**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 |

**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 |  | 0.08 |
| BO3.P7.part1.F |  | 0.08 |

Add 2.5 μL of lib master mix to each of the empty tubes, and then add 6.5 μL of “PreH” sample to each tube. 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.

1. 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.

1. 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) |  | 0.5 |
| H2O |  | 4.5 |

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

1. 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.
2. 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.
3. 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.
4. 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.
5. 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*×10μL(*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*× 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. Transfer the hybridization solution to the Binding Buffer/Beads and incubate 30 minutes at room temperature on a rotator. Collect the liquid at the bottom of the tube by briefly centrifuging.Pellet beads with magnetic particle stand for two minutes and remove supernatant.
8. Add 186 ml Wash Buffer 1(1× SSC, 0.1% SDS) to the beads. Incubate 10 minutes at room temperatureon a rotator. Collect the liquid at the bottom of the tube by briefly centrifuging.Pellet beads with magnetic particle stand for two minutes and remove the supernatant. Repeat step 8 one more time for a total of two washes. In the meantime, preheat Wash Buffer 2 (0.1× SSC, 0.1% SDS) to 45 °C.
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”.

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（NaON）.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 50-μL reaction |
| KAPA HiFitaq Ready Mix (2×) | 25 |  | 1× |
| Primer IS4 (10 μM) | 1 |  | 0.2μM |

1. Add 26μL of master mix, 23μLwell-mixed sample from step II-11, and 1 μL indexing primers to each tube. Mix well and amplify the samples using the following thermal profile: 98 °C for 45sec, 12 to 18 cycles of 98 °C for 15 sec, 65 °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. The pooled library should have 20 μL at concentration of 2 nM to 50 nM, which is 0.5 ng/μL to13ng/μLfor 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 |