Hybridization Capture for Targeted Methylation Sequencing

User Manual

Version 1.51 August 2021

For myBaits Custom Methyl-Seq (Catalog #s ending in “M.v5”)

Access additional manuals at arborbiosci.com/mybaits-manual

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Custom Methyl-Seq Protocol

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INTRODUCTION

myBaits® is an in-solution NGS library target enrichment system, compatible with Illumina®, Ion Torrent®, and essentially any amplifiable sequencing library. We use a versatile nucleic acid synthesis technology to produce biotinylated RNA "baits" that are complementary to your sequence targets. Baits and other reagents for NGS target enrichment are supplied with your myBaits kit. After enrichment with myBaits, libraries may then be sequenced on the aforementioned platforms, or further prepared for PacBio® or Oxford Nanopore Technologies® sequencing.

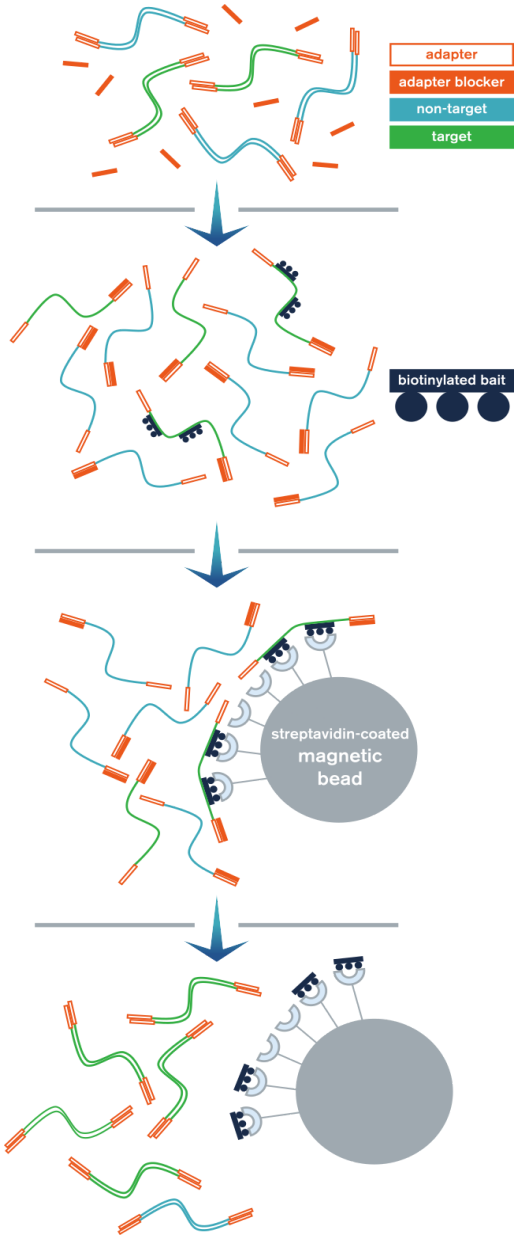
Procedure Overview

1. Amplified sequencing libraries made from bisulfite or enzymatically-converted DNA, adapter blockers, and other hybridization reagents are combined.

2. Libraries are denatured, allowing blockers to hybridize to adapters. Baits are then introduced and hybridized to targets for several hours.

3. Bait-target hybrids are bound to streptavidin-coated magnetic beads and pulled out of suspension with a magnet.

4. Most non-target DNA is washed away. The remaining library is then amplified and either taken directly to sequencing or further treated.



Arbor Biosciences products compatible with this manual

This manual is compatible with current generation myBaits Custom Methyl-Seq kits (Catalog numbers ending in "**M.v5"**). Visit arborbiosci.com/mybaits-manual to download alternative and previous manuals, or email techsupport@arbor.daicel.com for assistance.

Note for returning myBaits users: This reagent chemistry typically provides significantly higher target specificity (= percentage of on-target reads) compared to version 4 and earlier myBaits kits. If this is not desired for your application, please contact us for recommendations.

Kit components and stability

	Reagent	Cap color	Quantity (16 rxn)	Quantity (48 rxn)	Quantity (96 rxn)	Volume
Box 1 ***Store at 4˚C***	Hyb N	Red	1	2	4	500 µL
	Hyb S	Teal	1	2	4	750 µL
	Beads	Clear	1	2	4	1600 µL
	Binding Buffer	-	1	2	4	36 mL
	Wash Buffer	-	1	2	4	60 mL
Box 2 ***Store at -20˚C***	Hyb D	Yellow	1	2	4	190 µL
	Hyb R	Purple	1	2	4	70 µL
	Block C	Dark Green	1	2	4	130 µL
	Block O	Blue	1	2	4	270 µL
	Block X	Orange	2 × 9 µL	2 × 27 µL	4 × 27 µL	See adjacent
	Buffer E	Light Green	1	2	4	1600 µL
Box 3 ***Store at -80˚C***	Baits	White	2	6	12	50 µL (8 rxn)

At the recommended storage temperatures, myBaits kit components have a shelf life of one year.

It is strongly recommended that sub-aliquots of Baits are made in reaction sizes appropriate for your experiment plans to minimize freeze-thaw cycles.

REQUIREMENTS AND RECOMMENDATIONS

Input library

TYPE Use myBaits Custom Methyl-Seq with PCR-amplified and amplifiable NGS libraries generated from bisulfite- or enzymatically-converted nucleic acids in which non-methylated cytosines have been converted to uracils, and following PCR amplification these positions are now thymines. Compatible formats include Illumina TruSeq®-style, Illumina Nextera® Flex-style, Ion Torrent, or other libraries with universal adapter priming sites. Do NOT use myBaits with PCR-free libraries; additionally, myBaits are incompatible with libraries made using original Nextera or Nextera XT library preparation kits, or any library type containing biotin. Dual-indexed libraries are strongly recommended to reduce the hazard of mis-indexing induced by PCR jumping events.

AMOUNT For each Custom Methyl-Seq enrichment reaction, we recommend pooling 8 libraries, 200 ng each (for a total of 1.6 µg) concentrated to 7 µL with vacuum centrifugation or other means, and suspended in a nuclease-free buffer or water.

Blocking oligos

When ordering your myBaits kit, please indicate the sequencing library configuration you intend to enrich. The standard adapter blocking reagent provided with the kit (**Block X**) is compatible with Illumina TruSeq-style or Nextera-style libraries with single 6-12 bp or dual 6-12 bp indexing. For different adapter configurations, we recommend ordering Custom IDT® xGen® Blocking Oligos customized for your NGS library adapter sequences. At a concentration of 1 µg/µL, any custom adapter-blocking oligos can be used in lieu of myBaits Block X.

Equipment required

Item	Notes
50 mL tubes	1 per 44 reactions
Microcentrifuge tubes (1.5, 1.7, or 1.8 mL)	2 per 8 reactions
0.2 mL PCR strips with attached lids	2 per 8 reactions (e.g., VWR Cat# 93001-118)
Pipettors and tips for 0.5 - 500 µL	**Multichannel for 20 and 500 µL recommended**
† Thermal cycler with heated lid compatible with 0.2 mL strips	1 or 2
Magnetic particle collector for microcentrifuge tubes	1 (e.g., ThermoFisher Cat# 12321D)
Magnetic particle collector for 0.2 mL strips	1 (e.g., Permagen Cat# S500)
Vortex mixer and mini-centrifuge for tubes and strips	
Water bath or incubation oven at 63°C	
Heat block for microcentrifuge tubes at 60°C	

† *Ensure that the thermal cycler and strips allow no more than 4 µL of 30 µL volume evaporation overnight at 63°C*

Reagents required

Reagent	Notes
Nuclease-free ("NF") water	900 µL per reaction
PCR primers to amplify sequencing libraries after capture, e.g.:	
Illumina P5: AATGATACGGCGACCACCGA	2.5 uL @ 10 µM per reaction
Illumina P7: CAAGCAGAAGACGGCATACGA	2.5 uL @ 10 µM per reaction
PCR reagents for post-capture amplification	1 per reaction (e.g. Roche Cat# 07958927001)
PCR purification system, e.g., silica columns or SPRI beads	1 cleanup per reaction

PROCEDURE

PART 1: Hybridization setup

Sequencing libraries are mixed with various blocking nucleic acids, denatured, and then combined with other hybridization reagents (including baits). These hybridization reactions incubate for several hours to allow baits to encounter and hybridize with target library molecules.

M1.1 Prepare materials

Reagents	Equipment
Nuclease-free Water ("H2O")	Nuclease-free microcentrifuge tubes (×2)
Hyb N and Hyb S from Box 1	0.2 mL strips with attached lids (×1 per 8 reactions)
Hyb D and Hyb R from Box 2	Pipettors and tips; multichannel for 20 µL recommended
Block C, Block O, and Block X from Box 2	Vortex mixer and mini-centrifuge for above tube types
Baits from Box 3 **KEEP ON ICE**	Heat block set to 60°C
Libraries or library pools in 7 µL per reaction	Thermal cycler(s); 2 blocks recommended for 24 or more reactions

Program the thermal cycler:

Set lid temperature 5 to 10°C above each step temperature to minimize evaporation

Step	Temperature	Time
1	95°C	5m
2	63°C	5m
3	63°C	∞

M1.2 Hybridization Mix setup

1. Once the Hyb reagents have thawed, vortex them to homogenize and then briefly centrifuge.

   Heat Hyb N and Hyb S to 60°C and vortex to dissolve any precipitate present after thawing

2. Assemble the Hybridization Mix in a microcentrifuge (MC) tube, briefly vortex and briefly centrifuge to collect the sample. **The following volumes are already adjusted for pipetting error:**

Component	µL / Reaction	
Hyb N	9.25	
Hyb D	3.5	
Hyb S*	0.5	**Cloudiness caused by Hyb S addition will clear after step 3*
Hyb R	1.25	
H2O (round 1 / round 2)	1.1 / 4.4	*First enrichment round: 1.1 µL Second round: 4.4 µL*
Baits (round 1 / round 2)	4.4 / 1.1	*First enrichment round: 4.4 µL Second round: 1.1 µL*
TOTAL	**20**	

3. Incubate the Hybridization Mix at 60°C for 10 minutes in the heat block. Vortex occasionally to collect condensate. Remove from the heat block and let sit 5 minutes before proceeding.

4. For each capture reaction, aliquot 18.5 µL of Hybridization Mix to a 0.2 mL well/tube.

These reaction aliquots of Hybridization Mix are now referred to as "HYBs"

M1.3 Blockers Mix setup

1. Assemble the Blockers Mix **specific for your target taxon/taxa** in an appropriately-sized tube and mix by pipetting. **The following volumes are already adjusted for pipetting error:**

MOST TAXA	
Component	**µL / Reaction**
Block O	2.5
Block C	2.5
Block X	0.5
NF Water	-
TOTAL	**5.5**

PLANTS	
Component	**µL / Reaction**
Block O	5.0
Block C	-
Block X	0.5
NF Water	-
TOTAL	**5.5**

SALMONIDS	
Component	**µL / Reaction**
Block O	-
Block C	2.5
Block X	0.5
NF Water	2.5
TOTAL	**5.5**

2. For each capture reaction, aliquot 5 µL of Blockers Mix to a 0.2 mL well/tube.

3. Add 7 µL of individual or pooled libraries to each Blockers Mix aliquot and mix by pipetting.

These libraries mixed with Blockers Mix aliquots are now referred to as "LIBs"

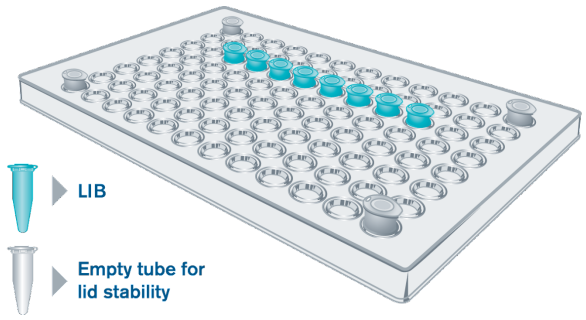
M1.4 Reaction assembly

Double-check the thermal program:

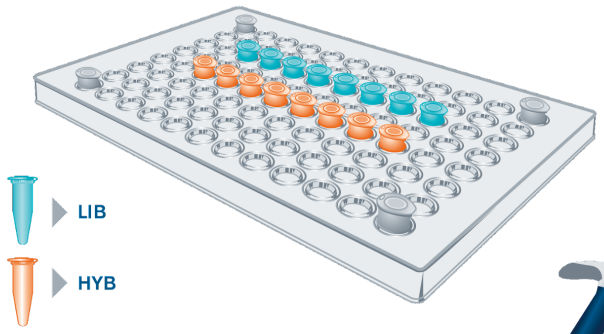
Set lid temperature 5 to 10°C above each step temperature to minimize evaporation

Step	Temperature	Time
1	95°C	5m
2	63°C	5m
3	63°C	∞

1. Put the **LIBs** in the thermal cycler, close the lid, and start the thermal program.

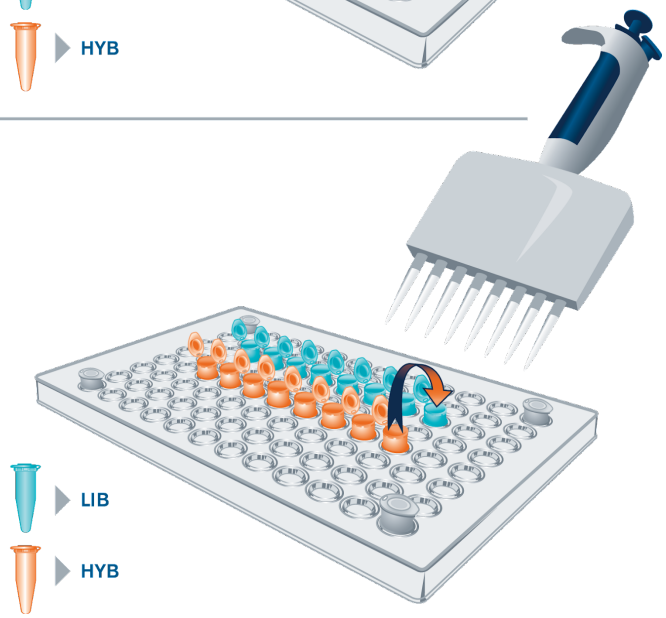


2. Once the cycler reaches 63°C during step 2, pause the program, put the **HYBs** in the thermal cycler, close the lid, and resume the program.



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.** Use a multichannel pipettor for easier execution. Gently homogenize by pipetting up and down 5 times.

4. Dispose of the HYB tubes. Briefly spin down the LIBs, return to the thermal cycler, close the lid, and allow the reactions to incubate overnight (16 to 24 hours).



PART 2: Bind and Wash ("Cleanup")

Bait-target hybrids are bound to streptavidin-coated magnetic beads, and then most non-target DNA is removed with several rounds of washing with a warm buffer. This is usually performed the day following completion of Part 1.

M2.1 Prepare materials

Start at least 90 minutes before intended hybridization stop time

Reagents
Hyb S (Box 1) *
Binding Buffer (Box 1) *
Wash Buffer (Box 1) *
Beads (Box 1)
Nuclease-free (NF) Water (up to 900 µL per reaction)

** Allow these reagents to come to room temperature before use; warm to 60°C and vortex to dissolve precipitate if necessary*

Equipment
Water bath or incubation oven set to 63°C
Receptacles for 50 mL tubes, 0.2 mL strips and microcentrifuge tubes compatible with above incubation device
Vortex mixer and mini-centrifuge for 0.2 mL strips and MC tubes
Magnetic particle collector(s) (MPC) for above strips and/or tubes

When using only a microcentrifuge (MC) tube-compatible MPC	When using a 0.2 mL tube-compatible MPC
Nuclease-free 50 mL tube, 1 per 44 cleanups	Nuclease-free 50 mL tube, 1 per 68 cleanups
Nuclease-free MC tubes, 1 per reaction	Nuclease-free 0.2 mL PCR strips with individually-attached lids, 1 vessel per reaction
Heat block set to 63°C	Thermal cycler set to 63°C
Pipettors and tips for 20 – 500 µL	Pipettors and tips for 20 – 200 µL; **multichannel pipettor strongly recommended**

M2.2 Wash Buffer X preparation

This step generates enough Wash Buffer X for 44 reactions in microcentrifuge ("MC") tube cleanup format, and 68 reactions in 0.2 mL cleanup format; scale up or down if needed.

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. Combine 400 µL Hyb S, 39.6 mL NF water and 10 mL Wash Buffer in a 50 mL tube. Vortex thoroughly, label "Wash Buffer X." *Wash Buffer X can be stored at 4°C for 1 month*
3. Heat the Wash Buffer X to the 63°C in the water bath or oven for at least 30 minutes before use.

M2.4 Bead preparation

 *Prepare beads immediately prior to use*

1. For each capture reaction, aliquot 30 µL beads to a microcentrifuge tube.
2. Pellet the beads in the MPC until the suspension is clear (1-2 minutes).
   Leaving the tubes on the magnet, remove and discard the supernatant.
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.
4. Repeat Step 3 above twice for a total of three washes.
5. Resuspend each washed bead aliquot in 70 µL Binding Buffer. If proceeding to washing in 0.2 mL format, transfer the aliquots to PCR strips.

   TIP: *Beads can be prepared in 8 (or fewer) reaction batches (240 µL) in a microcentrifuge tube. Multiply all volumes by the number of reactions in the batch; i.e., for 8 reactions-worth, wash with 1.6 mL and resuspend in 560 µL Binding Buffer, then aliquot 70 µL suspension to individual tubes.*

M2.5 Binding beads and hybrids

1. Heat the bead aliquots to 63°C for at least 2 minutes.
2. Transfer each capture reaction to the heated bead aliquots. Mix by pipetting.
3. Incubate the libraries + beads on the heat block or thermal cycler for 5 minutes. Agitate at the 2.5 minute mark by flicking or inverting the tubes to keep the beads suspended, followed by briefly centrifuging to collect.

M2.6 Bead washing

1. Pellet the beads with the MPC until the solution is clear. Remove and discard the supernatant.
2. Add 375 µL (MC tube format) or 180 µL (0.2 mL format) warmed Wash Buffer X to the beads, remove from the MPC, place on heat block for 15 seconds, and briefly vortex or mix by pipetting. Briefly centrifuge to collect.
3. Incubate for 5 minutes at 63°C in the heat block or thermal cycler. Agitate at the 2.5 minute mark via gentle vortexing and briefly centrifuge.
4. Repeat steps 1 through 3 two times for MC tube format (three washes total), or 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

Bead-bound enriched library is resuspended in Buffer E and amplified.

M3.1 Prepare materials

Reagents	Equipment
Buffer E (Box 2)	Tubes appropriate for PCR master mix assembly
PCR primers for amplifying libraries (e.g., P5 and P7)	Tubes or strips for 50 µL PCR amplification
PCR reagents for post-capture amplification	Pipettors and tips capable of 5 – 100 µL volumes
PCR purification system, e.g., silica columns or SPRI beads	Vortex mixer and mini-centrifuge for above tube types
Nuclease-free (NF) Water	Thermal cycler

M3.2 Enriched library recovery

1. Add 30 µL Buffer E to the washed beads and thoroughly resuspend by pipetting.
   Then, depending on your library amplification system, choose workflow A or B:

WORKFLOW A: ***When using KAPA HiFi HotStart or NEB Ultra II Q5 polymerase systems for amplification***	**WORKFLOW B:** ***When not using the polymerase systems for amplification in workflow A***
2A. Proceed directly to section H3.3 using this bead resuspension as template in amplification	2B. Incubate the suspension at 95°C for 5 minutes 3B. Immediately pellet the beads in the MPC and collect the supernatant containing the enriched libraries

M3.3 Library amplification

This is an example post-capture amplification using KAPA HiFi HotStart ReadyMix and Illumina libraries.

If this is the first time performing step M3.3, generate two of the following reactions per enrichment reaction (each with 15 µL enriched library as template). Otherwise, generate only one:

1. Assemble the following PCR master mix:

Component	Final Concentration	µL / Reaction	Sequence
NF Water	-	5	-
2X KAPA HiFi HotStart Ready Mix	1 X	25	-
P5 library primer (at 10 µM)	500 nM	2.5	AATGATACGGCGACCACCGA
P7 library primer (at 10 µM)	500 nM	2.5	CAAGCAGAAGACGGCATACGA
Enriched Library (on- or off-bead)	-	15 *	-
	TOTAL	**50**	**-**

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

M3.3 Library amplification (continued)

2. Cycle the reactions with the following thermal program:

Step	Temperature	Time		
1	98°C	2 minutes		
2	98°C	20 seconds	×14 or 10 cycles*	**First round of enrichment: 14 Second round of enrichment: 10*
3	60°C	30 seconds		
4	72°C	45 seconds		
5	72°C	5 minutes		
6	8°C	∞		

3. After amplification:
   - **If beads were included in the amplification reaction and you intend to use silica columns for purification**, pellet the beads first and purify only the supernatant.
   - Otherwise, purify the reaction using your preferred PCR cleanup (e.g., silica columns or SPRI beads).

M3.4 Perform a second round of enrichment

If this is the end of the first time through step M3.3 (one of two):

1. Combine both purified amplification reactions generated above and concentrate to 7 µL.
2. Repeat steps M1.1 through M3.3 using this once-enriched template as input.

If this is the end of your second time through step M3.3 (two of two), the enriched libraries are now ready for quantification, quality-assessment, and sequencing.

APPENDIX

MA1: Troubleshooting

During hybridization, my thermal cycler dropped below the hybridization temperature

You can expect a lower on-target read proportion and target read complexity for these libraries than if the temperature had remained where intended, but not outright enrichment failure. Shallow preliminary sequencing will determine whether targets are likely to be retrieved at sufficient coverage within budget.

My enriched and amplified library is not visible on electrophoresis gel or similar

Successful captures frequently yield a total mass of just a few nanograms even after re-amplification, which can be difficult to visualize with electrophoresis. This is most common when capturing especially small targets (<100 bp), or targets that are present at low frequency in the starting library (like those in degraded/ancient/environmental DNA), or if there is under-reamplification of the library post-capture. Often a few more cycles of library amplification will render the captured product sufficiently high in concentration to view with electrophoresis. Alternatively, determine with library qPCR whether the library is of sufficient mass for sequencing. If cycling is halted before reaching PCR plateau, the qPCR product can be visualized with electrophoresis to determine length distribution. Consult with your sequencing provider for library concentration and volume requirements.

My enriched and amplified library appears significantly longer than my original library, or has two peaks.

This may happen if the libraries are over-amplified and have formed 'daisy-chains' or 'bubbles' by experiencing cycles of denature-renature without template extension. These can be reverted to their original appearance in electrophoresis by applying three PCR cycles using regular library amplification.

I observe a high ratio of PCR duplicates in my enriched library sequence data.

Percent duplicates in sequencing data (i.e. "clonality" or "duplication rate") increases as you sequence deeper, and therefore it can only be fairly compared between experiments when the sequencing depth is normalized before analysis. Evaluate whether you have simply over-sequenced the libraries by plotting raw sequencing reads obtained on the X axis, and unique reads observed on the Y axis. If this **complexity curve** has plateaued, but you achieved sufficient unique reads, you sequenced more deeply than necessary. If it has not flattened, or you need to increase the total potential unique read yield of the library, **use more DNA per library preparation and/or more library per capture reaction. Avoid diluting baits before capture.** When working with heavily contaminated or damaged DNA target molecules, consider reducing temperatures used in all steps to improve capture sensitivity. Reducing PCR cycles when possible may also improve target coverage uniformity and complexity for a given sequencing depth, in some cases having an indirect effect on duplication rate. For more information about library complexity for any NGS application, we recommend Daley & Smith 2013 (doi: 10.1038/nmeth.2375).

MA2: myBaits Procedure Quick Guide - Custom Methyl-Seq Protocol

1. For each reaction, build the following Mixes; pipetting error is built in:

Hybridization Mix	
Component	**µL / Reaction**
Hyb N	9.25
Hyb D	3.5
Hyb S	0.5
Hyb R	1.25
Baits (round 1 / 2)	4.4 / 1.1
H2O (round 1 / 2)	1.1 / 4.4
TOTAL	**20**

Blockers Mix	
Component	**µL / Reaction**
Block X	0.5
Block C	2.5
Block O	2.5*
NF Water	0 †
TOTAL	5.5

*Plants: 5.0; Salmonids: 0
†Plants: 0; Salmonids: 2.5

2. After pre-warming the Hybridization mix for 10 minutes @ 60°C, for each reaction, aliquot **18.5 µL** of Hybridization Mix to their own tubes – now **"HYBs"**.
3. For each reaction, aliquot **5 µL** of Blockers Mix and then add **7 µL** of each library – now **"LIBs"**.
4. Incubate the LIBs in the thermal cycler for 5 minutes @ 95°C and then drop to 63°C. **Be sure to use a heated lid.**
5. Put the HYBs in the thermal cycler and warm to 63°C for 5 minutes.
6. Transfer **18 µL** of each HYB to each LIB, mix by pipetting, and incubate for 16-24 hours.
7. **1.5 hours before step 9**, prepare Wash Buffer X by combining 400 µL Hyb S, 39.6 mL nuclease-free molecular biology-grade water and 10 mL Wash Buffer in a 50 mL tube. Vortex thoroughly and warm to 63°C for at least 45 minutes.
8. Prepare 30 µL of beads per reaction by washing three times in 200 µL Binding Buffer. Resuspend the washed bead aliquots in 70 µL Binding Buffer and warm the suspensions to 63°C for at least 2 minutes.
9. Combine the warmed beads with the hybridization reactions and incubate for 5 minutes at 63°C, agitating at 2.5 minutes to keep beads suspended.
10. Pellet the beads and remove the supernatant. If using microcentrifuge tubes for cleanup, wash the beads three times with 375 µL warmed Wash Buffer X, incubating 5 minutes at 63°C. Wash four times with 180 µL washes if using a 96-well magnetic particle concentrator and 0.2 mL strips/tubes.
11. Resuspend the beads in 30 µL Buffer E and then use 15 µL of this in a 50 µL amplification reaction with KAPA® HiFi or NEB Ultra II Q5 polymerase systems. If not using these polymerase systems, instead elute the library from the beads by incubating the suspension for 5 minutes at 95°C, immediately pellet the beads, and then use 15 µL of the supernatant in a 50 µL amplification reaction. Do two amplifications if this is the first round of enrichment; do one if this is the second and last round of enrichment.
12. Purify the amplification reactions using silica columns or SPRI beads. **If using silica columns and beads were included in the amplification reaction, pellet the beads first and purify only the supernatant.**
13. If this is the end of your first round of enrichment: combine both post-capture amplifications and concentrate to 7 uL, then repeat steps 1-12. Otherwise, the enriched libraries are ready for QC and sequencing.