their storage location.

15 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.

Capture day 1 - preparing the hybridization

16 Thaw the following reagents at room temperature:
- the required capture probes (ready-to-use biotinylated single-stranded DNA probes)
- blocking oligos (BO4, BO6, BO8, BO11)
- blocking agent
- optional: human cot-DNA (depending on the probe sets used, see 'Blocker' field in input file)

Note

[Note]
If you do not know the required reagents and their volumes, thaw reagents only after reaching 24

17 Turn on the Hamilton robot and the connected devices beneath (three devices).

18 Log into the computer as user 'Hamilton' (password: 'hamilton').

19 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 'HomeBrew InSolution Capture' which can be found in the section 'InSolution Capture'. A dialog will ask to record the run. Click 'Ok' to save the run data for possible later run evaluation.

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

21 Choose "**Process 1**: **Hybridization setup**" as starting point from the menu and click 'Accept'.

Note

[Note]
The capture process can be started/ended at any of the points listed below:
Process 1: Hybridization set-up
Process 2: Overnight hybridization
Process 3: Post-Capture and washing
Process 4: qPCR template plate preparation
Process 5: Post-capture PCR
Process 6: Post Capture SPRI-bead clean-up

22 Choose "**Process 1**: **Hybridization Setup**" as ending point from the menu and click 'Accept'.

Note

[Note]
Starting and ending point are the same, because the overnight hybridization is currently not performed on the robot but in a thermal cycler.

23 Load the capture input file as described in go to step #3 and click "Ok".

24 A dialog will open displaying the composition of the blocker mix and probe sets required for the run. The corresponding file 'MastermixSummary.csv' can be found in: C:\Program Files (x86)\Hamilton\Logfiles. Print the file and save it in the respective capture 'in progress' folder on the public server (Z:\AncientDNA\captures\in_progress\in progress_YYYYMMDD_CapPl_XXX_XX). Click "Ok" to continue.

Note

[Note]
The calculation for the required mixes includes excess already.

[Documentation]
Attach the MastermixSummary.csv file to your Labfolder entry.

25 Briefly vortex and centrifuge the reagents mentioned in go to step #16. Prepare the mixes as indicated in the master mix file and transfer the master mixes to 2 ml screw capped tubes. If large volumes are required, split the master mixes into two tubes as indicated in the dialog and the MastermixSummary file.

Note

[Note]
The hybridization mix is very foamy and difficult to pipette. Carefully mix and intensely spin down the blocker mix before transferring it to 2 ml screw capped tubes. Make sure there are no bubbles remaining!

[Documentation]
Fill in the respective fields in Labfolder to document the reagents information.

Note

[Note]
Although the input volume of probe per capture reaction is always 5 µl, the final concentration of probes can differ among probe sets. The dialog will give you the exact volume and concentration required for each probe set. If necessary, dilute the probes with EB buffer so that their concentration matches the requirements.

26 Briefly centrifuge the capture sample plate from go to step #12.

27 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 (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 30-35: Place the capture sample plate on position 3.
- Track 43-49: Remove the caps from the tubes with the prepared mixes and put them into wells A1, B1, C1, etc. of the CPCA adapter (the black 24-well aluminum adapter for tubes) on position 4.

28 Start run by clicking the "Ok" button and follow the instructions.

Note

[Note]
Run time varies depending on number of probe sets (40-60 min).

Capture day 1 - finishing the hybridization

29 Turn on a thermal cycler to preheat the device. Set the block temperature to 65 °C and the lid temperature to 68 °C.

30 Once the robot gave notification that the protocol has finished, remove the capture sample plate containing the hybridization reaction from position 3 of track 30-35 and seal the plate carefully with peelable aluminum seal using the PlateLoc at 175°C for 1.5s.

31 Incubate the plate at 65 °C in the preheated thermal cycler (lid temperature set to 68° C). The default incubation time is ~20 hours, but may range from 16 to 72 hours.

Note

[Note]
In case you need to transport the capture sample plate to a thermo cycler located in another room, put the plate into a box without sealing it, as each round of sealing damages the rims of the wells. Transport carefully to avoid spilling of liquid!

[Documentation]
Fill in the fields in Labfolder detailing the incubation time.

32 Press "Ok" to finish the run. Clean up the robot as indicated by the protocol. Empty the plastic waste and wipe the metal part of the tip trash with Ethanol.

33 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.

Capture day 2 - preparing the post-hybridization workflow

34 Thaw one aliquot of each of the following reagents at room temperature:
- Equinox ready mix (2x)
- Oligos IS105 (100 µM) and IS109 (100 µM)

35 Prepare streptavidin beads:

35.1 Fully resuspend stock suspension of MyOne C1 beads by vortexing. Make sure no bead pellet remains.

35.2 Transfer 1,000 µl stock suspension of beads into an Eppendorf 1.5 ml Safe-lock tube. Pellet beads in magnetic rack, pipette off and discard supernatant. Add an additional 1,000 µl stock suspension of beads (2,000 µl used in total), pellet the beads and discard the supernatant.

35.3 Wash beads twice with 1,000 µl 1xBWT buffer and discard the wash buffer.

35.4 Resuspend beads in 1,000 µl 1xBWT. Add 17 ml 1xBWT buffer into a plastic reservoir and transfer the washed beads into the reservoir. Mix the bead suspension by thoroughly pipetting up and down until it looks homogenous.

35.5 Dispense 180 µl of bead suspension into each well of a 96-well MIDI plate using a multi-channel pipette.

36 Turn on the Hamilton robot and the connected components beneath (three components).

37 Log into the computer as user 'Hamilton' (password: 'hamilton').

38 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 'HomeBrew InSolution Capture' which can be found in the section 'InSolution Capture'. A dialog will ask to record the run. Click 'Ok' to save the run data for possible later run evaluation.

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

40 Choose "**Process 3: Post-Capture and washing**" as starting point from the menu and click 'Accept'.

41 Choose "**Process 6: Post Capture SPRI-bead clean-up**" as ending point from the menu and click 'Accept'.

42 Load the capture input file as described in go to step #3 and click "Ok".

43 Set the following parameters as indicated here:

Run Post Capture PCR on deck thermal cycler (ODTC)? →**YES**
Store the plate with purified libraries on the deck thermal cycler (ODTC) at the end of the method? →**NO**

Parameter selection:
Process 2. Hybridization:
Hybridization temperature: 65 °C (should be greyed out as this was performed outside of the robot)
Hybridization time: 16 hours (should be greyed out as this was performed outside of the robot)
Process 3. Capture and wash:
Post hybridization reload → checked (should be greyed out as this was performed outside of the robot)
Volume wash buffer 2: **200**
Number of hot wash steps: **3**

Process 5. Post Capture PCR:
Number of PCR cycles: capture round 1: **20 cycles**; capture round 2: **15 cycles**
Process 6. Clean-up of Captures:
Volume beads: **40 µl**
Sample transfer volume: **47 µl**
Volume elution buffer: **30 µl**
Sample bead separation time on magnet: **300 s**
Bead air-drying time: **600 s**
Using the metal lids: **checked** (if used, as per default)

Confirm your choice by clicking "Continue".

44 A dialog will open displaying the composition of the Equinox PCR master mix required for the run. The corresponding file 'MastermixSummary.csv' can be found in: C:\Program Files (x86)\Hamilton\Logfiles. Print the file and save it in the respective capture 'in progress' folder on the public server (Z:\AncientDNA\captures\in_progress\in progress_YYYYMMDD_CapPl_XXX_XX). Click "Ok" to continue.

Note

[Note]
The calculation for the required mixes includes excess already.

[Documentation]
Attach the master mix file to your Labfolder entry if working with less than a full capture plate (for a full plate, volumes are always the same).

45 Vortex and centrifuge the reagents listed in the table below. Prepare the PCR master mix as indicated in the master mix file by adding the primers to the Equinox ready mix provided in a 5 ml Eppendorf tube. Distribute the master mix to two 2 ml screw capped tubes by adding 1118.2 µl to each.

	Reagent	Volume (µl)	Volume per reaction (µl)	Final concentration
	Equinox ready mix (2x)	2150.4	20	1x
	IS105 (100 µM)	43	0.4	1 µM
	IS109 (100 µM)	43	0.4	1 µM

Note

[Note]
It is important to vortex the Equinox ready mix intensely after thawing.

[Note]
To save time, experienced users can proceed to the next step before the master mix is prepared. There will be enough time to prepare the mix while the robot is running.

[Documentation]
Fill in the respective fields in Labfolder to document the reagents information.

46 Set up the Hamilton deck as indicated by the robot dialog and confirm by clicking "Ok". This includes the following steps:

- Track -2-5: Place three MIDI plates to position 1, two Eppendorf full skirted 96 well plates (HSP plates) to position 3 (qPCR plates) and two Eppendorf full skirted 96 well plates (HSP plates) to position 4 (capture sample plates).
- 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).

Note

[Note]
The robot will give a warning that the tips required exceed the number of tip boxes available on the deck. Ignore and continue. Tips will be reloaded manually during the run.

- Track 30-35: Place a comfort lid on position 1. Make sure the label 'A' is positioned in the upper left corner. Place the MIDI plate with the MyOne C1 beads to position 4.
- Track 43-49: Remove the caps from the two 2 ml screw capped tubes with Equinox mix and put them into wells A1 and B1 of the CPCA adapter (the black 24-well aluminum adapter for tubes) on position 4. Add the black 96 well adapter to position 3.
- Track 53 and 54: 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.

	Track	Position	Reagent	Volume [ml]
	53	1	EtOH 80%	45.2
	53	2	empty	N/A
	53	3	empty (reserved for SPRI beads (38% PEG))	(7.2)
	53	4	spaceholder reservoir	N/A
	53	5	spaceholder reservoir	N/A
	54	1	Wash buffer 1	26.1
	54	2	Hot wash buffer (HWT)	36.6
	54	3	Hot wash buffer (HWT)	36.6
	54	4	Water	6.1
	54	5	EBT buffer	9.9

Note

[Note]
To prevent the SPRI beads from pelleting, leave them in the fridge until the run is paused for the refill of tip boxes.

The spaceholder reservoirs in track 53 (position 4 and 5) serve as a lid holder during the run. Place them into the correct position and remove the plastic lids.

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

47 Start run by clicking "Ok" and follow the instructions.

Note

[Note]
The total run time is ca. 4 h 45 min.

Capture day 2 - during the post-hybridization workflow

48 The robot will start preparing the MyOne C1 beads. After approx. 5 minutes, the robot will ask for the
post hybridization plate (capture plate from day 1). Remove the lid of the plate carefully **while it is still located in the thermo cycler**. Transfer the plate to position 3 of track 30-35. After transferring the plate, change gloves. Continue the run by clicking "Ok".

Note

[Note]
Check if wells in the post hybridization plate are equally filled. If evaporation occurred in specific wells, make a note in Labfolder.

49 After the amplification step, approx. 2.5 - 3 hours into the run, the robot will send an E-Mail notification that the pipette tips need to be refilled. For tip refilling, follow the instructions of the robot.

50 Gently shake the SPRI beads (38% PEG) until the beads have fully resuspended. Add ca. 12 ml SPRI bead suspension to a reservoir and place it to position 3 of track 53. Press "Ok" to continue the run.

Note

[Note]
If qPCR is performed directly after the run, this is a good moment to prepare the qPCR Mastermix. It is even possible to take out the qPCR plate now (track 43-49, position 3), put an empty spaceholder plate into the hamilton deck and perform the qPCR already on another robot.

Capture day 2 - finishing the post-hybridization workflow

51 The robot will send a notification via E-Mail that the run has finished. Remove the final capture plate from position 3 and the qPCR dilution plate from position 2 on track 30-35 and seal the plates carefully with peelable aluminum seal using the PlateLoc at 175°C for 1.0 s.

52 Store the plates in the fridge until they are used for NanoDrop/qPCR measurements and the next round of capture or pooling.

53 Take the MIDI plate with bead slurry from position 4 on track 36-42 and close it with a BioRad Microseal 'B' adhesive seal. Store in fridge until all quality checks have been performed and pooling of the capture plate is completed.

Note

[Note]
Print labels for the final capture plate, the qPCR dilution plate and the bead slurry plate including the capture plate ID, round 1/2, date and your initials.

Label a box with the capture plate ID, round 1 & 2, the date and your initials and store it in fridge until further use.

The MIDI plate containing bead slurry should be stored next to it in the fridge.

54 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.

55 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.

Capture library quantification using qPCR

56 Thaw the qPCR reagents (Maxima SYBR green qPCR mix, primers IS5 and IS6, one aliquot each) and the PCR plate containing the qPCR standard dilution (ranging from 10^2 to 10^8 copies/µl) at room temperature.

Note

[Note]
See MATERIALS for the preparation of a qPCR standard dilution.

57 Take the capture qPCR dilution plate from the fridge and centrifuge it briefly.

58 Turn on the Hamilton robot and the connected devices beneath (three devices).

59 Log into the computer as user 'Hamilton' (password: 'hamilton').

60 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 'qPCR setup SYBR Green' which can be found in the section 'qPCR'. A dialog will ask to record the run. Click 'Ok' to save the run data for possible later run evaluation.

61 Set up the Hamilton deck as indicated by the protocol and confirm by clicking "Ok":

- Load pipette tips as indicated by the robot 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 30-35: Place the Standard dilution plate to position 2 (column 1) and the capture qPCR dilution plate to position 3.
- Track 36-42: Add the silver 384 adapter to position 4 and put a fresh 384 qPCR white well plate on top of the adapter.

62 A dialog will open displaying the composition of the qPCR mix. The corresponding file 'MastermixSummary.csv' can be found in: C:\Program Files (x86)\Hamilton\Logfiles. Print the file and save it in the respective capture 'in progress' folder on the public server

(Z:\AncientDNA\captures\in_progress\in progress_YYYYMMDD_CapPl_XXX_XX). Click "Ok" to continue.

Note

[Note]
The calculation for the required mixes includes excess already.

[Documentation]
Attach the master mix file to your Labfolder entry if working with less than a full capture plate (for a full plate, volumes are always the same).

63 Briefly vortex and centrifuge the reagents required. Prepare the qPCR master mix as indicated in the master mix file by adding water and primers to the Maxima SYBR Green qPCR master mix provided
in a 5 ml Eppendorf tube and mix by vortexing and briefly centrifuge in a 5 ml table centrifuge. Distribute the master mix into two 2 ml screw capped tubes by adding 1189 µl to each.

	Reagent	Volume (µl)	Volume per reaction (µl)	Final concentration
	Maxima SYBR Green qPCR mix (2x)	1321.3	5	1x
	water	1030.6	3.9	
	IS5 (100 µM)	13.2	0.05	500 nM
	IS6 (100 µM)	13.2	0.05	500 nM

Note

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

64 Continue setting up the Hamilton deck as indicated by the protocol and confirm by clicking "Ok":

- Track 43-49: Place the 2 ml screw capped tubes containing master mix into the CPCA adapter (positions A1, B1).

65 Start run by clicking the "Ok" button and follow the instructions.

Note

[Note]
Run time is ca. 10 min.

66 Once the run has completed, remove the qPCR plate from position 4 on track 36-42 and seal it with optical foil using the BioRad sealer at 167 °C for 3.0 s (no frame). Briefly centrifuge the plate to make sure there are no bubbles left in the wells.

67 Transfer qPCR plate to room B4.15 and put it into one of the CFX384 Real-Time PCR systems. Perform the following steps using the BioRad CFX Manager software:

- Go to "Run setup", choose "user defined"
- from the "protocol" tab, choose "select existing", then "CFX_3StepAmp+Melt_SYB_10ul_assay.prcl" from "X:\public\AncientDNA\protocols\qPCR_Bravo\cycling program"
- from the "plate" tab, choose "select existing" and "96_Sample_Plate_384wells_SYBR_Hamilton.pltd" from "X:\public\AncientDNA\protocols\qPCR_Bravo\qPCR_platefile"

Note

[Documentation]
Note down the name of the qPCR machine used for cycling in your entry in Labfolder.

68 Start the cycling and save the file in the corresponding 'in progress' folder on the public server (Z:\AncientDNA\captures\in_progress\in progress_YYYYMMDD_CapPl_XXX_XX).

Note

[Note]
Cycling is performed using the following parameters:

	Step	Temperature °C	Time
	Initial denaturation	95	10 min
	Denaturation	95	30 s
	Annealing	60	30 s
	Extension (incl. measurement)	72	30 s
	Go to 2, 44 times		
	Denaturation curve (default)		5 min

The expected run time is ca. 2.5 hours.

69 Take out the qPCR dilution plate from position 3 on track 30-35 and seal the plate with peelable aluminum seal using the PlateLoc at 175 °C for 1.0 s. Put the plate back into the corresponding capture box.

70 Take out the qPCR standard plate and cover the filled wells with a 8-well striptube cap.

71 Press "Ok" to finish 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.

72 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.

73 After measurement, discard the 384 well qPCR plate. Perform data analysis on any computer connected to the network using the BioRad CFX Manager software.

Expected result

Expected library yields differ among probe sets and sample types. After one round of capture, they often range between 10^7 and 10^9 total molecules, with mitochondrial capture producing lower values than nuclear capture. After a second round of capture, yields increase, typically to a range between 10^9 and 10^10 molecules.

Capture library quantification using Nanodrop device

74 Take the final capture plate out of the fridge and centrifuge it briefly.

Note

[Note]
Capture library plates should always be stored in the fridge until the NanoDrop measurements and pooling are completed. In case a capture plate was frozen, mix all libraries by pipetting up and down using a multichannel pipette. Don't vortex the plate!

75 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.

76 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.

77 Blank the device by adding 1 µl of EBT 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.

78 Measure the concentrations of the final capture plate column-wise using the multi-channel pipette. Clean pedestal after every measurement with a soft tissue.

Expected result

Expected nanodrop values may vary depending on the probe sets and samples used. After one round of capture, they typically range from 75 to 150 ng / µl, but lower or higher values may occasionally occur. Nanodrop values are expected to be similar among sets of libraries from samples with similar properties that were captured with the same probe set.

79 Save the data in the corresponding 'in progress' folder on the public server (Z:\AncientDNA\captures\in_progress\in progress_YYYYMMDD_CapPl_XXX_XX).

Note

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

80 Put the final capture plate back to the corresponding box in the fridge.