

Enrichment protocol

For use with indexed Illumina TruSeq or NexTera short-read libraries

Before beginning

This protocol is based on the Arbor Daicel [myBaits v5](#) standard protocol, with some adjustments. The following reagents are needed for this protocol, along with the probeset synthesized using the Probe synthesis protocol. Contact Daicel Arbor Bioscience for a quote:

Reagent	Supplier	Catalogue #
myBaits Reagents	Daicel Arbor Bioscience	NA
KAPA HiFi HotStart ReadyMix	Roche	07958935001
P5 library amplification primer = ATGATACGGCGACCACCGA	IDT	NA
P7 library amplification primer = CAAGCAGAAGACGGCATACGA	IDT	NA
PCR purification method	Various	NA

Equipment

Beyond standard plasticware and pipettes, the facility performing this protocol will require:

Thermal cycler with a heated lid that can accommodate 0.2mL strip tubes

Magnetic tube racks (one for 1.5mL tubes, another for 0.2mL strip tubes)

Vortexer and picofuge for tubes and strips

Oven (preferable) or water bath set to 62 °C

Enrichment Protocol

Day 1

Hybridization Master Mix

1. Determine the volume of 100ng/ μ L biotinylated RNA probeset for all reactions. 3 μ L per reaction is sufficient for this protocol. Dilute RNA probeset to this volume at a concentration of 100ng/ μ L using nuclease-free H₂O.
2. Assemble a master mix using the following volumes per reaction. The excess for pipette error is already accounted for.

Reagent	μ L per reaction
Hyb N	4.63 μ L
Hyb D	1.75 μ L
Hyb S	0.25 μ L
Hyb R	0.63 μ L
100ng/ μ L probeset	2.75 μ L
Total:	10 μ L

3. Dispense 9.5 μ L of master mix into 0.2mL PCR strips, one for each reaction being performed. These tubes will, from here, be referred to as the HYBs.
4. Incubate the HYBs at 60°C for 10m.
5. After incubating, remove and let sit at room temperature for 5m.

Library Master Mix

1. Assemble a master mix using the following volumes per reaction. The excess for pipette error is already accounted for. If performing enrichment on plants, replace Block C volume with more Block O. If performing enrichment on salmonids, replace Block O volume with nuclease-free H₂O.

Reagent	μ L per reaction
Block X	0.25 μ L
Block C	1.25 μ L
Block O	1.25 μ L
Total:	2.75 μ L

2. Dispense 2.5 μ L per reaction into 0.2mL PCR strips, one for each indexed library.
3. Add 3.5 μ L of the indexed library into its corresponding tube, then mix well and spin briefly on a microfuge. These tubes will, from here, be referred to as the LIBs.

4. Incubate the LIBs on the thermal cycler for 5 minutes at 95°C, then drop to 62°C, the hybridization temperature.
 - a. *****IMPORTANT***** After the 95°C incubation, the reactions **MUST** remain as close to the hybridization temperature as possible for the entirety of the protocol. Deviations will result in a loss of specificity due to non-specific binding and, therefore, lower enrichment efficiency. While it is impossible to eliminate it completely during later steps, make sure to minimize the time the reactions spend off the heating block.
5. Incubate the HYBs for 5min at the hybridization temperature alongside the LIBs, after the 95°C step.
6. While still on the heating block, transfer 9uL of the HYBs into the tubes containing the LIBs. Mix by pipetting up and down 10 times. Use a multichannel pipette whenever possible when processing multiple samples in parallel.
 - a. Ensure not to deposit liquid on the sides of the tubes during this step. If this happens, spin the tubes very briefly with a microfuge and immediately replace on thermal cycler.
7. Incubate at 62°C for 16-24 hours.

Day 2

Wash Buffer Preparation

1. Dissolve any precipitate in wash buffer and Hyb S. Warm slightly if necessary.
2. Prepare 4 aliquots (1 for each wash) of the following mix, multiplying by the number of reactions. Excess for pipette error is already accounted for:

Reagent	μL per reaction
Hyb S	1.68μL
Wash Buffer	52.5μL
H2O	166.32μL
Total:	220.5μL

3. Place the aliquots in an oven or water bath set to 62°C for at least 30m before using.
 - a. If using an oven to heat these wash aliquots, it is ideal to also put any racks/reagent reservoirs used in the washing step in at the same time. This ensures that these do not take too much of the heat from the samples when they're being used for washing, allowing the samples to stay closer to the hybridization temperature. Ideally, the oven should contain all aliquots of the wash buffer, the magnetic rack for PCR tubes, a plastic rack for general handling after supernatant removal, and the reagent reservoir for holding wash buffer.

Streptavidin Bead Preparation

1. Aliquot 15uL per reaction of streptavidin-coated beads into a 1.5mL microcentrifuge tube.

- a. Up to 16 reactions worth of beads can be prepared in a single tube (240uL beads) due to binding buffer volume during washes. If preparing for more reactions, adjust accordingly.
2. Pellet the beads in a magnetic rack for 1.5mL microcentrifuge tubes.
3. Remove supernatant.
4. Add 100uL binding buffer per reaction to beads.
5. Mix thoroughly by vortexing.
6. Pellet beads on magnetic rack.
7. Remove supernatant
8. Repeat steps 4-7 twice for a total of three washes.
9. Resuspend the beads in 35uL binding buffer per reaction.
10. Transfer 35uL of bead suspension to 0.2mL strip tubes, one per reaction.

Capture

1. Incubate strip tubes with beads on heating block at 62°C for 2m.
2. Transfer each hybridization reaction to its corresponding bead tube using a multichannel pipette while still on the heating block. Mix by pipetting up and down 10 times.
 - a. Transferring reactions to tubes that contain the beads, rather than the other way around, can help reduce non-specific binding, as any DNA adsorbed to the side of the plastic tube will be left behind.
3. Incubate the combined reactions at 62°C for 5m.
 - a. After 2.5m, gently agitate the reactions by flicking the strips of tubes, then very briefly centrifuge in a picofuge. Do this as quickly as possible to avoid excessive cooling of the samples.
 - b. Ensure liquid is not present in the cap when replacing tubes onto the thermal cycler, as the heated lid will be at a higher temperature than the block and may cause dissociation of probes and target molecules.

Washing

1. Remove the magnetic rack for PCR tubes from the 62°C oven if it is heating there.
2. Pellet beads on magnetic rack.
3. Remove supernatant
 - a. This supernatant can optionally be saved to be re-enriched with the same or different probes.
4. Remove the plastic rack from the oven and move the samples from the magnetic rack to this. Replace the magnetic rack in the oven.
5. Remove the wash buffer aliquot and reagent reservoir from the oven. Pour wash buffer into the reagent reservoir.
6. Using a multichannel pipette, add 180uL warmed wash buffer to each tube containing streptavidin-coated beads and sample.
7. Mix samples by flicking and briefly centrifuging, then replace onto the heating block.
8. Return the reagent reservoir and plastic rack to the oven.
9. Incubate 5m on the heating block at 62°C for 5m.

- a. After 2.5m, gently agitate the reactions by flicking the strips of tubes, then very briefly centrifuge in a picofuge. Do this as quickly as possible to avoid excessive cooling of the samples.
 - b. Ensure liquid is not present in the cap when replacing tubes onto the thermal cycler, as the heated lid will be at a higher temperature than the block and may cause dissociation of probes and target molecules.
10. Remove the magnetic rack for PCR tubes from the oven if it is heating there.
11. Pellet beads on magnetic rack.
12. Remove supernatant
 - a. Ensure when removing supernatant not to disturb beads. It's better to leave a few μL of wash buffer in the bottom of each tube than to remove beads with bound probes.
13. Repeat steps 4-12 three times for a total of four washes.
14. Briefly centrifuge the remaining bead pellet and replace onto magnetic rack. After separating, remove all remaining liquid at the bottom of the tube. Here, it is often inevitable to take up a small amount of beads – minimize it as much as possible.
15. Resuspend beads in 15 μL Buffer E

Library Amplification

1. Set up the following master mix multiplied by the number of reactions. Pipette error is accounted for:

Reagent	μL per reaction
2X KAPA HiFi HS RM	26.25 μL
10 μM P5 Lib Amp Primer	2.63 μL
10 μM P7 Lib Amp Primer	2.63 μL
H ₂ O	5.25 μL
Total:	36.76 μL

P5 Primer = AATGATACGGCGACCACCGA

P7 Primer = CAAGCAGAAGACGGCATACGA

2. Dispense 35 μL master mix to 0.2mL PCR tubes, one per enrichment reaction.
3. Add the entire bead suspension of each reaction directly to its corresponding amplification reaction. Mix thoroughly.

4. Run PCR according to the following cycling conditions:

Temperature	Time	Cycles
98°C	2m	
98°C	20s	8 to 14*
60°C	30s	
72°C	45s	
72°C	5m	
10°C	∞	

*Minimize cycles to avoid overamplification. The number of cycles to reach concentration requirements may exceed 14.

5. Purify amplified DNA using your cleanup method of choice.
- If doing column purification, ensure to separate beads and only purify the supernatant. If using beads such as AMPure XP, this is unnecessary.
6. These libraries are now ready to be assessed and processed using any standard sequencing workflow.