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Targeted hybridization capture for phylogenomics

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We use this protocol and it's working

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Abstract

Ultra-Conserved Elements (UCEs) are genomic regions that remain highly conserved across distantly related taxa. **By sequencing both UCEs and their flanking regions, we can reconstruct evolutionary histories and investigate population-level relationships across a wide range of organisms.** Hybridization capture, the core method used here, is versatile and can also be applied to genotyping, gene discovery, rare variant detection, indel and copy number variation analysis, as well as exome sequencing.

We developed this protocol to resolve phylogenetic relationships within the superfamily Chalcidoidea and, more broadly, to study insects of the order Hymenoptera.

Our protocol combines and optimizes two key steps, using commercial kits from NEB and Daicel Arbor Biosciences:
1) **Genomic library construction from very small amounts of DNA**.
2) **Target enrichment to selectively capture libraries containing the UCEs of interest** (with pools of 16 specimens per reaction).

We successfully applied this approach to both ethanol-preserved and dry specimens, ranging from 0.1 cm to several centimeters in size, including rare and historical museum material (type specimens).

DNA was extracted using the Qiagen's DNeasy Blood & Tissue Kit. An average of 30 ng of input DNA was used for library preparation, but samples with DNA quantities below the Qubit detection limit were also successfully processed.

The protocol is scalable, enabling the processing of batches of up to 96 samples in parallel for sequencing on Illumina platforms (e.g., MiSeq, NovaSeq X+).

To minimize costs, we use half the reagents of an NEBNext Ultra II DNA Library Prep Kit, effectively doubling its capacity to produce 192 libraries per kit, and we streamlined several steps to reduce handling time.

For multiplexing, each library is uniquely indexed prior to hybridization capture. We designed 32 P1 adapters and 24 P2 adapters (Illumina-compatible), allowing up to 768 uniquely indexed libraries to be sequenced simultaneously.

Attachments

XLSX

Sequences_adapters_P..

15KB

Materials

Equipment and consumables :

Note

All equipment and consumables described here correspond to those available in our laboratory, but they may be substituted with equivalent alternatives.

1. Pipette Tips (10µl - 200µl - 1000µl)
2. Single-Channel Pipettes (10µl - 200µl - 1000µl)
3. Multi-Channel Pipettes (10µl - 200µl)
4. Multipette E3/E3x electronic multi-dispenser pipette (Eppendorf)
5. Combitips advanced dispenser tips (Eppendorf)
6. Vortex
7. Plate and tube centrifuge (Eppendorf)
8. 0.65 ml Bioruptor® Pico Microtubes (Diagenode)
9. Tube holder for 0.65 ml tubes - Bioruptor® Pico (Diagenode)
10. Bioruptor® Pico (Diagenode)
11. Tube DNA LoBind 1,5 ml PCR clean (Eppendorf)
12. PCR plate without skirt, 96 well, transparent, High Profile, 200µl, PCR Performance Tested, PP (Sarstedt)
13. Invitrogen™ DynaMag™-2 Magnet (Invitrogen)
14. SPRIPlate 96R Ring Super Magnet Plate (Beckman Coulter)
15. Sealing film Adhesive PCR film 4TITUDE (Dutscher)
16. Adhesive Aluminium Film 131×83.6mm (Dutscher)
17. Thermocycler MasterCycler (Eppendorf)
18. Reaction tube, 1.5 ml, PP (Sarstedt)
19. Tube with conical bottom 15 ml (bulk) Falcon® (Dutscher)
20. Tube with conical bottom 50 ml (bulk) Falcon® (Dutscher)
21. Qubit assay tubes (Invitrogen)
22. Qubit 4 Fluorometer (Thermo Fisher)
23. Axygen® 0.2 mL Thin Wall PCR Tubes with Flat Cap, Clear, Nonsterile (Fisher)
24. ThermoMixer® C (Eppendorf)
25. Agilent 2100 Bioanalyzer (Agilent)
26. LightCycler®480 (Roche)

Reagents :

Note

For each reagent, refer to the Safety Data Sheet (SDS) for information on proper handling and disposal.

1. NaCl (Merck)
2. Tris-Cl (Merck)
3. Buffer EB (Qiagen)
4. Ethanol absolute
5. AMPure XP Reagent (Beckman Coulter)
6. NEBNext® Ultra™ II DNA Library Prep Kit for Illumina® (NEB E7645L)
7. Nuclease-Free Water (Qiagen)
8. Qubit dsDNA HS Assay Kit
9. myBaits UCE Hymenoptera 1.5Kv1 (Daicel Arbor Biosciences)
10. 2X KAPA HiFi HotStart ReadyMix (Roche)
11. High Sensitivity DNA Chip (Agilent)
12. Lib Quantification Kit Illumina/Universal (Roche)

Troubleshooting

Safety warnings

All these manipulations are performed with filter tips, dedicated pipettes and according to the principles of forward motion and good laboratory practice. Refer to the procedures of the laboratories used.

Before You Start :

1 **Preparing tagged adapters :**

Note

Homemade tagged adapters will be used during library preparation. Each tagged P1 and P2 adapter consists of two oligonucleotides (Forward and Reverse) hybridized together. The oligonucleotides were ordered from IDT (Integrated DNA Technologies). The sequences of all adapters are provided in the attached file *"Sequences_adapters_P1andP2_protocols.io"* available in the description tab of this protocol.

1.1 **Prepare annealing buffer and store it at 4°C :**

AB buffer 10X, [M] 500 millimolar (mM) NaCl , [M] 100 millimolar (mM) Tris-Cl , (pH 7.5-8.0)

1.2 **Prepare the oligonucleotides at a concentration of 100µM :**

1. Centrifuge the lyophilized oligo tubes thoroughly
2. Resuspend the lyophilized oligos at [M] 100 micromolar (µM) in ultrapure H2O by adding the volume of H2O indicated on the sheet provided by IDT,
3. Incubate the tubes at 4 °C overnight,
4. The next day, vortex and centrifuge the tubes thoroughly before proceeding to quantification.

1.3 **Prepare each oligonucleotide pair at a final concentration of 25 µM :**

1. Vortex and centrifuge the resuspended 100µM oligos thoroughly,
2. Pool the oligo pairs (Forward/Reverse) into 1.5 ml non-stick tubes as follows:

25 µL of Forward oligo (100µM)
25 µL of Reverse oligo (100µM)
10 µL of AB Buffer (10X)
40 µL of ultra pure H2O

3. Vortex and centrifuge the pools thoroughly, then distribute 50 µL of each pool into 2 × 0.2 ml tubes or into a PCR plate,
4. Close the tubes tightly or seal the plate with PCR-specific film.

1.4 **Hybridization of oligonucleotide pairs to generate adapters :**

1. Place the tubes or plate in a thermal cycler, with the heated lid set to ≥ 75°C, and run the following program :

00:02:00 at 95 °C
00:01:00 at 95 °C (15 cycles with -1.7°C / cycle)
00:01:00 at 69 °C (40 cycles with -1°C / cycle)
Hold at 10 °C

2. Vortex and centrifuge the tubes or plate containing the hybridized oligos,
3. Check the concentration of the adapters by measuring 2 µL of a 1:15 dilution using the Qubit™ dsDNA HS Assay kit, following the manufacturer's instructions.

1.5 **Adjustment of adapter concentrations to 10 µM :**

1. Dilute the AB buffer 10 times (1:10) in ultrapure water to obtain 1X AB buffer,
2. Adjust adapter concentration to [M] 10 micromolar (µM) in 1X AB buffer, with a final volume of 100 µL. The AB buffer prevents the dissociation of the two strands of the adapters.
3. Vortex and centrifuge the adapters thoroughly, then aliquot them into 0.2 mL tubes or tube strips for storage at [M] 10 micromolar (µM) .
4. Store the aliquots at -20 °C ,
5. Adapters will be used at [M] 1.5 micromolar (µM) in this protocol.

Part I : Library preparation (using the NEBNext® Ultra™ II DNA Library Prep Kit)

2 **Normalize DNA for DNA shearing :**

Prepare 100 µL of each 96 DNA samples in 96 × 0.65 mL microtubes (Diagenode). An average of 30 ng of input DNA is used for library preparation, but samples with DNA quantities below the Qubit detection limit were also successfully processed.

Note

The microtubes can be stored at 4 °C overnight or at -20 °C for up to one month before shearing.

3 **DNA Shearing (using the Diagenode Bioruptor Pico) :**

1. Keep the microtubes at 4 °C or on ice throughout the shearing step,
2. Briefly vortex and centrifuge the microtubes,
3. Remove any bubbles you may see in the microtubes,
4. Install the microtubes in the 0.5/0.65ml tube holder,
5. To obtain ~400 bp fragments, select the following program: 15 s ON / 90 s OFF for 8 cycles.
6. Keep the microtubes at 4 °C or on ice after shearing,

Note

The sheared DNA can be stored at 4 °C overnight or at -20 °C for up to one month before the next step.

4 **DNA Reconcentration (optional) :**

Note

For samples with more than 10 ng of input DNA for shearing, we use half of the shearing volume (50 µL) as input for the next step (End Prep). For samples with less than 10 ng of input DNA, we reconcentrate the entire DNA volume to 50 µL before proceeding. Therefore, the step detailed below is only required for certain samples.

1. Let the Beckman Coulter™ Agencourt AMPure XP beads stand for 00:30:00 at Room temperature
2. Bring the fragmented DNA microtubes to Room temperature
3. Vortex the beads thouroughtly,
4. Transfer the 100 µL of fragmented DNA into 1.5mL DNA LoBind tubes or into the wells of a 96-well plate,

5. Add 100 µL of Ampure beads to reach a final volume of 200 µL ,
6. Mix thoroughly by pipetting,
7. Incubate for 00:05:00 at Room temperature ,
8. Prepare fresh 70% EtOH (for 96 tubes: mix 30 mL of EtOHabs + 13.8 mL of H2OmiliQ),
9. Move tubes or plate into a magnet rack for 00:05:00 or until the supernatant appear clear,
10. Remove and discard the supernatant without disturbing beads,
11. Add 200 µL of 70% fresh EtOH into the tubes or plate without removing the tubes or plate from the magnetic rack ,
12. Incubate for 00:00:30 at Room temperature ,
13. Remove and discard EtOH,
14. Repeat steps 10 to 12 for a total of two washes,
15. Dry tubes or plate with beads for 00:08:00 at Room temperature to ensure all traces of EtOH are removed. **WARNING :** Do not over-dry the beads, as this will significantly decrease elution efficiency,
16. Add 50 µL of EB Buffer (Qiagen) without removing the tubes or plate from the magnetic rack,
17. Remove tubes or plate from magnetic rack and mix well by pipetting,
18. Incubate for 00:02:00 at Room temperature ,
19. Move tubes or plate into a magnet rack for 00:05:00 or until the supernatant appear clear,
20. Transfer 50 µL of supernatant into wells of a 96-well plate for the next step.

Note

For samples where fragmented DNA did not require reconcentration, transfer 50 µL of DNA in the plate and keep the remaining 50 µL as a back-up (store it at -20 °C).

5 **End Prep :**

1. Thaw NEBNext Ultra II End Prep Reaction Buffer and keep NEBNext Ultra II End Prep Enzyme Mix On ice ,
2. Briefly vortex and centrifuge the NEBNext Ultra II End Prep Reaction Buffer,

3. Prepare the End Prep mix for 96 samples (a volume for 100 samples is calculated here to ensure sufficient volume is available) :

350 µL of NEBNext Ultra II End Prep Reaction Buffer (or 3.5 µL per sample)
150 µL of NEBNext Ultra II End Prep Enzyme Mix (or 1.5 µL per sample)

4. Vortex thoroughly and centrifuge the mix,
5. Add 5 µL of End Prep Mix in each well of the 96-well plate containing fragmented DNA,
6. Add 5 µL of EB Buffer (Qiagen) in each well of the 96-well plate to reach a final volume of 60 µL ,
7. Seal the plate with PCR-specific foil,
8. Briefly vortex and centrifuge the plate,
9. Place the plate in a thermal cycler with the heated lid set to ≥ 75°C, and run the following program :

00:30:00 at 20 °C
00:30:00 at 65 °C
Hold at 10 °C

Note

If necessary, samples can be stored at -20 °C ; however, a slight loss in yield (~20%) may be observed. NEB recommends proceeding with adapter ligation before stopping.

6 **Adapters Ligation :**

Note

In this protocol, we use homemade P1 and P2 adapters (compatible with Illumina sequencing) that are not included in the NEBNext® Ultra™ II DNA Library Prep Kit. To prepare them, refer to the "Before You Start" section of this protocol.

1. Let the Beckman Coulter™ Agencourt AMPure XP beads stand for 00:30:00 at Room temperature ,
2. Thaw the [M] 1.5 micromolar (µM) P1 and P2 adapters at Room temperature , then briefly vortex and centrifuge before use,
3. Thaw the NEBNext Ultra II Ligation Master Mix. Gently vortex and centrifuge before use,
4. Thaw the NEBNext Ultra II Ligation Enhancer. Vortex and centrifuge before use,
5. Keep the reagents on a 0 °C cooling rack during mix preparation.
6. Prepare the Ligation mix for 96 samples (a volume for 100 samples is calculated here to ensure sufficient volume is available) :

1500 µL of NEBNext Ultra II Ligation master mix (or 15 µL per sample)
50 µL of NEBNext Ultra II enhancer (or 0.5 µL per sample)

7. Gently vortex and centrifuge the mix, then keep it on the 0 °C cooling rack.
8. Remove the plate containing 60 µL of DNA (from the end prep step) from the thermocycler and place it on a 0 °C rack,
9. Add 15.5 µL of Ligation mix in each well of the 96-well plate. Note that the mix is very viscous,
10. Add 15.5 µL of EB Buffer (Qiagen) in each well of the 96-well plate to reach a final volume of 91 µL ,
11. Add 1.25 µL of P1 adapters (1.5µM) and 1.25 µL of P2 adapters (1.5 µM) to each well (final reaction volume = 93.5 µL).

For 96 libraries, we use the following layout :

	1	2	3	4	5	6	7	8	9	10	11	12
A	P1-1	P1-9	P1-1	P1-9	P1-1	P1-9	P1-1	P1-9	P1-1	P1-9	P1-1	P1-9
B	P1-2	P1-10	P1-2	P1-10	P1-2	P1-10	P1-2	P1-10	P1-2	P1-10	P1-2	P1-10
C	P1-3	P1-11	P1-3	P1-11	P1-3	P1-11	P1-3	P1-11	P1-3	P1-11	P1-3	P1-11
D	P1-4	P1-12	P1-4	P1-12	P1-4	P1-12	P1-4	P1-12	P1-4	P1-12	P1-4	P1-12
E	P1-5	P1-13	P1-5	P1-13	P1-5	P1-13	P1-5	P1-13	P1-5	P1-13	P1-5	P1-13
F	P1-6	P1-14	P1-6	P1-14	P1-6	P1-14	P1-6	P1-14	P1-6	P1-14	P1-6	P1-14
G	P1-7	P1-15	P1-7	P1-15	P1-7	P1-15	P1-7	P1-15	P1-7	P1-15	P1-7	P1-15
H	P1-8	P1-16	P1-8	P1-16	P1-8	P1-16	P1-8	P1-16	P1-8	P1-16	P1-8	P1-16
	P2-1		P2-2		P2-3		P2-4		P2-5		P2-6	

12. Seal the plate with aluminium foil,
13. Briefly vortex and centrifuge the plate,
14. Place in a thermal cycler, without heated lid, and run the following program :

00:15:00 at 20 °C
Hold at 10 °C

Note

If bead purification cannot be performed immediately, store the samples at -20 °C (e.g., during lunch break or overnight).

7 **Cleanup of Adapter-ligated DNA :**

1. Let the Beckman Coulter™ Agencourt AMPure XP beads stand for 00:30:00 at Room temperature ,
2. If needed thaw and centrifuge the plate containing the ligated DNA,
3. Vortex the beads thoroughly,
4. Transfer 93.5 µL of ligated DNA into 0.2 mL PCR tube strips or into a 96-well plate,
5. Add 75 µL (0.8X) of Ampure beads to reach a final volume of 168.5 µL ,
6. Mix thoroughly by pipetting (or vortexing),
7. Incubate for 00:05:00 at Room temperature ,
8. Prepare fresh 70% EtOH (for 96 tubes: 30 mL of EtOHabs + 13.8 mL of H2OmiliQ),
9. Move tubes or plate into a magnet rack for 00:05:00 or until the supernatant appear clear,
10. Remove and discard the supernatant without disturbing beads,
11. Add 200 µL of 70% fresh EtOH into the tubes or plate without removing the tubes or plate from the magnetic rack ,
12. Incubate for 00:00:30 at Room temperature ,
13. Remove and discard EtOH,
14. Repeat steps 11 to 13 for a total of two washes,
15. Dry tubes or plate with beads for 00:08:00 at Room temperature to ensure all traces of EtOH are removed. **WARNING** : Do not over dry the beads as this will significantly decrease elution efficiency,
16. Add 15 µL of EB Buffer (Qiagen) without removing the tubes or plate from the magnetic rack,
17. Remove tubes or plate from magnetic rack and mix well by pipetting,

18. Incubate for 00:02:00 at Room temperature ,
19. Move tubes or plate into a magnet rack for 00:05:00 or until the supernatant appear clear,
20. Transfer 15 µL of supernatant in a 96-well plate for the next step.

Note

If necessary, the plate can be stored at -20 °C

8 **PCR Enrichment of Adapter-ligated DNA :**

Note

For the enrichment PCR, we use 10µM primers compatible with the adapters used previously.

Forward 5'-AATGATACGGCGACCACCGA-3'
Reverse 5'-CAAGCAGAAGACGGCATACGA-3'

1. Thaw the NEBNext Ultra II Q5 master mix. **Do not** vortex, but gently centrifuge before use,
2. If needed, thaw and centrifuge the plate containing the purified Adapter-ligated DNA,
3. If needed, thaw, vortex and centrifuge the PCR primers Forward and Reverse
[M] 10 micromolar (µM)
4. Prepare the Enrichment PCR mix for 96 samples (a volume for 100 samples is calculated here to ensure sufficient volume is available) :

1250 µL of NEBNext Ultra II Q5 master mix (or 12.5 µL per sample)
100 µL of Primer Forward 10µM (or 1 µL per sample)
100 µL of Primer Reverse 10µM (or 1 µL per sample)

5. Gently vortex and centrifuge the mix,
6. Add 14.5 µL of Enrichment PCR mix to each well of the 96-well plate that contains the purified Adapter-ligated DNA,
7. Then, add 14.5 µL of EB Buffer (Qiagen) to each well of the 96-well plate to reach a final volume of 44 µL .

8. Seal the plate with PCR-specific foil,
9. Briefly vortex and centrifuge the plate,
10. Place the plate in a thermal cycler, with the heated lid set to ≥ 75°C, and run the following program :

00:00:30 at 98 °C
Followed by 15 cycles :
00:00:10 at 98 °C
00:01:15 at 65 °C
And a final extension :
00:05:00 at 65 °C
Hold at 10 °C

Note

If necessary, samples can be stored at -20 °C

9 **Quantification of the amplified libraries :**

1. If needed, thaw the amplified libraries, vortex and centrifuge the plate,
2. Quantify 2 µL from each well using the Qubit™ dsDNA HS Assay kit, following the manufacturer's instructions.

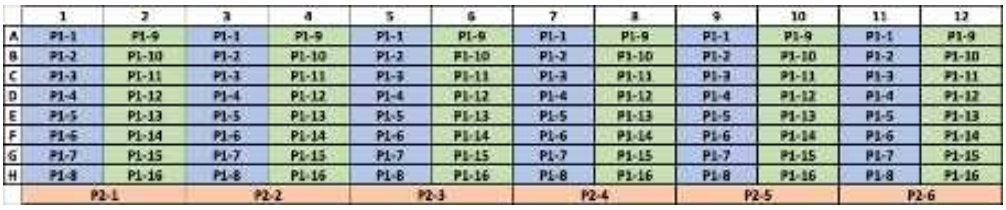
10 **Equimolar pooling and bead-based re-concentration of amplified libraries :**

Note

At this stage, the libraries (one per sample) could be sequenced. However, to enrich them for fragments containing only UCEs, a probe hybridization capture step will be performed.
Before capture, the libraries will be pooled in groups of 16 (100 ng per sample) to generate a total of six pools.

1. Let the Beckman Coulter™ Agencourt AMPure XP beads stand for 00:30:00 at Room temperature ,
2. Pool the libraries in groups of 16 (100 ng each) into six 1.5 mL Eppendorf Low-Bind tubes, for example following the plate layout below:

Pool 1 = columns 1 & 2
Pool 2 = columns 3 & 4
...
Pool 6 = columns 11 & 12



	1	2	3	4	5	6	7	8	9	10	11	12
A	P1-1	P1-9	P1-1	P1-9	P1-1	P1-9	P1-1	P1-9	P1-1	P1-9	P1-1	P1-9
B	P1-2	P1-10	P1-2	P1-10	P1-2	P1-10	P1-2	P1-10	P1-2	P1-10	P1-2	P1-10
C	P1-3	P1-11	P1-3	P1-11	P1-3	P1-11	P1-3	P1-11	P1-3	P1-11	P1-3	P1-11
D	P1-4	P1-12	P1-4	P1-12	P1-4	P1-12	P1-4	P1-12	P1-4	P1-12	P1-4	P1-12
E	P1-5	P1-13	P1-5	P1-13	P1-5	P1-13	P1-5	P1-13	P1-5	P1-13	P1-5	P1-13
F	P1-6	P1-14	P1-6	P1-14	P1-6	P1-14	P1-6	P1-14	P1-6	P1-14	P1-6	P1-14
G	P1-7	P1-15	P1-7	P1-15	P1-7	P1-15	P1-7	P1-15	P1-7	P1-15	P1-7	P1-15
H	P1-8	P1-16	P1-8	P1-16	P1-8	P1-16	P1-8	P1-16	P1-8	P1-16	P1-8	P1-16
	P2-1		P2-2		P2-3		P2-4		P2-5		P2-6	

3. Measure the exact volume of each pool,
4. Vortex the beads thoroughly,
5. Add 0.8 V of Ampure beads for 1 V of libraries pool,
6. Mix thoroughly by pipetting or vortexing,
7. Incubate for 00:05:00 at Room temperature ,
8. Prepare fresh 70% EtOH (for 6 tubes: 2 mL of EtOHabs + 920 µL of H2OmiliQ),
9. Move tubes into a magnet rack for 00:05:00 or until the supernatant appear clear,
10. Remove and discard the supernatant without disturbing beads,
11. Add 200 µL of 70% fresh EtOH into the tubes without removing them from the magnetic rack ,
12. Incubate for 00:00:30 at Room temperature ,
13. Remove and discard EtOH,
14. Repeat steps 11 to 13 for a total of two washes,
15. Dry tubes with beads for 00:08:00 at Room temperature to ensure all traces of EtOH are removed. **WARNING :** Do not over dry the beads as this will significantly decrease elution efficiency,
16. Add 10 µL of EB Buffer (Qiagen) without removing the tubes from the magnetic rack,
17. Remove the tubes from the magnetic rack and mix thoroughly by pipetting,
18. Incubate for 00:02:00 at Room temperature ,
19. Move tubes back to the magnet rack for 00:05:00 or until the supernatant appear clear,

20. Transfer 10 µL of supernatant into new, unused Eppendorf Low-Bind 1.5 ml tubes,
21. Briefly vortex and centrifuge the tubes,
22. Quantify 1 µL of each pool using the Qubit™ dsDNA HS Assay kit, following the manufacturer's instructions.
23. In 0.2 mL low-bind tubes (here labeled "LIB 1" to "LIB 6"), normalize 7 µl of each pool to obtain 100-500 ng of material for the probe hybridization capture protocol.

Note

If necessary, samples can be stored at 4 °C overnight or at -20 °C for up to 1 month.

At this stage, 96 individual genomic DNA libraries have been constructed, tagged, and pooled into six groups of 16. Using a commercial kit from Daicel Arbor Biosciences (myBaits UCE Hymenoptera 1.5Kv1), each pool will now be enriched for the target sequences (UCEs). This kit contains complementary oligonucleotides to the UCEs, transcribed into biotinylated RNA, which serve as probes for hybridization. The baits to capture UCEs were designed by Faircloth et al. 2015. doi: 10.1111/1755-0998.12328

Part II : Probe hybridization capture of libraries (using the myBaits® hybridization capture kit)

11 **Hybridization :**

Note

The protocol described here follows the recommendations of version v5.03 (June 2023) of the *Hybridization Capture for Targeted NGS Manual* from Daicel Arbor Biosciences. Please ensure compatibility with the version of the kit you are using. If necessary, the most recent version can be downloaded from the manufacturer's website.

In this step, sequencing libraries are mixed with various blocking nucleic acids, denatured, and then combined with a mixture of hybridization reagents (including baits). The hybridization reactions are then incubated for several hours to allow the baits to encounter and hybridize with the target UCEs.

1. Preheat a thermomixer to 60 °C ,

2. Thaw the following reagents and keep them at Room temperature. Vortex and centrifuge before use. If HybN and/or HybS remain cloudy, place them at 60 °C for 00:05:00 until the crystals are completely dissolved :
   Hyb N
   Hyb S
   Hyb D
   Hyb R
   Block C
   Block O
   Block X
3. Thaw the baits and keep them On ice
4. Prepare the hybridization mix for 6 pools :

55.5 µL of Hyb N (or 9.25 µL per pool)
21 µL of Hyb D (or 3.5 µL per pool)
3 µL of Hyb S (or 0.5 µL per pool)
7.5 µL of Hyb R (or 1.25 µL per pool)
33 µL of Baits (or 5.5 µL per pool)

5. Gently vortex and centrifuge the mix,
6. Incubate the mix at 60 °C for 00:10:00 in the thermomixer. Remove the mixture from the heat block and allow it to return to Room temperature for 00:05:00 ,
7. For each capture reaction (6 in total here), aliquot 18.5 µL of Hybridization mix into 0.2 mL low-bind tubes (here labeled "HYB 1" to "HYB 6"),
8. Prepare the blockers mix for 6 pools :

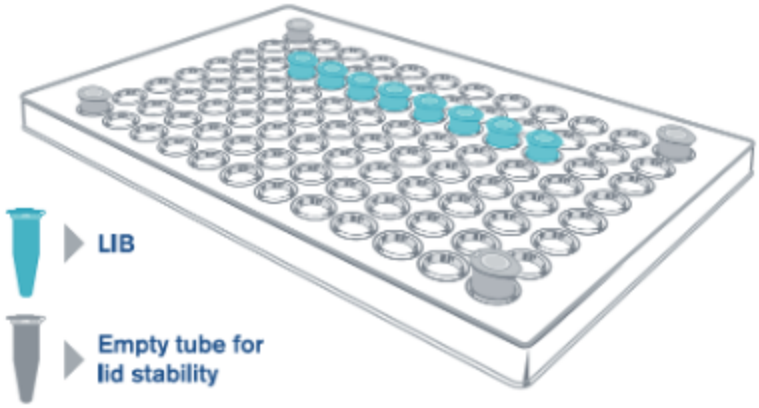
15 µL of Block O (or 2.5 µL per pool)
15 µL of Block C (or 2.5 µL per pool)
3 µL of Block X (or 0.5 µL per pool)

9. Gently vortex and centrifuge the mix,
10. Thaw the tubes containing 7 µL of each previously prepared library pool (here "LIB 1" to "LIB 6"),

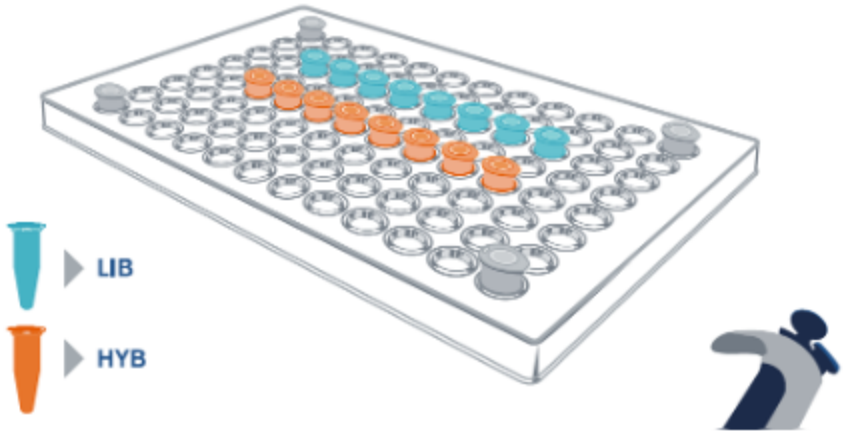
11. Add 5 µL of blockers mix to each "LIB" tubes and mix by pipetting (total volume 12 µL),
12. Preheat a thermocycler, with the heated lid set to ≥ 75°C, and program it with the following thermal cycling conditions :

00:05:00 at 95 °C (step 1)
00:05:00 at 65 °C (step 2)
Hold at 65 °C

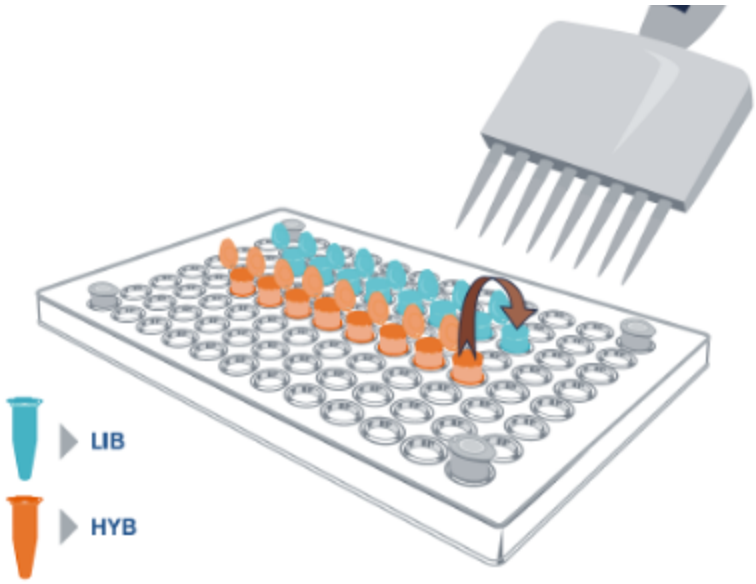
13. Put the "LIB" tubes in the thermal cycler, close the lid, and start the program,



14. Once the cycler reaches the hybridization temperature (65°C, step 2), pause the program, put the "HYB" tubes in the thermocycler, close the lid, and resume the program,



15. Once step 2 of the program is complete, and while keeping all tubes in the thermocycler, pipette 18 µL from each "HYB" tubes into the corresponding "LIB" tubes. Use a multichannel pipette to facilitate the process. Gently homogenize by pipetting up and down 5 times,



16. Dispose of the "HYB" tubes, close the lid, and allow the reactions to incubate at the hybridization temperature (65 °C) for 16:00:00 to 24:00:00 ,

Note

WARNING: The process must NOT be stopped here — it is essential to proceed immediately to the next step.

12 **Bind and wash :**

Note

In this step, bait–target hybrids are bound to streptavidin-coated magnetic beads, and most non-target DNA is removed through several rounds of washing with pre-warmed buffer. This procedure is typically performed the day after completion of the hybridization step.

12.1 **Preparation of Wash Buffer X:**

1. Preheat a thermomixer to 65 °C ,
2. Thaw the following reagents and keep them at Room temperature . Vortex and centrifuge before use (if HybS remain cloudy, place it at 60 °C for 00:05:00 until the crystals are completely dissolved) :
   Hyb S
   Binding Buffer
   Wash Buffer
   Streptavidin Beads
   Buffer E
3. In a 15 ml tube, prepare Wash Buffer X for 6 pools :

54 µL of Hyb S (or 9 µL per pool)
5400 µL of Ultra Pure H20 (or 900 µL per pool)
1362 µL of Wash Buffer (or 227 µL per pool)

4. Aliquot Wash Buffer X into 6 × 1.5 mL tubes and incubate at 65 °C for 00:30:00 before use.

12.2 **Preparation of Streptavidin-Coated Magnetic Beads :**

1. For each capture reaction (6 here), aliquot 30 µL of beads into 1.5mL Eppendorf Low-Bind tubes (6 here),
2. Pellet the beads on a magnetic rack for 00:02:00 until the supernatant appear clear. While keeping the tubes on the magnet, carefully remove and discard the supernatant,
3. Add 200 µL of Binding Buffer to each bead aliquot. Remove the tubes from the magnet, vortex to resuspend the beads and centrifuge briefly. Place tubes into the magnetic rack and pellet the beads; remove and discard the supernatant,
4. Repeat step 3 above twice for a total of three washes,
5. Resuspend each washed bead aliquot in 70 µL of Binding Buffer.

12.3 **Binding of Bait–Target Hybrids to Streptavidin-Coated Beads :**

1. Heat the bead aliquots in a thermomixer at 65 °C for at least 00:02:00
2. Transfer each capture reaction to the heated bead aliquots. Mix by pipetting,
3. Incubate the tubes containing libraries and beads on the thermomixer at 65 °C for 00:05:00. Agitate at the 2.5 minute mark by flicking or inverting the tubes to keep the beads suspended, followed by briefly centrifuging.

12.4 **Washing of Beads :**

1. Pellet the beads into a magnetic rack for 00:02:00 until the supernatant appear clear. While keeping the tubes on the magnet, remove and discard the supernatant,
2. Add 375 µL of of warmed Wash Buffer X to the beads, remove tubes from magnetic rack and briefly vortex them,
3. Place the tubes immediately in a thermomixer and incubate for 00:05:00 at 65 °C (with 00:00:10 of shaking every 00:02:00 at 900 rpm),
4. Pellet the beads into a magnetic rack for 00:02:00 until the supernatant appear clear. While keeping the tubes on the magnet, remove and discard the supernatant,
5. Repeat step 2 to 4 twice for a total of three washes,
6. After the final pelleting step, carefully remove as much liquid as possible without disturbing the bead pellet.

13 **Resuspension and Amplification of Libraries :**

1. Thaw the 2X KAPA HiFi HotStart Ready Mix at Room temperature ,
2. Thaw the [M] 10 micromolar (µM) forward and reverse PCR primers (See Part I Section 8) at Room temperature
3. Add 30 µL of Buffer E to the libraries+beads from the previous step and thoroughly resuspend the beads by pipetting,

Note

The beads can be kept resuspended in Buffer E at 4 °C until the next day or at -20 °C for up to one month before proceeding to the next step.

4. Transfer 15 µL of each library+bead resuspension twice into a PCR plate. The goal is to perform two PCR reactions for each library+bead pool to increase the diversity of fragments used during amplification,
5. Prepare the amplification mix for 12 samples (6 library+bead resuspension x2), a volume for 13 samples is calculated here to ensure sufficient volume is available :

325 µL of 2X KAPA HiFi HotStart Ready Mix (or 25 µL per sample)
65 µL of Ultra Pure H20 (or 5 µL per sample)
32.5 µL of Primer Forward 10µM (or 2.5 µL per sample)
32.5 µL of Primer Reverse 10µM (or 2.5 µL per sample)

6. Gently vortex and centrifuge the mix,
7. Add 35 µL of amplification mix to each well of the plate containing 15 µL of library-bead resuspension,
8. Seal the plate with PCR-specific foil,
9. Briefly vortex and centrifuge the plate,
10. Place the plate in a thermal cycler with the heated lid set to ≥ 75°C, and run the following program :

00:02:00 at 98 °C
Followed by 14 cycles :
00:00:20 at 98 °C

00:00:30 at 65 °C
00:00:45 at 72 °C
And a final extension :
00:05:00 at 72 °C
Hold at 10 °C

14 **PCR clean-up using AMPure XP beads :**

Note

Before performing the PCR clean-up, the streptavidin beads must be removed as detailed below.

1. Let the Beckman Coulter™ Agencourt AMPure XP beads stand for 00:30:00 at Room temperature ,
2. Place the PCR plate on a magnetic rack for 00:05:00 to pellet the streptavidin beads,
3. **DO NOT DISCARD THE SUPERNATANT**, instead, transfer and pool the supernatants (library by library) into 1.5 mL low-bind tubes. Here, since two 50 µL enrichment PCR reactions were performed per library, a total of 100 µL of supernatant is recovered for each library.
4. Vortex the AMPure beads thoroughly,
5. Add 100 µL of AMPure Beads (1X) in each tubes containing 100 µL of PCR products to reach a final volume of 200 µL ,
6. Mix well by pipetting or vortexing,
7. Incubate for 00:05:00 at Room temperature ,
8. Prepare fresh 70% EtOH (for 6 tubes: 2 mL of EtOHabs + 920 µL of H2OmiliQ),
9. Move tubes into a magnetic rack for 00:05:00 or until the supernatant appears clear,
10. Remove and discard the supernatant without disturbing the beads,
11. Add 200 µL of 70% fresh EtOH into the tubes without removing them from the magnetic rack ,
12. Incubate for 00:00:30 at Room temperature ,
13. Remove and discard EtOH,
14. Repeat steps 12 to 14 for a total of two washes,

15. Dry tubes with beads for 00:08:00 at Room temperature to ensure all traces of EtOH are removed. **WARNING** : Do not over dry the beads as this will significantly decrease elution efficiency,
16. Add 25 µL of EB Buffer (Qiagen) without removing the tubes from the magnetic rack,
17. Remove tubes from magnetic rack and mix well by pipetting or vortexing,
18. Incubate for 00:02:00 at Room temperature ,
19. Move tubes back to the magnetic rack for 00:05:00 or until the supernatant appears clear,
20. Transfer 25 µL of supernatant in new, unused Eppendorf Low-Bind 1.5 ml tubes.
21. Quantify 2 µL of each library using the Qubit™ dsDNA HS Assay kit, following the manufacturer's instructions.

Library Quality and Quantity Assessment :

15 **Quality control by microfluidic electrophoresis :**

1. Take the reagents from the Agilent High Sensitivity DNA kit out of the fridge 30 minutes before use,
2. Load 1 µl of undiluted libraries onto an High Sensitivity DNA Chip and run it in a Agilent 2100 Bioanalyzer, following the supplier's recommendations.
3. An average library size of 520 bp is expected (400 bp insert + 120 bp adapters).

Note

Microfluidic electrophoresis separates NGS library fragments based on size, enabling validation of the construct size and verification of the absence of short fragments (e.g., adapter dimers). This technique also provides an approximate estimate of library concentration. However, a more accurate quantification method must be used before performing equimolar pooling of NGS libraries for sequencing.

16 **Quantity Control by qPCR :**

Note

Before NGS libraries are loaded onto the sequencer, they must be quantified and normalized to ensure that each library is sequenced to the desired depth and yields the required number of reads.
Quantification by qPCR is performed by amplifying DNA fragments containing the P1 and P2 adapters. Because the PCR primers are designed to anneal specifically to the adapter sequences, the qPCR assay detects only properly adapted, amplifiable libraries capable of cluster formation during sequencing. However, qPCR can also amplify adapter dimers. Therefore, it is critical to verify their absence by microfluidic electrophoresis (e.g., Bioanalyzer) prior to quantification.

This step is carried out using the 'Lib Quantification Kit Illumina/Universal' commercialized by Roche on a LightCycler® 480, following the manufacturer's instructions.

The final library concentration is calculated using the following formula : Final library concentration = [Length of qPCR DNA standard fragments (bp) / Average lengths of library fragments (bp)] x Library concentration determined by qPCR

Note that In the 'Lib Quantification Kit Illumina/Universal', Length of qPCR DNA standard fragments = 452bp

17 **Equimolar pooling of libraries for Illumina sequencing :**

Based on the qPCR quantification results, mix the libraries equimolarly according to the requirements of the Illumina sequencer to be used.

Protocol references

The baits used in this protocol were designed by Faircloth et al. 2015.
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We used this protocol to capture UCEs from (micro-)hymenoptera but also moths (with another set of baits) :
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