N.B.

It is very important to avoid potential cross-contamination. The following tips should be followed:

- *Clean your bench with bleach (84) every time after you did DNA extraction or fish dissection. Clean your pipettors, rack and bench space with bleach once a while.*
- *Aliquot all reagents into appropriate amount, so not to contaminate the whole tube of reagents.*
- *Make sure you have enough filtered tips, and never use tips without filter.*
- ***Centrifuge the tube every time before you open a lid.*** *Keep you finger off the inside of the lid. If you suspect that your gloves are contaminated, change it.*
- *Leave a space between tubes on rack, if you are not using multichannel pipettors.*
- *When pipetting, do it smoothly, don't pump it too fast. Keep the tip submerged just under the solution, so not push the liquid to overflow.*
- *When adding samples, set the tip against the wall of the tube.*
- *Move your tubes to an up row or switch the orientation of your tubes after adding samples to keep track which one has been added to.*
- *Keep focused; please don't talk to others when they are working on sample prep.*

Gene capture for target sequencing

Introduction

This protocol is based on the user manual of "MYbaits-manual-v3" 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

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

Gene capture procedure

I. Hybridization

1. For regular samples, set the following program on a thermal cycler: 95 °C for 5 min, 65 °C for 5 min, 65 °C for 300 min, 60 °C for 300 min, 55 °C for 300 min, 50 °C for 300 min, and 50 °C forever.
   It is ok to keep the samples on the machine at 50 °C for a few hours after the program ends.
   If working with eDNA or ancient DNA, set the following program on a thermal cycler: 95 °C for 5 min, 65 °C for 5 min, 65 °C for 600 min, 60 °C for 600 min, 55 °C for 600 min, 50 °C for 600 min, and 50 °C forever.

2. Prepare **HYB Baits Mix** for the number of samples needed as follow:

Reagent	Volume (µL) per sample	× *n*
HYB#1/20X SSPE	7	
HYB#2/0.5 M EDTA	0.3	
HYB#3/50× Denhardt's	3	
HYB#4/**10%** SDS	0.3	
SUPERase•In (20U/µl)	1	
RNA Baits (MYselect) * don't dilute	0.3	
H_2O	8	
Total		***No need to prep mix for extra samples***

   Mix the reagents by pipetting gently, and collect the liquid at the bottom of the tube by briefly centrifuging. Add 19 µL of **Hyb Mix** to each of empty tubes. Set aside in a refrigerator until step 4.
   Thoroughly vortex HYB #1 before use, and bring HYB #4 to room temperature to fully dissolve its SDS before use.
   Use 0.3 µL of baits per sample. If you have multiplexed samples, adjust the amount of baits and the H_2O to be used accordingly.

3. Prepare **Lib Mix** as follow for the number of samples needed:

Reagent	Volume (µL) per sample	× n
Block#1/Human Cot1 (1 µg/µL)	2.5	
BO1.P5.F (200 µM)	0.5	
BO3.P7.part1.F (200 µM)	0.5	
Total		***No need to prep mix for extra samples***

   Mix the sample by pipetting gently. Collect the liquid at the bottom of the tube by briefly centrifuging. Add 3 µL of the mixture to each of the empty tubes labeled

with sample name, and then add 7 µL of "PreH" sample (adjust according to conc., usually 100ng ~ 500ng in total) to each tube (*Jak: use at least 100ng or as much as you have if there is less than 100ng*). Mix the sample by pipetting gently. Collect the liquid at the bottom of the tube by briefly centrifuging. Set aside in a refrigerator until step 4.
If you have to add a lot of "PreH" samples, bring down the volume use vacuum centrifuge first.

4. Transfer the tube containing the **Lib Mix** to the thermocycler and start the program set in step 1. This will denature the DNA library for 5 minutes at 95 °C.

5. Once the thermocycler program reaches step 2 (temperature = 65 °C), transfer the tube containing the **HYB Baits Mix** to the thermocycler. Leave the **Lib Mix** in the thermocycler. This will pre-warm the Hybridization Master Mix for 5 minutes at 65 °C.

6. While keeping tubes at 65 °C, transfer all of the **HYB Baits Mix** and to **Lib Mix** and mix via pipetting up and down.
Use a 20-200 µL multichannel pipettor for easier execution.

7. Keep the hybridize solution on the thermal cycler until the program end. Depending on the application, hybridization time may need some optimization (*MYcroarray: between 16 to 48 hours*).

II. Bind to beads and wash
Start ~90 minutes before intended hybridization stop-time
STRONGLY RECOMMENDED: Multichannel pipettor for 20-200 µL

1. Add $n \times 10$ µL (n is the number of samples) of streptavidin MyOne (Invitrogen cat# 65002) or M270 beads (Invitrogen cat#: 653-06) to 200 µL tubes. Each tube should hold no more than 180 µL beads. *For multiplexed samples, use 30 µL of beads per multiplexed sample.*

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

3. Add 200 µL Binding Buffer (at RT) to beads to wash. Mix the beads by pipetting gently. Place on magnetic particle stand for two minutes to pellet the beads and remove and discard supernatant. *Discard any loose beads that are not tightly attracted to the wall of the tube.*

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 labeled with sample names, and

then add 20 µL resuspended beads to those.

7. Incubate for 2 min at last hybridizing temp, e.g., 50 °C.

8. Transfer the hybridization solution to the Binding Buffer/Beads and incubate 30 minutes at last hybridizing temp, e.g. 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 or as soon as the beads are clear and remove supernatant **completely**. *While incubating, preheat the same number tubes of Wash Buffer 2 (0.1× SSC, 0.1% SDS, 190 µL each tube) in a thermal cycler at the hybridizing temp (e.g.,* 50 °C*) for at least 10 min.*

9. Add 186 µL 50 °C Wash Buffer 2 (0.1× SSC, 0.1% SDS) to the beads, mix the beads gently using pipettor. Incubate for 10 minutes at 50 °C on a thermal cycler. Take out the tubes and collect the liquid at the bottom of the tube by briefly centrifuging. Pellet beads with magnetic particle stand for two minutes and remove supernatant.

10. Repeat step 9 two times for a total of three 50 °C washes.

11. Wash one more time with TE buffer at room temperature.

12. Add 35 µL TE buffer to beads, label as "sample name + 1st cap". Put in -20 °C freezer.

III. Pre-hybridization PCR (off-beads amplification; for the second Gene Capture)

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×)	13.75		1×
Primer IS7 (10 µM)	0.55		0.3 µM
Primer IS8 (10 µM)	0.55		0.3 µM
Total		***No need to prep mix for extra samples***	

Add 13.5 µL of master mix, 11.5 µL well-mixed sample from step II-12 (make sure you take the beads with the TE buffer) to tube labeled with sample name. Mix well, collect the liquid at the bottom of the tube by briefly centrifuging, 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 (12 – 18 cycles).

2. Cleanup the PCR product using the MagNA bead method. Elute the DNA using 25 μL of TE buffer and transfer it to a new tube labeled as "sample name + preH2".

3. Load 1 μL of PCR product to a mini agarose gene to check the size of the captured library. The band should be barely visible.

IV. Do a second gene capture use the product of III 2 as template. Label the products as "sample name + 2nd cap".

V. Post-hybridization indexing PCR (off-beads amplification; for NGS)

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×)	13.75		1×
Primer IS4 (10 μM)	0.55		0.2 μM
Total		***No need to prep mix for extra samples***	

Add 13 μL of master mix, 0.5 μL indexing primers (P7 indexing primer) and 11.5 μL well-mixed sample (make sure you take the beads with the TE buffer) from step IV off the second gene capture to each tube labeled with sample name. Mix well, collect the liquid at the bottom of the tube by briefly centrifuging, 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 (usually 12 – 14, no more than 16 cycles).

2. Cleanup the PCR product using the MagNA bead method. Elute the DNA using 25 μL of TE buffer and transfer it to a new tube labeled as "sample name + Ind".

3. Load 1 μL of PCR product to a mini agarose gene to check the size of the captured library. The band should be barely visible.

V. Pooling multiple samples for sequencing

1. Determined the DNA concentration of "Ind" samples using Nanodrop 3300. The concentration of the samples should be around 0.1 - 0.9 ng/μL. ***Make sure your reads are within the range of standard curve. Accuracy is very important for pooling samples equimolarly.***

2. Pool all samples in equimolar ratios.

3. Quantify the pooled library using nanodrop 3300 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	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT-Pho
BO2.P5.R	AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCATT-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

Wash Buffer 2: 0.1X SSC, 0.1% SDS + tween 0.1%?

Make sure the base composition of the index is balanced for the inline index and the P7 adapter index. Use all 48 inline index and make different combination. If use less than 30 P7 index, try one of the following picks. If use more than 30 P7 index, use one of the 30 picks, and then add other index in a random order.

Here are some lists of 10 balanced P7 index:

Set I: 27, 100, 208, 236, 336, 526, 527, 533, 685, 702
Set II: 1, 2, 68, 432, 436, 442, 554, 647, 687, 693
Set III: 10, 138, 254, 423, 435, 440, 527, 551, 639, 679

Here are some lists of 15 balanced P7 index:

Set I: 1, 10, 89, 138, 254, 423, 435, 440, 527, 549, 551, 625, 639, 679, 711
Set II: 1, 10, 93, 233, 236, 285, 325, 517, 530, 533, 571, 654, 679, 687, 696
Set III: 2, 7, 9, 11, 288, 420, 435, 524, 526, 530, 559, 676, 684, 693, 695

Here are some lists of 20 balanced P7 index:

Set I: 1, 2, 4, 124, 130, 255, 347, 420, 434, 435, 441, 522, 526, 527, 547, 647, 668, 686, 691, 693
Set II: 2, 3, 12, 93, 138, 237, 255, 309, 388, 419, 527, 533, 549, 558, 589, 625, 639, 695, 706, 711
Set III: 1, 2, 7, 9, 11, 288, 335, 420, 431, 435, 524, 526, 530, 547, 559, 647, 676, 684, 693, 695

Here are some lists of 25 balanced P7 index:

Set I: 3, 7, 10, 13, 26, 200, 229, 235, 288, 325, 339, 381, 432, 487, 517, 522, 527, 530, 546, 609, 686, 687, 688, 703, 709
Set II: 1, 9, 20, 100, 129, 229, 332, 336, 383, 407, 421, 435, 440, 443, 513, 526, 527, 533, 546, 603, 628, 676, 684, 700, 706
Set III: 1, 8, 10, 11, 16, 129, 219, 254, 332, 334, 337, 362, 400, 517, 522, 526, 527, 565, 567, 654, 679, 683, 685, 691, 710

Here are some lists of 30 balanced P7 index:

Set I: 1, 7, 10, 16, 50, 100, 129, 130, 255, 336, 339, 383, 388, 419, 431, 513, 517, 526, 527, 533, 547, 588, 617, 633, 639, 650, 688, 693, 695, 707
Set II: 2, 3, 4, 7, 27, 89, 134, 229, 255, 303, 313, 332, 388, 435, 436, 527, 530, 533, 546, 571, 582, 587, 604, 609, 620, 638, 672, 686, 693, 707
Set III: 1, 2, 4, 13, 27, 208, 219, 229, 237, 332, 340, 389, 407, 431, 435, 438, 482, 515, 526, 527, 530, 554, 567, 603, 633, 647, 678, 686, 687, 693