Hybridization Capture for Targeted NGS

Manual
Version 4.0 April 2018

Get the latest manual at www.arborbiosci.com/mybaits-manual

FOR RESEARCH USE ONLY. Not intended for diagnostic use.

5840 Interface Drive, Suite 101
Ann Arbor, MI 48103 (USA)
+1 (734) 998-0751
info@arborbiosci.com

CONTENTS

INTRODUCTION

myBaits® is an in-solution NGS library target enrichment system, compatible with Illumina®, Ion Torrent®, and many other sequencing library types. We use a versatile nucleic acid synthesis technology to make biotinylated RNA "baits" that are complementary to your sequence targets. Baits and other reagents for NGS target enrichment are supplied with the myBaits kit.

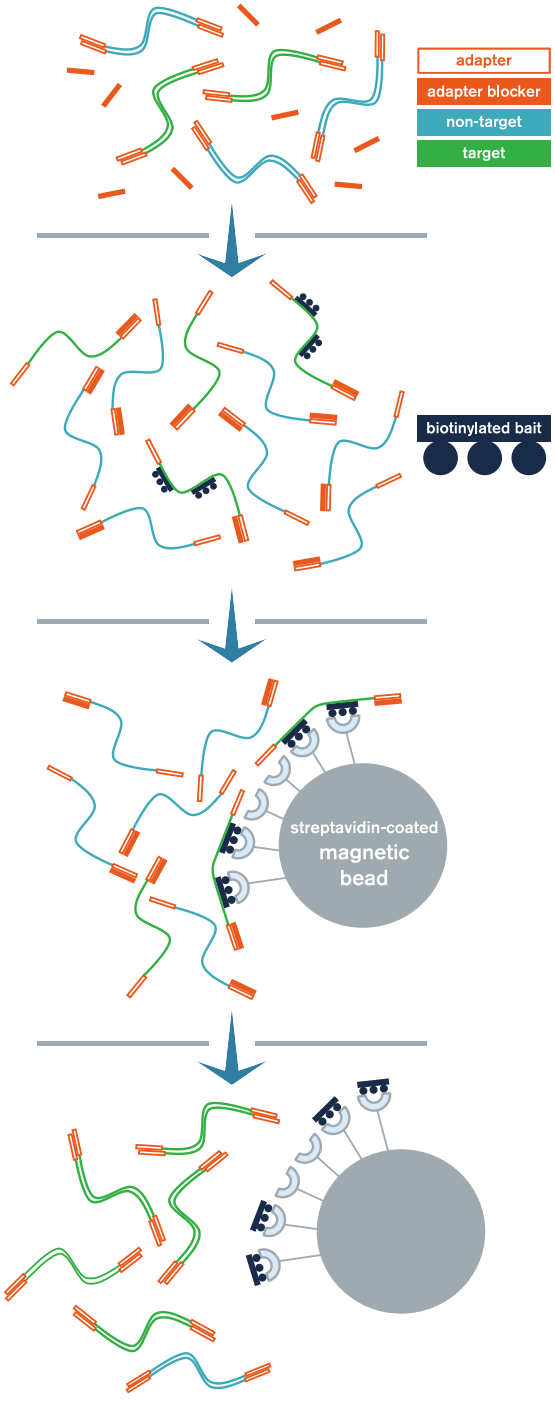
Procedure overview

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
300*** 301***	myBaits Custom	Custom baits designed and synthesized for your specific targets of interest
302***	myBaits WGE	Baits manufactured using a DNA sample as template, such as whole genomic DNA
303***	myBaits Mito	Pre-designed baits targeting the mitochondrial genomes of various taxonomic groups
305*** 306***	myBaits UCE	Pre-designed baits targeting ultraconserved genomic elements of various taxonomic groups

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

Changes since myBaits manual version 3.02

- New reagent names and formulations
- Streptavidin-coated magnetic beads ("Beads") are now included in the kit
- Pre-warming of Hybridization Mix is required before subsequent steps
- Microcentrifuge tube-scale cleanups use less Wash Buffer volume

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 C	Green	50 µL	130 µL
	Block O	Blue	50 µL	130 µL
	Block A	Orange	30 µL	30 µL
Box 3 ***Store at -80˚C***	Baits	White	44 µL / 8 rxn	44 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

Library specifications

- Each enrichment reaction has space for 7 µL library input, and 100-500 ng total library per reaction is recommended. Many libraries will require concentration prior hybridization, and should be ready well in advance of setting up the hybridization reactions.

Experimental design

Due to the wide variety of target, non-target, and library complexities compatible with myBaits, it is not possible to predict the conditions for *optimal* enrichment performance for a given combination of libraries and custom baits without trial use evaluations. The following are general experiment guidelines that have proven effective over thousands of enrichment experiments.

- The myBaits system is compatible with most sequencing libraries, including Illumina TruSeq-style and Ion Torrent formats. **Blockers specific to your library type and index configuration are included in your kit as Block A.**

  To couple myBaits with libraries prepared using the Illumina Nextera® kits, see pre-treatment instructions in Appendix section A2.

- Amplifying libraries before enrichment is strongly recommended – PCR-free libraries tend to perform poorly in most circumstances.
- Enrich 100-500 ng of library, as quantified using an intercalating dye assay or library qPCR. Fewer than 1 ng and as many as 3 µg of total library mass may also be viable.
- Multiple libraries can be pooled into individual capture reactions. If pooling, begin with 4 libraries per reaction, 125 ng each.
- Dual-indexed libraries are strongly recommended in general, and especially when pooling multiple libraries per capture reaction, to reduce the rate of mis-indexing induced by jumping events during pooled amplification.
- For most applications, **use 65°C for all relevant steps** in the myBaits procedure. Do not exceed 68°C. Use reduced temperatures when the expected bait-target divergence exceeds 5%, starting with 62°C.
- For most applications, **hybridize overnight**. Short hybridizations (<1.5-4 hr) are possible, but maximum potential library target proportion and complexity usually occurs with 12-16 hr hybridization periods.

Enriching low-copy, degraded and/or contaminated DNA libraries

- For libraries with a substantial anticipated amount of non-target templates (e.g., ancient, forensic, or environmental samples), use as much library as possible up to 2 µg.
- Pooling multiple libraries per enrichment reaction is not recommended. If libraries must be pooled, equilibrate by the target constituent rather than the total amount to reduce sample dropout.
- For libraries where the majority of target insert molecules are shorter than 80 bp and/or are heavily damaged, use 60°C temperatures for all relevant steps of the myBaits procedure. Optimal conditions will require trials.
- Libraries with especially low proportions of target DNA, such as heavily-contaminated ancient DNA libraries, may benefit from hybridizing 36-48 hours, or longer in some cases.
  - Two rounds of capture are strongly recommended for these situations; consult with us for experiment design advice.

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)
- Baits (Box 3) ***Keep on ice***
- Sequencing libraries to be enriched, in a final volume of 7 µL per reaction

Equipment:

- 1.7 mL nuclease-free low bind tubes (×2)
- Low-bind 0.2mL 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

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	Hybridization Temp.	5m
3	Hybridization Temp.	∞

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
Block A	0.5
Block C	2.5
Block O	2.5
TOTAL	**5.5**

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.

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

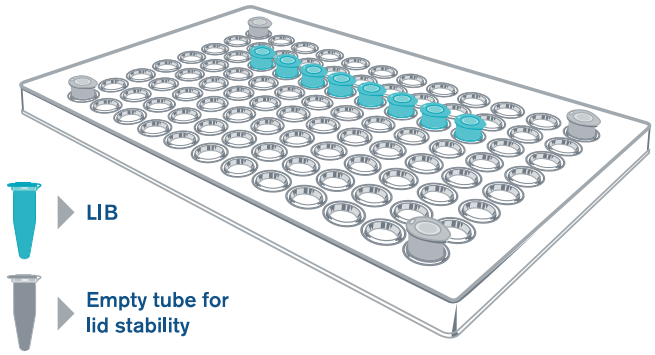
1.4 Reaction assembly

Double-check the thermal program:

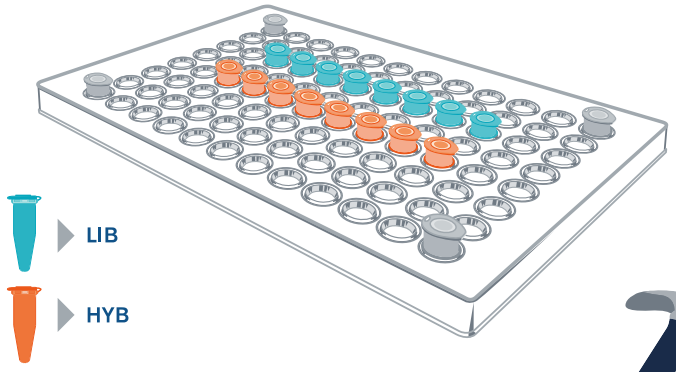
Include a heated lid

Step	Temperature	Time
1	95°C	5m
2	Hybridization Temp.	5m
3	Hybridization Temp.	∞

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

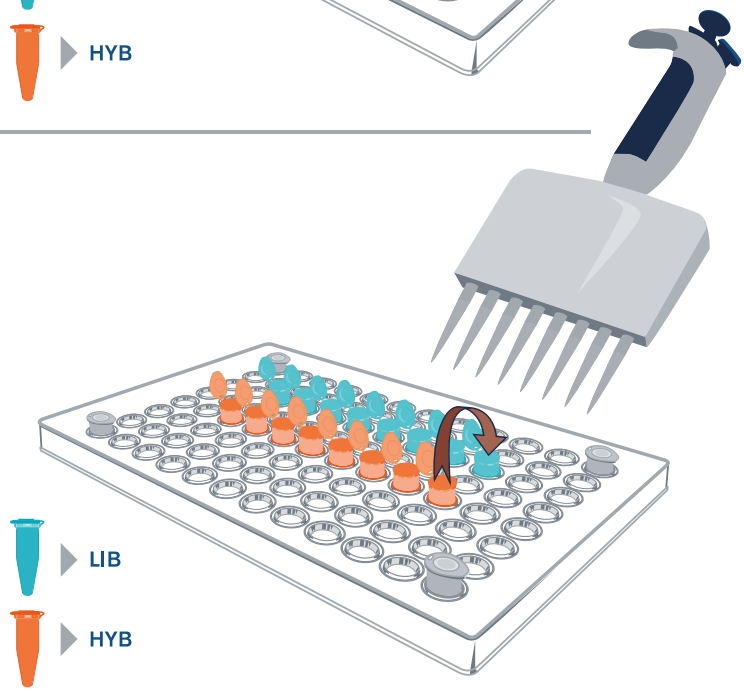


2. Once the cycler reaches the hybridization temperature during step 2, pause the program, put the **HYBs** in the thermal cycler, close the lid, and resume the program.



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

4. Dispose of the HYB tubes. Briefly spi down the LIBs, return to the thermal cycler, close the lid, and allow the reactions to incubate at the hybridization temperature for your chosen time (e.g., 16 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 these 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 to the intended bind and wash temperature
- Vortex mixer
- Mini centrifuge for 2 mL and 0.2 mL size tubes/strips
- Magnetic particle concentrator(s) ("MPC") for ~1.8mL 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 hybridization temperature 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 hybridization temperature (e.g., 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 30 minutes. Agitate every 10 minutes 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 10 minutes at the hybridization temperature in the heat block or thermal cycler. Agitate every 3 minutes 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	× 8 to 14 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
† Use the fewest cycles required for minimum sequencing requirements

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.

APPENDIX

A1: 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 amplified enriched library is not visible on electrophoresis gel or similar

After enrichment and amplification, aliquots of some libraries will not be sufficiently concentrated to visualize with standard electrophoresis methods. This does not necessarily indicate that the enrichment procedure failed. Successful captures frequently yield a total mass of just a few nanograms even after re-amplification. Invisible enriched libraries are typically the result of capturing especially small targets (<100 bp), or targets that were present at low frequency in the starting library (like those in degraded/ancient/environmental DNA), or under-reamplification of the library post-capture. Often a few more cycles of library amplification will render the captured product sufficiently high concentration to view with electrophoresis. However, we recommend quantification with library quantitative PCR, and visualization of the qPCR product prior to reaching amplification plateau, to determine sequenceable mass and length distribution of the captured library. This will also tell you whether it is necessary to amplify the library with more cycles before sequencing. Consult with your sequencing provider for library concentration and volume requirements for templating.

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

The total number of unique sequences available in an enriched library is mostly driven by starting molecular complexity and the capture sensitivity of a bait set. Each PCR cycle used during indexing and post-capture amplifications can induce representation bias between templates. **Percent duplicates** (aka "clonality" or "duplication rate"), on the other hand, is determined by sequencing depth, and 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 building a 2D plot with raw sequencing reads obtained on the X axis, and unique on-target reads observed on the Y axis. If this **complexity curve** has plateaued, but you achieved sufficient unique reads, you sequenced more deeply than was necessary. If it has not plateaued, 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.** If you are working with heavily contaminated or damaged DNA target molecules, consider reducing temperatures used in all steps to improve capture sensitivity. Reducing PCR cycles where you can might also improve target coverage uniformity and complexity for a given sequencing depth, in some cases having an indirect effect on duplication rate as measured. For more information about library complexity for any NGS application, we recommend Daley & Smith 2013 (doi: 10.1038/nmeth.2375).

A2: Pre-treating libraries made with Nextera® kits

We have observed that unmodified, index-amplified libraries made with the Nextera kits show higher streptavidin affinity than standard libraries. This renders them incompatible with myBaits. The following procedure amplifies Nextera libraries a few cycles with universal primers, and then depletes them of remaining streptavidin-affinity molecules, making them compatible with myBaits.

A2.1 Prepare materials

Reagents

- 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 library amplification (e.g., KAPA HiFi HotStart ReadyMix, Kapa Biosystems)
- Dynabeads® MyOne™ Streptavidin C1 Beads (30 µL per library amplification)
- Salt Solution: 1M NaCl, 10mM Tris-HCl pH 7.5 (200 µL per library amplification)
- PCR purification materials of your choice (e.g., silica columns or SPRI beads system)

Equipment:

- Strips or tubes appropriate for 50 µL amplification reactions (1 vessel per library)
- Thermal cycler compatible with the vessels above
- A magnetic particle concentrator and compatible tubes or strips (1 vessel per library)
- Pipettors and tips for 20 µL – 200 µL volumes
- Vortex mixer, mini centrifuge, etc.

A2.2 Amplification of indexed Nextera libraries

We recommend using a polymerase with reduced GC and length bias, such as KAPA® HiFi (Kapa Biosystems; see Quail *et al.* 2012; doi:10.1038/nmeth.1814), and the universal primers designed to amplify Illumina sequencing libraries without overwriting the index sequences (see Reagents section above).

Suggested mastermix and thermal program for amplifying Nextera libraries with universal primers

Component	Final conc.	µL per reaction
2X KAPA HiFi HotStart ReadyMix	1 X	25
P5-side primer (at 10 µM)	500 nM	2.5
P7-side primer (at 10 µM)	500 nM	2.5
200 ng Indexed Nextera Library		Up to 20
NF Water		To 50 µL
TOTAL		**50**

Step	Temp.	Time	
1	98°C	2 m	
2	98°C	20 s	× 4 cycles
3	60°C	30 s	
4	72°C	60 s	
5	72°C	5 m	
6	8°C	∞	

A2.3 Prepare streptavidin magnetic beads

While the reactions in A2.2 are cycling, prepare streptavidin-coated magnetic beads for removal of residual streptavidin-affinity molecules prior to purification.

1. For each amplification reaction, aliquot 30 µL Dynabeads MyOne Streptavidin C1 beads to a vessel compatible with your magnetic particle concentrator (**"MPC"**).
2. Pellet the beads in the MPC until the suspension is clear (~1-2 minutes). Leaving the tubes on the MPC, remove and discard the supernatant.
3. Add 100 µL Salt Solution to each bead aliquot. Vortex 3 seconds and centrifuge briefly. Pellet in the MPC until clear, remove and discard the supernatant.
4. Repeat step 3 once for a total of two washes.

A2.4 Deplete residual streptavidin-affinity molecules

Here, residual streptavidin-affinity molecules are removed from the amplification reactions by binding them to the beads prepared in A2.3 and removing the supernatant for purification and use with myBaits.

1. Once the PCR program (A2.2) is complete, remove the reactions from the thermal cycler.
2. Transfer each PCR reaction to a washed aliquot of C1 beads prepared above.
3. Mix the beads and PCR reaction by pipetting up and down until all beads are suspended.
4. Incubate the suspension at room temperature for 15 minutes.
5. Pellet the beads in the MPC.
6. Remove and purify the supernatant using silica columns, SPRI beads or similar cleanup method.

The final purified library is now myBaits-compatible and can be treated like a normal sequencing library.

A3: myBaits 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**
Block A	0.5
Block C	2.5
Block O	2.5
TOTAL	**5.5**

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 the hybridization temperature (e.g., 65°C). **Be sure to use a heated lid.**
5. Put the HYBs in the thermal cycler and warm to the hybridization temperature 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 the hybridization temperature 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 the hybridization temperature for at least 2 minutes.
9. Combine the warmed beads with the hybridization reactions and incubate for 30 minutes at the hybridization temperature, agitating every 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 10 minutes at the hybridization temperature. 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.