Wheat Exome v1 Kit
Hybridization Capture for Targeted NGS

User Manual
Version 1.53 June 2023

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Wheat Exome v1 Kit Protocol

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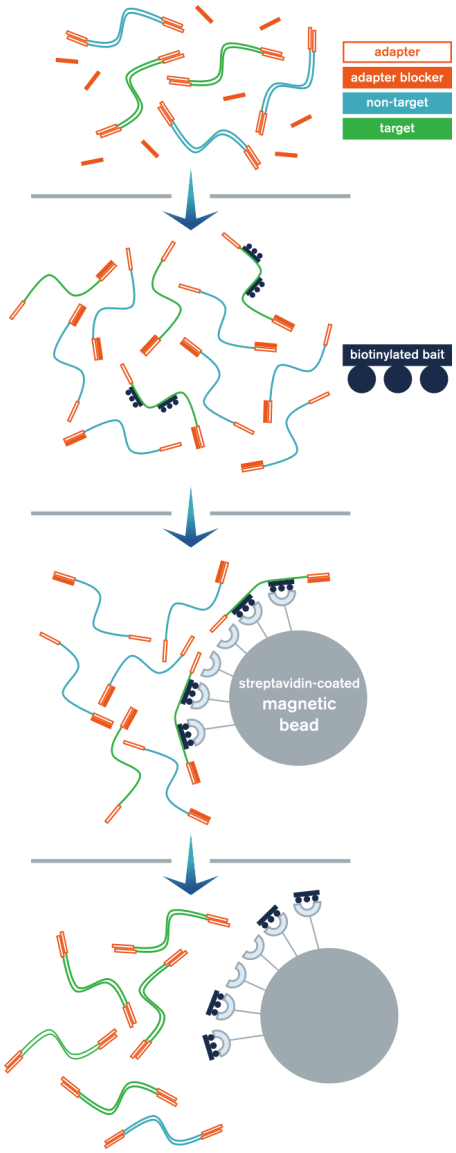
INTRODUCTION

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 sequence targets, here roughly 200 Mbp of exon and exon-proximate space as defined by the version 1.1 annotation set of the IWGSC Chinese Spring reference genome sequence (version 1). 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 library, 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 Arbor Biosciences catalog numbers starting with 3091. Visit arborbiosci.com/mybaits-manual to download alternative and previous manuals, or email techsupport@arbor.daicel.com for assistance.

Changes since myBaits Wheat Exome v1 Manual version 1.52 (March 2022)

- Updated **Requirements and Recommendations**, washing tips, post-capture QC, and Troubleshooting
- Reagent cap colors are updated (contents are unchanged)

Changes since myBaits Wheat Exome v1 Manual version 0.9 (August 2019)

- **Block X** is now supplied and users are no longer required to supply their own blockers
- **Buffer E** is now supplied for post-cleanup bead resuspension

Kit components and stability

Box	Reagent	Cap color		Volume per kit	
		Lot# **23**XXXX	Lot# **22**XXXX	**8 Reaction**	**48+ Reaction**
Box #1 *Store at 4˚C*	Hyb N	Red	Red	400 µL	1000 µL
	Hyb S	Blue	Teal	1500 µL	1500 µL
	Beads	Clear	Clear	550 µL	1600 µL
	Binding Buffer	Clear	Clear	12 mL	36 mL
	Wash Buffer	Clear	Clear	20 mL	60 mL
Box #2 *Store at -20˚C*	Hyb D	Yellow	Yellow	140 µL	400 µL
	Hyb R	Purple	Purple	50 µL	150 µL
	Block O	Blue	Blue	90 µL	270 µL
	Block X	Orange	Orange	18 µL	106 µL
	Buffer E	Clear	Light Green	550 µL	1600 µL
Box #3 *Store at -80˚C*	Baits	Clear	White	50 µL per 8 rxns	

myBaits kits are shipped at ambient temperature and are stable during transit. If the kit boxes are stored upon arrival at the temperatures listed above, the reagents expire one year from the date they were received.

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

REQUIREMENTS AND RECOMMENDATIONS

Input library

DNA SOURCE Libraries can be made from diploid, tetraploid, or hexaploid wheat and relatives (barley, rye, etc). Prior to library preparation, the DNA solution should be free of color, non-viscous, and contain low RNA contamination.

DNA TO LIBRARY PREP We recommend using between 100 and 300 ng non-sheared (for library preparation protocols that use enzymatic shearing) or mechanically-sheared total DNA quantified with Qubit™ (or similar). Mechanically-sheared input should contain fragments falling between 400 and 700 bp.

LIBRARY PREPARATION myBaits is compatible with any PCR-compatible linear library structure, and blockers included in the kit are compatible with most Illumina configurations. Prior to capture, libraries should be PCR-amplified for 4-8 cycles. Final insert lengths should fall between 300 and 700 bp with a mode between 450 and 550 bp (not including adapters). Uniquely dual-indexed libraries are strongly recommended to reduce the hazard of mis-indexing induced by PCR jumping events. It is NOT recommended to 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.

AMOUNT For each enrichment reaction, pool 8 libraries, 1 µg each (as quantified with e.g. Qubit), for a total 8 µg pool. Then, concentrate this volume to 7 µL with vacuum centrifugation or other means, suspended in a nuclease-free buffer or water. If the starting library pool is larger than 50 µL, first perform a buffer exchange using e.g. SPRI or silica columns to bring the volume down to 30 µL prior to vacuum centrifugation to 7 µL.

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 65°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 65°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

Pools of 8 sequencing libraries (1 µg each, for a total of 8 µg) are mixed with various blocking nucleic acids, denatured, and then combined with other hybridization reagents (including baits). These hybridization reactions incubate overnight to allow baits to encounter and hybridize with target library molecules.

W1.1 Prepare materials

Reagents	Equipment
Hyb N and Hyb S from Box 1	Nuclease-free microcentrifuge tubes (×2)
Hyb D and Hyb R from Box 2	0.2 mL strips with attached lids (×1 per 8 reactions)
Block O and Block X from Box 2	Pipettors and tips; multichannel for 20 µL recommended
Baits from Box 3 **KEEP ON ICE**	Vortex mixer and mini-centrifuge for above tube types
Libraries in pools of 8, 1 µg each, in 7 µL per reaction	Heat block set to 60°C
	Thermal cycler(s); 2 blocks recommended for >23 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	65°C	5m
3	65°C	∞

W1.2 Hybridization Mix setup

1. Once the Hyb reagents have thawed, vortex 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 the contents to collect. **The following volumes are already adjusted for pipetting error:**

Component	µL / Reaction
Hyb N	9.25
Hyb D	3.5
Hyb S*	0.5
Hyb R	1.25
Baits	5.5
TOTAL	**20**

**Cloudiness caused by Hyb S addition will clear after step 3*

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

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"

W1.3 Blockers Mix setup

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

Component	µL / Reaction
Block O	5.4
Block X	2.1
NF Water	-
TOTAL	**7.5**

2. For each capture reaction, aliquot 7 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"

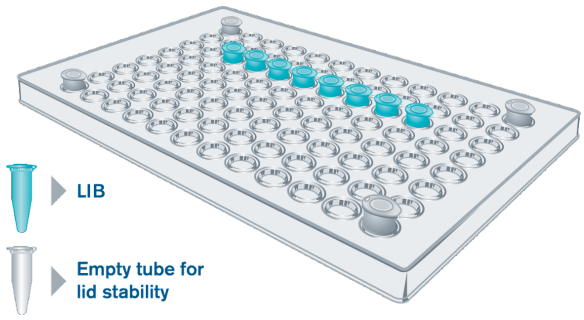
W1.4 Reaction assembly

Double-check the thermal program:

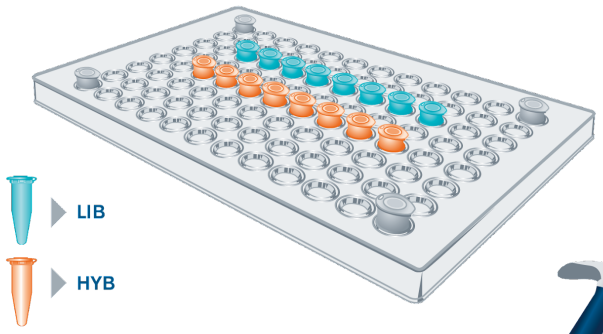
Set lid temperature 5 to 10°C above each step temperature to minimize evaporation

Step	Temperature	Time
1	95°C	5m
2	65°C	5m
3	65°C	∞

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

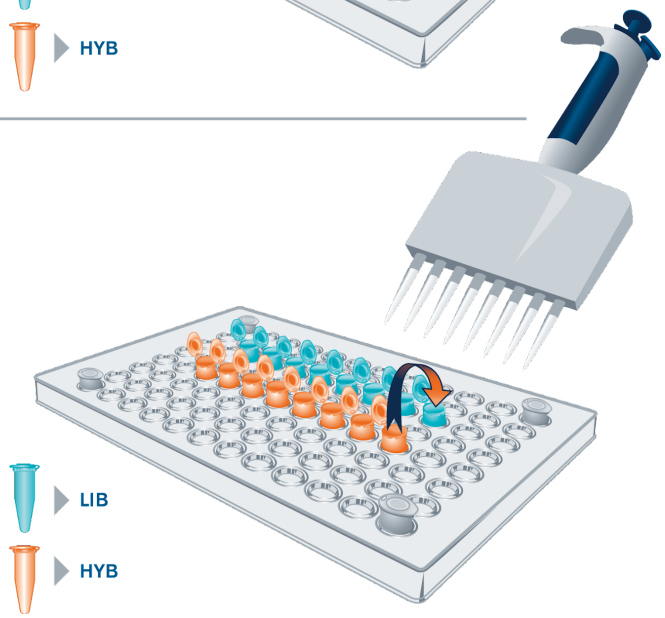


2. Once the cycler reaches 65°C during step 2, pause the program, put the **HYBs** in the thermal cycler, close the lid, and resume the program.



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 for between 18 and 24 hours.



PART 2: Bind and Wash ("Cleanup")

After the 18-24 hour incubation, 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.

W2.1 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 65°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 65°C	Thermal cycler set to 65°C
Pipettors and tips for 20 – 500 µL	Pipettors and tips for 20 – 200 µL; **multichannel pipettor strongly recommended**

W2.2 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 65°C in the water bath or oven for at least 30 minutes before use.

W2.3 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. Place tube in the MPC and pellet beads; 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.*

W2.4 Binding beads and hybrids

1. Heat the bead aliquots to 65°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.

W2.5 Bead washing

1. Pellet beads with the MPC until the solution is clear. Remove and discard the supernatant.
2. **Leaving the MPC on the bench for all wash steps**, 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 the mixture.
3. Incubate for 5 minutes at 65°C in the heat block or thermal cycler. Agitate at the 2.5 minute mark via gentle vortexing and then 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 liquid as possible without touching the bead pellet.**

PART 3: Library Resuspension, Amplification, and Sequencing

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

W3.1 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

W3.2 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 S3.3 using this bead resuspension as the 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

W3.3 Library amplification

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

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.*

W3.3 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	
3	60°C	30 seconds	×10 cycles
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).

   The enriched libraries are now ready for quantification, quality-assessment, and sequencing. Post-capture yields are typically

W3.4 Post-capture QC and Sequencing

- Captured libraries quantified with Qubit (or similar) should be between 100 and 300 ng total mass.
- Visualize the libraries using e.g. TapeStation or Bioanalyzer (Agilent). Captured library lengths should be on average 325 bp + the length of the adapters.
- If total library yield post-capture is 100 ng or greater, and it meets the indicated length specifications, take the libraries to sequencing. Shallow screen-sequencing is recommended to ensure the raw reads on-target is 60% or better.
- Once mass, length, and specificity are confirmed within-spec as described above, take the libraries to deeper sequencing. To sequence the targets in hexaploid wheat to 30X unique average unique read depth, we recommend 18 Gbp (60 M read-pairs) PE150 sequencing per library (144 Gbp or 480 M read-pairs per pool of 8 libraries). Tetraploid and diploid wheat require 12 or 6 Gbp PE150 sequencing, respectively.

APPENDIX

WA1: Troubleshooting

Post-capture, my libraries were low yield and/or short

If after capture the total library mass was <100ng, or if the mean insert length (not including adapters) is 325 bp or lower, repeat the capture but with 20% more library mass. Consider increasing the mean insert length of the libraries prior to capture as well, either by size-selecting prior to or after pooling for capture, or repeating library preparation with a more appropriate shearing regiment.

My on-target read percentage (before PCR duplicate collapse) is lower than 60%

Ensure that 8 micrograms total library material (as measured with quantitative PCR or intercalating dye such as) has been used per enrichment reaction. Lower input amounts and/or use of other adapter-blocking oligos than Block X may reduce percent on-target significantly. If the total post-capture library mass was >200 ng, typically sequencing the library more deeply is the best route to obtaining sufficient coverage depth.

During hybridization, my thermal cycler dropped below 65°C

This kit should return between 50 and 70% reads on-target when enriching libraries made from hexaploid wheat. If the temperature drops during hybridization, you can expect a lower on-target read proportion and target read complexity, but not outright enrichment failure. Shallow preliminary sequencing will determine whether targets are likely to be retrieved at sufficient coverage within budget.

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

Sequencing 18 Gbp per library using PE150 protocol (i.e., 60 M read-pairs per library) on Illumina platforms typically yields fewer than 30% PCR duplicates following alignment to the IWGSC Chinese Spring reference genome assembly. If you observe higher PCR duplicate rate at 18 Gbp (or lower) sequencing depth, ensure 1 ug of library as measured with verified library quantitative PCR was used per enrichment reaction. Also ensure the libraries used the manufacturer's recommended nanograms input DNA per prep.

WA2: myBaits Procedure Quick Guide - Wheat Exome V1

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	5.5
TOTAL	**20**

Blockers Mix	
Component	**µL / Reaction**
Block X	2.1
Block O	5.4
NF Water	0 †
TOTAL	7.5

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 **7 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 65°C. **Be sure to use a heated lid set to 5 to 10 °C higher than each step temperature.**
5. Put the HYBs in the thermal cycler and warm to 65°C for 5 minutes.
6. Transfer **18 µL** of each HYB to each LIB, mix by pipetting, and incubate for between 18 and 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 65°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 65°C for at least 2 minutes.
9. Combine the warmed beads with the hybridization reactions and incubate for 5 minutes at 65°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 65°C. Wash four times with 180 µL washes if using a 96-well magnetic particle concentrator and 0.2 mL strips/tubes. Leave the MPC on the bench for all steps (i.e., do not place it on the thermal cycler/hot block).
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.
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.**