The following procedure is a slight modification from the Arbor Scientific "Hybridization Capture for Targeted NGS" protocol, v4.01 (April 2018). The unique elements are highlighted and primarily focus on the combining probe sets from different mybaits kits.

Part 1: Hybridization

A) Materials required (*when removing reagents from freezer/fridge – only remove what is needed for your reactions*). All reagent names refer to materials provided in Arbor myBaits kits.

- Hyb reagents (Boxes 1 (4ºC) and 2 (–20ºC)
- Block reagents (Box 2)
- Baits (Box 3 (–80ºC) (Aliquot to 12 µl) ***Keep on ice***
- Sequencing libraries to be enriched, in a final volume of 7 µL per reaction
- 1.7 mL nuclease-free low bind tubes (×2)
- Low-bind 0.2 mL tubes with individual caps (×2 per reaction)
- Pipettors and tips (multichannel pipette 20 µL)

- Stoichiometrically combined libraries of similar size. Each combined set of libraries (ca. 24 libraries per hyp-seq reaction) will be run through as one hybridization reaction. They should have 100-500ng total DNA. Small libraries (< 300bp including the 140 bp of adapters) and larger libraries (ca. 350-700 bp including adapters) should be pooled and used in the separate hybridization reactions. Once combined, use the speedvac to concentrate the set down to 7 µl total volume.

B) Hybridization mix setup

1. Thaw the Hyb reagents (Boxes 1 and 2), vortex to homogenize, and centrifuge briefly. (Note: If Hyb N and/or Hyb S have visible precipitate after thawing, heat them to 60°C and vortex until the precipitate dissolves)
2. **For the Baits**, combine different probe sets based on the number of probes per set. In our case it was 2:1 mixture of Angiosperms-353 (80K probes) to Niklov1827 (40K probes)
3. Assemble the Hybridization Mix in a 0.2 ml low bind tube for less than 8 reactions or a 1.5 ml tube for larger numbers of reactions.

Component	**µL per reaction**	**µL for four reactions**
Hyb N	9.25	37
Hyb D	3.5	14
Hyb S	0.5	2
Hyb R	1.25	5
Baits	5.5	22
TOTAL	**20**	**80**

Note: *Introduction of Hyb S will cause cloudiness; mixture will clarify after step 3*

4. Incubate the Hybridization Mix at 60°C for 10 min in the heat block (use heated lid), vortexing occasionally to collect condensed evaporate from the tube lid. Remove the mix from the heat block, briefly spin down and allow to sit at room temperature for 5 min.
5. For each capture reaction, aliquot 18.5 µL of Hybridization Mix to a 0.2 mL tube. These now called **HYB**.

C) Blockers Mix setup

1. Assemble the Blockers Mix in a 0.2 ml nobind tube and mix by pipetting.

Component	**µL per reaction**	**µL per 4 reactions**
Block A	0.5	2
Block C	2.5	10
Block O	2.5	10
TOTAL	**5.5**	**22**

2. For each capture reaction, aliquot 5 µL of Blockers Mix to a low-bind 0.2 mL tube.
3. Add 7 µL of library (100 – 500 ng recommended) to each Blockers Mix aliquot and mix by pipetting. The resulting mix will be referred as **LIB**.

D) Reaction assembly

Thermal program for thermal cycler (Use heated lid)

Step	**Temperature**	**Time**
1	95°C	5 m
2	Hybridization Temp. (65°C)	5 m
3	Hybridization Temp. (65°C)	∞

1. Put the **LIB**s in the thermal cycler, close the lid, and start the thermal program.
2. Once the cycler reaches the hybridization temperature during step 2, pause the program, put the **HYBs** in the thermal cycler, close the lid, and resume the program.
3. After step 2 of the program is complete, leaving all tubes in the thermal cycler, **pipette 18 µL of each HYB to each LIB** with multichannel pipette. Gently homogenize by pipetting up and down 5 times.
4. Dispose of the HYB tubes. Briefly spin down the **LIB**s, return to the thermal cycler, close the lid, and allow the reactions to incubate at the hybridization temperature (heated lid on) for your chosen time. For this study we used 24 hours.

Part 2: Bind and Wash (Cleanup)

A) Begin assembly of materials at least 90 min before the end of the hyb reaction
B) Materials required

Note: Bring the solutions to room temperature prior to use. Warm gently to dissolve precipitate if necessary.

- Hyb S
- Binding Buffer
- Wash Buffer
- Arbor Beads (Streptavidin bound)
- Nuclease free sterile water (up to 900 µL per cleanup)
- 10 mM Tris-Cl, 0.05% TWEEN-20 solution (**pH 8.0-8.5**)
- Magnetic particle concentrator(s) (“MPC”) for 0.2 ml PCR strips/plates
- Bring incubator and water bath to 65°C
- 50 ml nuclease free tube

B) Wash Buffer X preparation

1. Thaw and thoroughly homogenize Wash Buffer and HYB S prior to aliquoting in order to dissolve any visible precipitate; warm slightly if necessary.
2. For each enrichment reaction combine the following in a 1.5ml NF sterile tube, vortex, and label "Wash Buffer X."

Reagents	**1 reaction**	**4 reactions**
HYB S	9 µl	36 µl
NF water	900 µl	3600 µl
Wash Buffer	227 µl	908 µl

C) Bead preparation

Note: Prepare beads immediately prior to use

1. For each capture reaction, aliquot 30 µL Beads to a low-bind 1.7 mL tube.
2. Pellet the beads in the MPC until the suspension is clear (1-2 min). Leaving the tubes on the magnet, **remove and discard the supernatant without disturbing beads**.
3. Add 200 µL Binding Buffer to each bead aliquot. Vortex to resuspend the beads and centrifuge briefly. Pellet in the MPC, **remove and discard the supernatant without disturbing the beads**.
4. Repeat Step 3 above twice for a total of **three washes**.
5. Resuspend each bead aliquot in 70 µL Binding Buffer.
6. Transfer the bead aliquot to 0.2 ml plate tubes for 96-well process and MPC style magnets. Other options are available here – see the original myBaits protocol.

D) Binding beads and hybrids

1. Heat the bead aliquots (sealed in their 0.2 ml well) to the hybridization temperature (65°C) for at least 2 min in thermal cycler.
2. Transfer each capture reaction to the heated bead aliquots. Mix by pipetting. Seal the tops to the tubes (strip cap lids work well).
3. Incubate the libraries+beads on the thermal cycler for 5 min. Agitate at the 2.5-minute mark by pipetting (briefly centrifuging to collect if necessary).
4. After the 5 min pellet the beads with the MPC until the solution is clear. **Remove and discard the supernatant without disturbing the beads**. Immediately move to the next step.

E) Bead washing

1. Remove samples from the MPC add 180 µL warmed Wash Buffer X to the beads, mix by pipetting. If necessary, briefly centrifuge to collect.
2. Incubate for 5 min at the hybridization temperature in the heat block or thermal cycler. Agitate at the 2.5-minute mark via pipetting (briefly centrifuge if necessary).
3. Pellet the beads with the MPC and discard supernatant without disturbing the bead portions.
4. Repeat steps 1 through 3 three times for 0.2 mL format (four washes total). After the last wash and pelleting, **remove as much fluid as possible without touching the bead pellet.**

Part 3: Library resuspension and amplification

A) Materials required

- 10 mM Tris-Cl, 0.05% TWEEN-20 solution (pH 8.0-8.5)
- Reagents for library amplification using universal primers
- PCR purification system, SPRI beads

B) Enriched library resuspension

1. Add 30 µL of 10 mM Tris-Cl, 0.05% TWEEN-20 solution (pH 8.0 – 8.5) to the washed beads and thoroughly resuspend by pipetting.

Note, beads can be frozen at –20C if you are not moving on to amplification immediately.

C) Library amplification

1. For each sample, assemble the following PCR master mix:

Component	**Final Concentration**	**µL per reaction**
NF Water	-	8.75
2X KAPA HiFi HotStart ReadyMix	1 X	25
IDT xGEN amp primers (20um)	500 nM	1.25
Enriched Library (**pellet the beads before pulling off the 15 µl aliquot**)	-	15*
TOTAL		**50**

Remaining bead-bound library can be stored at –20°C for several months.

2. Amplify the reactions with the program below. Note, the number of cycles needed can be highly variable and can be influenced by the sequencing provider's requirements and the sequencing platform. For our HiSeq 4000 runs with Novogene, we used 14 initial cycles of PCR, then paused the PCR program at 4C to quickly run a Qubit dsDNA HS estimate concentration and then ran additional cycles to reach our desired concentration (we targeted around 4-10ng/µl – which gave us > 2 µM libraries after SPRI cleanup):
   Use calculated temperature setting

Step	**Temperature**	**Time**	
1	98°C	2 minutes	
2	98°C	20 seconds	# of cycles to be determined. In our case, 17+ cycles ultimately used.
3	60°C	30 seconds	
4	72°C	length-dependent*	
5	72°C	5 minutes	
6	8°C	∞	

*Extension time can be library size dependent (when in doubt, a slightly longer time is ok)
< 500 bp average: 30s

500 to 700 bp: 45s
> 700 bp: 1m

3. Purify the reaction using your preferred PCR cleanup (e.g., SPRI beads or Column cleanup). In our hands both worked, but the SPRI cleanup recovered a higher faction of the DNA. The enriched libraries are now ready for sequencing.

SPRI bead purification using ABM magnetic beads (these were performed in 96-well format).

- Add 90 µl of room temp and resuspended ABM SPRI beads to the 50 µl PCR reaction (1.8 SPRI : 1 PCR)
- Pipette up and down 10 times to mix and incubate at room temp for 5 min
- Place on the MPC until beads have cleared from the solution (2-5 min typically).
- Carefully remove and discard supernatant without taking up any beads. At this step it may be hard not to accidentally pickup beads – so you can leave a bit of liquid behind if needed.
- Keeping the tubes on the MPC, add 200 µl of freshly made 70% ETOH, incubate 30 s and remove and discard supernatant. Beads are not so easily disturbed now and you can remove all liquid.
- Repeat one more wash with 200 µl 70% ETOH
- Air dry beads 1 minute
- Remove the plate from the MPC and elute the DNA from beads with 30 µl of 0.1X of TE (1X TE [10 mM Tris, 1 mM EDTA, pH8] diluted 1:10). If the concentration is a concern, you could recover the DNA in a lesser volume of 0.1X TE.
- Pellet beads with MPC and transfer the newly suspended DNA a clean tube.
- Store at –20C or –80C.