Expert Wheat Exome V1 Enrichment Kit

Manual
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INTRODUCTION

myBaits® is an in-solution NGS library target enrichment system, compatible with Illumina®, Ion Torrent®, and many other sequencing library types. Arbor Biosciences uses a versatile nucleic acid synthesis technology to make biotinylated RNA "baits" that are complementary to target sequences. Probes ("baits") and other reagents for NGS target enrichment are supplied with the myBaits kit.

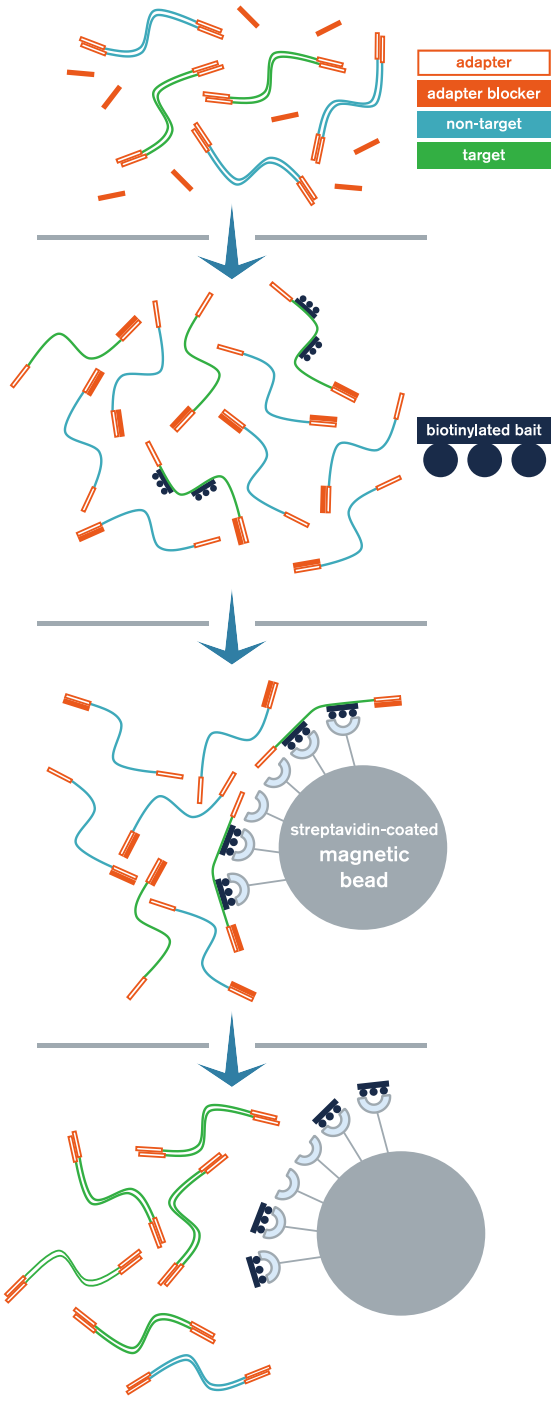
Procedure overview

1. Sequencing library, adapter blockers, and other hybridization reagents are combined

2. Libraries are denatured and cooled to allow blockers to hybridize to adapters, and then baits are introduced and allowed to hybridize to targets for several hours

3. Bait-target hybrids are bound to streptavidin-coated magnetic beads and sequestered with a magnet

4. Most non-target DNA is washed away, and the remaining library is amplified



Arbor Biosciences products compatible with this manual

Catalog #	Description	Includes
3091**	myBaits® Expert Wheat Exome V1	Probes and reagents for hybridization capture of the wheat exome in NGS libraries

Contact info@arborbiosci.com, or visit our website, for product manuals not listed above

Notable differences from the standard myBaits procedure

- One microgram (1 µg) of each NGS library per sample is required
- Eight (8) NGS libraries are pooled per enrichment reaction (total 8 µg per pool) in 5 µL
- IDT xGen® Universal Blockers are strongly recommended to be used instead of Block A
- Block C is omitted from each reaction, and Block O is doubled

Kit components and stability

	Reagent	Cap color	Volume (16 rxn)	Volume (48+ rxn)
Box 1 ***Store at 4˚C***	Hyb N	Red	175 µL	500 µL
	Hyb S	Teal	750 µL	750 µL
	Beads	-	550 µL	1600 µL
	Binding Buffer	-	12 mL	36 mL
	Wash Buffer	-	20 mL	60 mL
Box 2 ***Store at -20˚C***	Hyb D	Yellow	70 µL	190 µL
	Hyb R	Purple	25 µL	70 µL
	Block O	Blue	100 µL	260 µL
	Block A	Orange	40 µL	100 µL
Box 3 ***Store at -80˚C***	Baits	White	50 µL / 8 rxn	50 µL / 8 rxn

At the recommended storage temperatures, myBaits kit components have a shelf life of 1 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

Equipment required

- Nuclease-free (**NF**) 50 mL, 1.7 mL (or similar) microcentrifuge low-bind and 0.2 mL low-bind tubes, e.g., Axygen MAXYmum Recovery™ tubes
- For 96-well format Bind and Wash procedure (see Part 2), 0.2 mL PCR strips with individually-attached lids
- Pipettors and tips capable of pipetting 0.5 µL – 500 µL
- Thermal cycler with heated lid compatible with chosen 0.2 mL tubes
  
  Ensure that the chosen combination of thermal cycler and 0.2 mL tubes does not allow more than 4 µL of 30 µL volume evaporation overnight at 65°C
- Magnetic particle concentrator for microcentrifuge tubes (e.g., Life Technologies DynaMag™-2, #123-210) and/or 96-well magnetic particle concentrator (e.g., Permagen® 96-well Ring Magnet Plate S500 or similar)
- Vortex mixer
- Mini centrifuge with adapters for 1.5–1.8 mL and 0.2 mL tubes/strips
- Water bath or incubation oven capable of 65°C
- Heat block capable of 65°C
- **STRONGLY RECOMMENDED:**
  - Multichannel pipettor capable of 20 µL volume for hybridization setup
  - Multichannel pipettor capable of up to 200 µL volume for 96-well format cleanups

Reagents required

- Nuclease-free (**NF**) molecular biology-grade water (up to 900 µL per enrichment reaction)
- 10 mM Tris-Cl, 0.05% TWEEN®-20 solution (pH 8.0-8.5) (30 µL per enrichment reaction)
- PCR primers for amplifying your sequencing libraries after capture, e.g., the "reamp" primers described in Meyer & Kircher 2010 (doi:10.1101:pdb.prot5448) for Illumina libraries
- PCR reagents for post-capture amplification (e.g., KAPA® HiFi HotStart ReadyMix, Kapa Biosystems)
- PCR purification system, e.g., silica columns or SPRI beads
- **STRONGLY RECOMMENDED:**
  - IDT xGen® Universal Blockers:
    - Cat# 107547* for 6 or 8 bp dual-indexed TruSeq-style libraries
    - Cat# 108110* for 10 bp dual-indexed TruSeq-style libraries
    - Cat# 107958* for dual-indexed Nextera libraries

Library specifications

- Each enrichment reaction should be performed on a pool of 8 libraries, 1 µg each, in 5 µL.

PROCEDURE

PART 1: Hybridization setup

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

1.1 Prepare materials

Gather these components:

Reagents:

- Hyb reagents (Boxes 1 and 2)
- Block reagents (Box 2)
- IDT xGen® Universal Blockers suitable for your library type, 2 µL per reaction
- Baits (Box 3) ***Keep on ice***
- Pool(s) of 8 NGS libraries, 1 ug each (8 ug total), in a volume of 5 µL per pool

Equipment:

- 1.7 mL (or similar) nuclease-free low bind tubes (×2)
- Low-bind 0.2 mL tubes with individual caps (×2 per reaction)
- Pipettors and tips; **multichannel pipettor for pipetting up to 20 µL recommended**
- Vortex mixer
- Thermal cycler; 2 blocks recommended for 24 or more reactions
- Heat block set to 60°C

Program the thermal cycler:

Include a heated lid for all steps to keep evaporation to a minimum.

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

1.2 Hybridization Mix setup

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

   If Hyb N and/or Hyb S have visible precipitate even after thawing, heat them to 60°C and vortex until the precipitate dissolves.

2. Assemble the Hybridization Mix in a 1.7 mL tube, briefly vortex and briefly centrifuge to collect. **The following volumes are already adjusted for pipetting error:**

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

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

3. Incubate the Hybridization Mix at 60°C for 10 minutes in the heat block, vortexing occasionally to collect condensed evaporate from the tube lid. Remove the mix from the heat block and allow to sit at room temperature for 5 minutes.

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

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

1.3 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 per Reaction
IDT xGen® Universal Blockers†	2.1
Block O	5.4
TOTAL	**7.5**

   † *Use 2.1 µL Block A if xGen® blockers are not available*

2. For each capture reaction, aliquot 7 µL of Blockers Mix to a low-bind 0.2 mL tube.

3. Add 5 µL of the pool of eight libraries to each Blockers Mix aliquot and mix by pipetting.

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

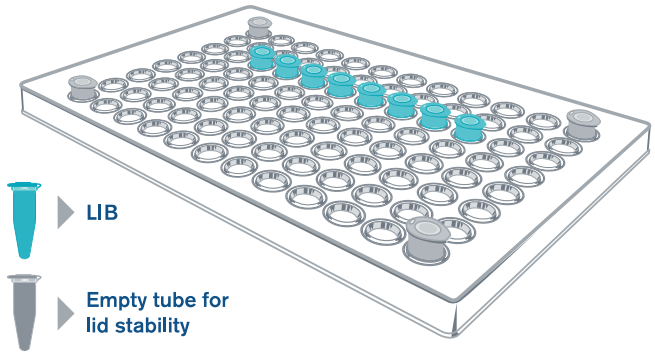
1.4 Reaction assembly

Double-check the thermal program:

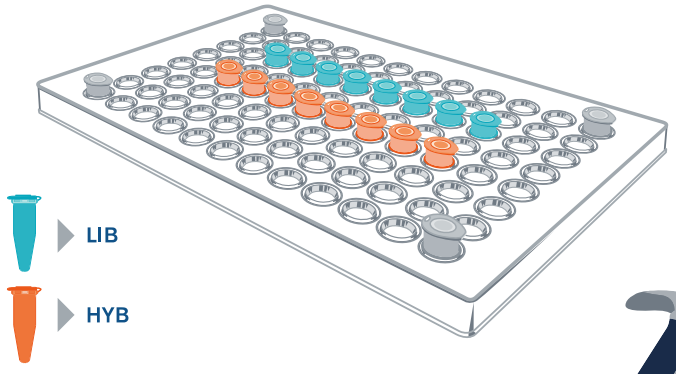
Include a heated lid

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.

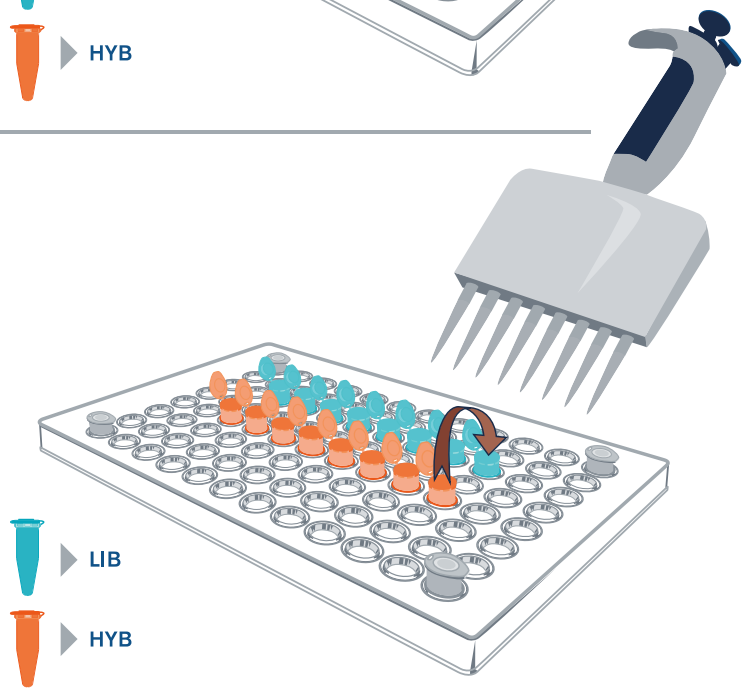


2. Once the cycler reaches 65°C during step 2, pause the program, put the **HYBs** in the thermal cycler, close the lid, and resume the program.



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 at 65°C overnight (15-20 hours).



PART 2: Bind and Wash ("Cleanup")

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

2.1 Prepare materials

Start at least 90 minutes before intended hybridization stop time.

Gather the following components:

Reagents:

- Hyb S
- Binding Buffer
- Wash Buffer

  Bring the solutions above to room temperature prior to use; warm gently to dissolve precipitate if necessary
- Beads
- NF Water (up to 900 µL per cleanup)
- 10mM Tris-Cl, 0.05% TWEEN-20 solution (pH 8.0-8.5)

Equipment:

- Water bath or incubation oven set 65°C
- Heat block (for microcentrifuge tube cleanup) or thermal cycler (for 0.2 mL strips/ plates cleanup) set to 65°C
- Vortex mixer
- Mini centrifuge for 2 mL and 0.2 mL size tubes/strips
- Magnetic particle concentrator(s) **("MPC")** for 1.8 mL tubes and/or 0.2 mL PCR strips/plates

When using only a microcentrifuge tube-compatible MPC	***When using a 0.2 mL vessel-compatible MPC***
• Nuclease-free (NF) low-bind 1.7 mL tubes, 1 vessel per cleanup • Heat block set to the bind and wash temperature(s) • Pipettors and tips for 20 – 500 µL • NF 50 mL tube, 1 per 44 cleanups	• Nuclease-free 0.2mL PCR strips with individually-attached lids, 1 vessel per cleanup • Thermal cycler set to the bind and wash temperature(s) • Pipettors and tips for 20 – 200 µL; multichannel pipettor strongly recommended • NF 50 mL tube, 1 per 68 cleanups

2.2 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. Wash Buffer X can be stored at 4°C for 1 month.

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."
3. Heat the Wash Buffer X to the 65°C in the water bath or oven for at least 30 minutes before use.

2.3 Bead preparation

 *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 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 1.7 mL 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.*

2.4 Binding beads and hybrids

1. Heat the bead aliquots to the 65°C for at least 2 minutes in the heat block or thermal cycler.
2. Transfer each capture reaction to the heated bead aliquots. Mix by pipetting.
3. Incubate the libraries + beads on the hot 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.

2.5 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, and briefly vortex or mix by pipetting. Briefly centrifuge to collect.
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 briefly centrifuging.
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

Here, bead-bound enriched library is resuspended in Tris-TWEEN solution, and then either taken directly to amplification while bound to beads, or heat-denatured from the baits and then amplified.

3.1 Prepare materials

Gather the following components:

Reagents:

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

Equipment:

- Tubes appropriate for PCR master mix assembly
- Vessels for 50 µL PCR amplification, e.g., 0.2 mL PCR strips or plates
- Pipettors and tips capable of 5 – 100 µL volumes
- Vortex mixer
- Mini centrifuge for 2 mL and 0.2 mL size tubes/strips
- Thermal cycler

3.2 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. Then, depending on your amplification system:

When using KAPA HiFi HotStart polymerase for amplification	***When using a different polymerase system for amplification***
2. Proceed directly to section 3.3 using this bead resuspension as template in amplification	2. Incubate the suspension at 95°C for 5 minutes 3. Immediately pellet the beads in the MPC, and take only the supernatant

3.3 Library amplification

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

1. Assemble the following PCR master mix:

Component	Final Concentration	µL per reaction
NF Water	-	5
2X KAPA HiFi HotStart ReadyMix	1 X	25
Forward library primer (at 10 µM)	500 nM	2.5
Reverse library primer (at 10 µM)	500 nM	2.5
Enriched Library (on- or off-bead)	-	15*
TOTAL		**50**

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

3.3 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	× 10 cycles
3	60°C	30 seconds	
4	72°C	length-dependent*	
5	72°C	5 minutes	
6	8°C	∞	

* For libraries
<500 bp average: 30s
500 to 700 bp: 45s
>700 bp: 1m

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 sequencing.

4. Sequencing depth: 18 Gbp of PE150 Illumina sequencing per library (144 Gbp per capture pool) typically yields >30X average unique read coverage across the ~200 Mbp target space in hexaploid wheat.

APPENDIX

A1: Troubleshooting

My on-target percentage (before PCR duplicate collapse) is lower than 50%

Ensure that 8 micrograms total library material (as measured with quantitative PCR or intercalating dye such as) has been used per enrichment reaction, and that xGen blockers were used. Lower input amounts and/or use of other adapter-blocking oligos will reduce percent on-target significantly.

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.

A2: myBaits Expert Wheat Exome V1 Procedure Quick Guide

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

Hybridization Mix	
Component	**µL per 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 per Reaction**
xGen® Blockers	2.1
Block O	5.4
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 **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 65°C. **Be sure to use a heated lid.**
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 16-24 hours.
7. **1.5 hours before step 9**, prepare Wash Buffer X by combining 400 µL HYB #4, 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.
11. Resuspend the beads in 30 µL of 10 mM Tris-Cl, 0.05% TWEEN®-20 (pH 8-8.5) and then use 15 µL of this in a 50 µL amplification reaction with KAPA® HiFi DNA polymerase. **Following amplification, pellet the beads and purify only the supernatant.**
12. If not using KAPA HiFi polymerase, elute the library from the beads by incubating the suspension for 5 minutes at 95°C. Pellet the beads and then use 15 µL of the supernatant in a 50 µL amplification reaction.