Gene capture for target sequencing

Introduction

This protocol is based on the user manual of "MYselect" kit with modifications to suit the needs for capturing divergent species. Particularly, the temperature and salt concentration for the hybridization and wash steps are optimized. Because in phylogenetic studies, the target species often is not the same as the species used to design the baits, the hybridization conditions need to be relaxed and optimized for different similarity between the baits and the target. The following table shows the melting temperature (Tm) of the hybridization at different salt concentration and different similarity between the DNA baits and target sequences.

SSC Conc.	Na^+ (M)	Tm 100% identity	Tm 95% identity	Tm 90% identity	Tm 85% identity	Tm 80% identity	Tm 75% identity	Tm 70% identity	Tm 65% identity	Tm 60% identity
20X	3.3	109	102	95	88	81	74	67	60	53
10X	1.65	104	97	90	83	76	69	62	55	48
5X	0.825	99	92	85	78	71	64	57	50	43
2X	0.33	92	85	78	71	64	57	50	43	36
1X	0.165	87	80	73	66	59	52	45	38	31
0.2X	0.033	75	68	61	54	47	40	33	26	19
0.1X	0.0165	70	63	56	49	42	35	28	21	14

*** add changes for capturing closely related species**

Gene capture procedure

I. Hybridization

1. Set the following program on a thermal cycler: 95 °C for 5 min, 65 °C for 3 min, 65 °C for 2 min, 65 °C for 660 min, 60 °C for 660 min, 55 °C for 660 min, 50 °C for 660 min, and 50 °C forever.

2. Prepare lib master mix as follow for the number of samples needed:

Reagent	× *n*	Volume (µL) per sample
Block#1/Human Cot1 (1 µg/µL)		2.5
BO1.P5.F		0.25
BO3.P7.part1.F		0.25

Add 2.5 µL of lib master mix to each of the empty tubes, and then add 6.5 µL of "PreH" sample (adjust according to conc.) to each tube (use at least 100ng). Mix the sample by vertexing. Collect the liquid at the bottom of the tube by briefly centrifuging. Set aside in a refrigerator until step 5.

3. Prepare Hybridization master mix for the desired number of samples as follow:

Reagent	× *n*	Volume (µL) per sample
HYB#1/20X SSPE		10
HYB#2/0.5 M EDTA		0.4
HYB#3/50× Denhardt's		4
HYB#4/1% SDS		4

Mix the reagents by vertexing, and collect the liquid at the bottom of the tube by briefly centrifuging. Set aside in a refrigerator until step 5.

4. Prepare the bait mix for the number of samples needed as follow:

Reagent	× *n*	Volume (µL) per sample
SUPERase•In (20U/µl)		1
RNA Baits (MYselect) * don't dilute		1
H_2O		4

Mix the reagents by using a pipette. Set aside until step 5.

5. Transfer the tube containing the Library Master Mix to the thermocycler and start

the program set in step 1. This will denature the DNA library for 5 minutes at 95 °C.

6. Once the thermocycler program reaches step 2 (temperature = 65 °C), transfer the tube containing the Hybridization Master Mix to the thermocycler. Leave the Library Master Mix in the thermocycler. This will pre-warm the Hybridization Master Mix for 3 minutes at 65 °C.

7. Once the thermocycler program reaches step 3, transfer the tube containing the Capture Baits Master Mix to the thermocycler. Leave all other tubes in the thermocycler. This will pre-warm the Capture Bait Master Mix for 2 minutes at 65 °C.

8. While keeping tubes at 65 °C, transfer 13 µl of Hybridization Master Mix and 7 µl of Library Master Mix to Capture Baits Master Mix and mix via pipetting up and down.

9. Keep the hybridize solution on the thermal cycler until the program end. Depending on the application, hybridization time may need some optimization.

II. Bind to beads and wash

1. Add $n \times 10$ µL (*n* is the number of samples) of streptavidin M270 beads (Invitrogen cat#: 653-06) to a 200 µL or 1.5 mL tube according to the total volume of the beads.

2. Pellet beads using a magnetic particle stand and discard the supernatant.

3. Add 200 µL Binding Buffer (at RT) to beads to wash. Vortex tube for 5-10 seconds, place on magnetic particle stand for two minutes to pellet the beads and remove and discard supernatant.

4. Repeat step 3 twice for a total of three washes.

5. Resuspend the beads in $n \times 20$ µL Binding Buffer, add 1 µL 10% Tween.

6. Add 180 µL Binding Buffer to empty 200 µL tubes, and then add 20 µL resuspended beads to those.

7. Incubate for 2 min at 50 C.

8. Transfer the hybridization solution to the Binding Buffer/Beads and incubate 30 minutes at last hybridizing temp 50 C on a rotator. Collect the liquid at the bottom of the tube by briefly centrifuging. Pellet beads quickly with magnetic particle stand for two minutes and remove supernatant completely.

9. Add 186 ml 45 °C Wash Buffer 2 (0.1× SSC, 0.1% SDS) to the beads. Incubate for

10 minutes at 45 °C on a thermal cycler. Take out the tubes and pellet beads with magnetic particle stand for two minutes and remove supernatant.

10. Repeat step 9 two times for a total of three 45 °C washes. After the last wash make sure all additional buffer is removed.

11. Add 50 µL water to beads, label as "sample name + cap". Put in -20 C freezer.

III. Post-hybridization indexing PCR (off-beads amplification)

We use off-beads amplification (Fisher et al., 2011). This avoids the need to denature and elute the captured target from the baits using sodium hydroxide. The procedure is less problematic and results in more captured products.

1. Prepare a master mix as the follow for the number of samples needed.

Reagent	Volume (µL) per sample	× *n*	Final concentration in 25-µL reaction
KAPA HiFi taq Ready Mix (2×)	12.5		1×
Primer IS4 (10 µM)	0.5		0.2 µM

1. Add 13 µL of master mix, 11.5 µL well-mixed sample from step II-11, and 0.5 µL indexing primers to each tube. Mix well and amplify the samples using the following thermal profile: 98 °C for 45 sec, 12 to 18 cycles of 98 °C for 15 sec, 60 °C for 30 sec and 72 °C for 45 sec, followed by 72 °C for 1 min, and hold at 4 °C for 10 min. The number of PCR cycles can be adjusted according to the starting material used to construct the library.

2. Load 3 µL of PCR product to a mini agarose gene to check the size of the captured library. The band should be barely visible.

3. Cleanup the PCR product using the SPRI bead method. Elute the DNA using 20 µL of water and transfer it to a new tube labeled as "sample name + Ind".

IV. Pooling multiple samples for sequencing

1. Determined the DNA concentration of "Ind" samples using Qubit2. The concentration of the samples should be around 0.1 - 0.9 ng/µL.

2. Pool all samples in equimolar ratios.

3. Quantify the pooled library using q-PCR and nanodrop before sequencing. The pooled library should have 20 µL at concentration of 2 nM to 50 nM, which is 0.5 ng/µL to 13 ng/µL for DNA ~ 500 bp.

Sequences of blocking oligos
Pho indicates a 3'-phosphate

Name	Sequences
BO1.P5.F	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACG ACGCTCTTCCGATCT-Pho
BO2.P5.R	AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGG TGGTCGCCGTATCATT-Pho
BO3.P7.part1.F	AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-Pho
BO4.P7.part1.R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-Pho
BO5.P7.part2.F	ATCTCGTATGCCGTCTTCTGCTTG-Pho
BO6.P7.part2.R	CAAGCAGAAGACGGCATACGAGAT-Pho

DNA Clean-Up Using MagNA Beads
(modified from Rohland and Reich, 2012)

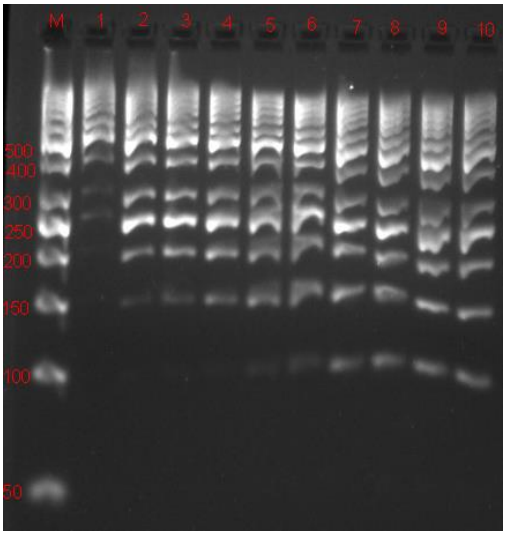

Fig.1. 4% agarose gels showing DNA ladder (GeneRuler 50 bp #SM0373) cleaned up with different amount of MagNA Buffer added (lane descriptions are listed in table 1).

Table 1

Lane	1	2	3	4	5	6	7	8	9	10
Sample(μL)	20	20	20	20	20	20	20	20	20	20
MagNA buffer (μL)	10	15	18	24	30	36	42	48	54	60
MagNA buffer/Sample	0.5x	0.75x	0.9x	1.2x	1.5x	1.8x	2.1x	2.4x	2.7x	3.0x

DNA clean-up procedure

1. First add 30 μL MagNA beads (see next page for preparing MagNA beads mixture) in an empty tube, dry the beads using magnetic plate.

2. Add sheared samples and MagNA buffer to the dried beads (samples : MagNA buffer = 1 : 0.75, e.g., 20uL sample need add 15uL MagNA buffer for cleaning off 100bp fragment). Vortex the tubes for several times.

3. Let the tube stand for 5 min at room temperature. Collect the liquid at the bottom of the tube by briefly centrifuging.

4. Place the tube on a 96-well ring magnetic plate, and let it stand for 5 min or more until the beads separated from the solution. Pipette off and discard the supernatant without touching the beads.

5. Leave the tube on the magnetic rack, and wash the beads by adding 186 μL of freshly prepared 70% ethanol. Let stand for 1 min and remove the supernatant. *Keep the tube on the magnetic rack, do not disturb the beads!!!

6. Repeat Step 5 one more time.

7. Remove residual traces of ethanol. Let the beads air-dry for 5 min at room temperature without caps.

8. Add 20 μL of nuclease free water or EBT to the wells and seal the tube with caps. Remove the tube from the magnetic rack, and resuspend the beads by repeated vertexing or pipetting. Let it stand for 1 min, and then collect the liquid in the bottom of the wells by briefly centrifuging. Occasionally the beads may appear clumpy after vortexing; this does not have a negative effect on DNA recovery.

9. Place the tube back on the magnetic rack, let stand for 1 min, and transfer the supernatant to a new tube. Carryover of small amounts of beads will not inhibit subsequent reactions.

1. Recipe for 100 ml MagNA beads (18% PEG8000, 2.5 M NaCl)

Reagent	Amount	Final concentration
5% Sera-Mag Magnetic Speed-beads (FisherSci, cat.#: 09-981-123)	2 mL	0.1%
PEG8000 (cat #: Fisher BP233-1)	18 g	18%
5M NaCl	50 mL	2.5 M
1M Tris-Cl	1 mL	10 nM
0.5M EDTA	200 uL	1 mM
add H_2O to 100 mL		

Note: beads contain sodium azide, wash beads 2x with water or TE before addition.

2. Recipe for 100 ml MagNA Buffer (18% PEG8000, 2.5 M NaCl）

Reagent	Amount	Final concentration
PEG8000 (cat #: Fisher BP233-1)	18g	18%
5M NaCl	50mL	2.5M
1M Tris-Cl	1mL	10nM
0.5M EDTA	200uL	1mM
add H_2O to 100 mL		