# Hybridization Capture for Targeted NGS

## User Manual

**Version 5.03 June 2023**

For myBaits Custom DNA-Seq
myBaits Custom RNA-Seq
myBaits Expert (excluding Wheat Exome and Human Affinities)

Access additional manuals at arborbiosci.com/mybaits-manual

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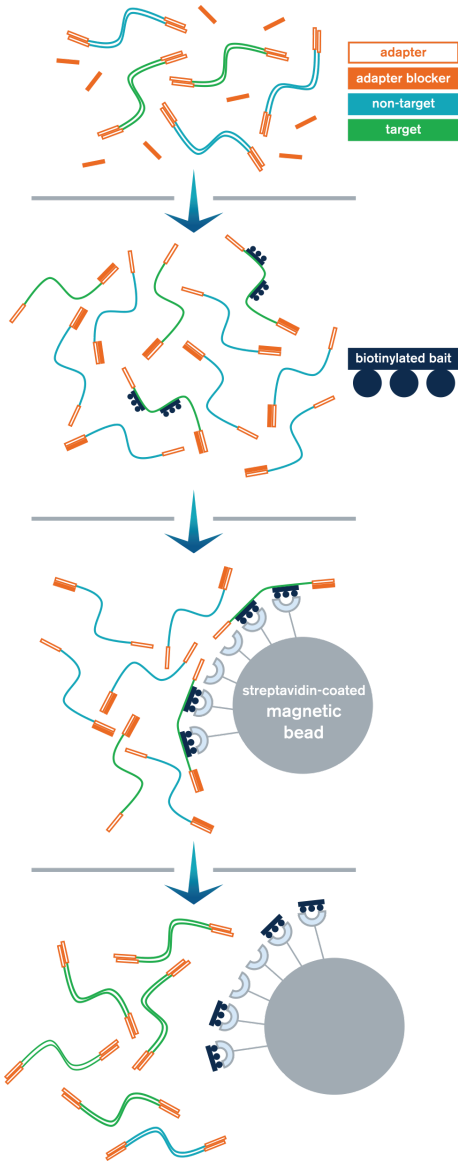
# INTRODUCTION

myBaits® is an in-solution NGS library target enrichment system, compatible with Illumina®, Ion Torrent®, and essentially any linear and 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 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.



## Daicel Arbor Biosciences products compatible with this manual

This manual is compatible with current generation myBaits Custom DNA-Seq, Custom RNA-Seq, and Expert kits, but not Custom Methyl-Seq, Expert Wheat Exome, or Expert Human Affinities kits. Visit arborbiosci.com/mybaits-manual to download other manuals, or email techsupport@arbor.daicel.com for assistance. See Page 4 to select the correct protocol within this manual to follow for your application.

## Changes since myBaits manual version 5.02

- Reagent cap colors are updated (contents are unchanged); minor text changes

## Changes since myBaits manual version 4

- Three distinct myBaits protocols are included for different applications (see Page 4)
- **Block X** replaces Block A and typically provides improved specificity
- **Buffer E** is now supplied for post-cleanup bead resuspension

**Note for returning users**: Version 5 reagent chemistry typically provides significantly higher target specificity (= percentage of on-target reads) compared to version 4 and earlier kits.

## Kit components and stability

| Box | Reagent | Cap color | | Volume per kit | | |
|---|---|---|---|---|---|---|
| | | Lot# **23**XXXX | Lot# **22**XXXX | **8 Reaction** | **16 Reaction** | **48+ Reaction** |
| Box #1<br>*Store at 4˚C* | **Hyb N** | Red | Red | 400 µL | 400 µL | 1000 µL |
| | **Hyb S** | Blue | Teal | 1500 µL | 1500 µL | 1500 µL |
| | **Beads** | Clear | Clear | 550 µL | 550 µL | 1600 µL |
| | **Binding Buffer** | Clear | Clear | 12 mL | 12 mL | 36 mL |
| | **Wash Buffer** | Clear | Clear | 20 mL | 20 mL | 60 mL |
| Box #2<br>*Store at -20˚C* | **Hyb D** | Yellow | Yellow | 140 µL | 140 µL | 400 µL |
| | **Hyb R** | Purple | Purple | 50 µL | 50 µL | 150 µL |
| | **Block C** | Green | Dark Green | 50 µL | 50 µL | 130 µL |
| | **Block O** | Blue | Blue | 90 µL | 90 µL | 270 µL |
| | **Block X** | Orange | Orange | 5 µL | 9 µL | 27 µL |
| | **Buffer E** | Clear | Light Green | 550 µL | 550 µL | 1600 µL |
| Box #3<br>*Store at -80˚C* | **Baits** | Clear | White | 50 µL per 8 reactions | | |

**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.

# IMPORTANT
# READ BEFORE PROCEEDING

## CHOOSE A PROTOCOL

This manual includes three separate myBaits protocols. Please select from the list below depending on the nature of your sequencing libraries. If you are unsure which protocol to follow, email techsupport@arbor.daicel.com for consultation with one of our NGS scientists.

Note that these are suggested starting points. Identification of optimal parameters for enrichment with a specific bait set and library will require testing and evaluation. These protocols have worked well across many bait set and library combinations.

### Standard Begins on Page 5

For most targeted sequencing applications

Recommended when enriching NGS libraries that:

- derive from high-quality genomic DNA, with target inserts of 80-1,000 bp in length,
- contain a mean target sequence GC content of >25%,
- are free of substantial (>50%) contamination from non-target organisms, and
- do not have extensive base substitutions (from e.g. deamination or bisulfite conversion)

### High Sensitivity Begins on Page 17

For high background contamination, ancient DNA, high bait-target divergence, OR short target sequences

Recommended when enriching NGS libraries that have ANY of the following characteristics:

- derive from DNA samples heavily contaminated with exogenous DNA (e.g. pathogen targets in host DNA background, ancient DNA, environmental DNA),
- contain mostly target sequences shorter than 80 bp,
- contain a mean target sequence GC content of <25%, or
- contain targets that are on average ≥25% divergent from the bait sequences

### Long Insert Begins on Page 29

For target enrichment of NGS libraries containing inserts 1-10 kilobase pairs in length

Recommended when enriching NGS libraries that:

- are dominated by inserts longer than 1,000 bp, and
- can be amplified with universal adapter primers prior to platform-specific library prep (e.g. PacBio or Nanopore library prep)

# Standard Protocol

For most targeted sequencing applications

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# REQUIREMENTS AND RECOMMENDATIONS

## Input library

**TYPE** Use myBaits with PCR-amplified and amplifiable NGS libraries, including Illumina TruSeq®-style, Illumina Nextera® Flex-style, Ion Torrent, or other libraries with universal adapter priming sites. 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. Dual-indexed libraries are strongly recommended to reduce the hazard of mis-indexing induced by PCR jumping events.

**VOLUME** Each myBaits target enrichment reaction has space for 7 µL total NGS library volume. Many pools will require concentration to 7 µL with vacuum centrifugation or other means. Complete this in advance of your myBaits experiments. Libraries should be suspended in nuclease-free buffer or water.

**MASS** A wide range of total library mass can be successfully enriched with myBaits (nanograms to micrograms). However, best results are seen when 100 ng or more total mass is used per enrichment reaction. It is strongly recommended that library qPCR is used to measure library mass, rather than e.g. dye assay or electrophoresis. See note below on default recommendations for mass input for library pools.

**POOLING** Optimal pooling parameters (both in terms of number of libraries and total mass per library) will vary between library types and bait sets, and will require trials to identify. However, many configurations should work well. To minimize variation in capture performance among pooled samples, only pool libraries based on comparable anticipated bait-genome sequence identity (i.e, taxon), DNA quality, starting DNA amount, library insert length, and relative target constituent vs. background. Pool equal amounts of each library. We recommend that when libraries derive from organisms with genome sizes smaller than 5 Gbp, pool eight libraries, 250 ng each, for a total of 2 µg per enrichment reaction. For genomes 5 Gbp or larger, pool eight libraries, 1 µg each for a total of 8 µg.

## 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

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.

### S1.1 Choose a hybridization temperature ($T_H$)

**65°C**

When bait-target sequence divergence is expected to be less than 10%

**62°C**

When bait-target sequence divergence is expected to be 10 to 15%

**60°C**

When bait-target sequence divergence is expected to be 15 to 25%

### S1.2 Prepare materials

| Reagents |
|---|
| Hyb N and Hyb S from Box 1 |
| Hyb D and Hyb R from Box 2 |
| Block C, Block O, and Block X from Box 2 |
| Baits from Box 3 **KEEP ON ICE** |
| Libraries or library pools in 7 µL per reaction |

| Equipment |
|---|
| Nuclease-free microcentrifuge tubes (×2) |
| 0.2 mL strips with attached lids (×1 per 8 reactions) |
| Pipettors and tips; multichannel for 20 µL recommended |
| Vortex mixer and mini-centrifuge for above tube types |
| Heat block set to 60°C |
| 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** | $T_H$ | 5m |
| **3** | $T_H$ | ∞ |

## S1.3 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"***

## S1.4 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"***

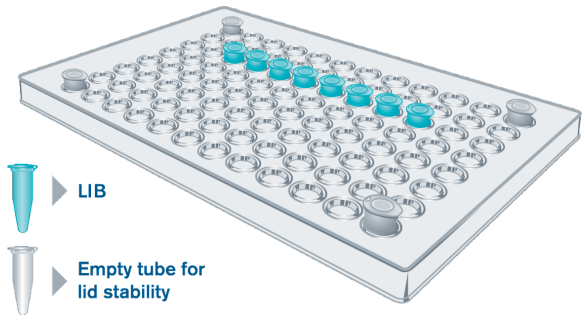
## S1.5 Reaction assembly

**Double-check the thermal program:**

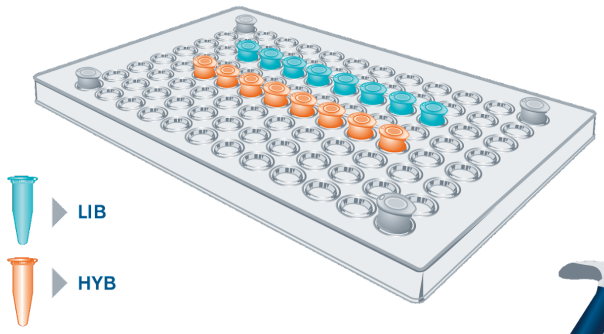
*Set lid temperature 5 to 10°C above each step temperature to minimize evaporation*

| Step | Temperature | Time |
|---|---|---|
| 1 | 95°C | 5m |
| 2 | $T_H$ | 5m |
| 3 | $T_H$ | ∞ |

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

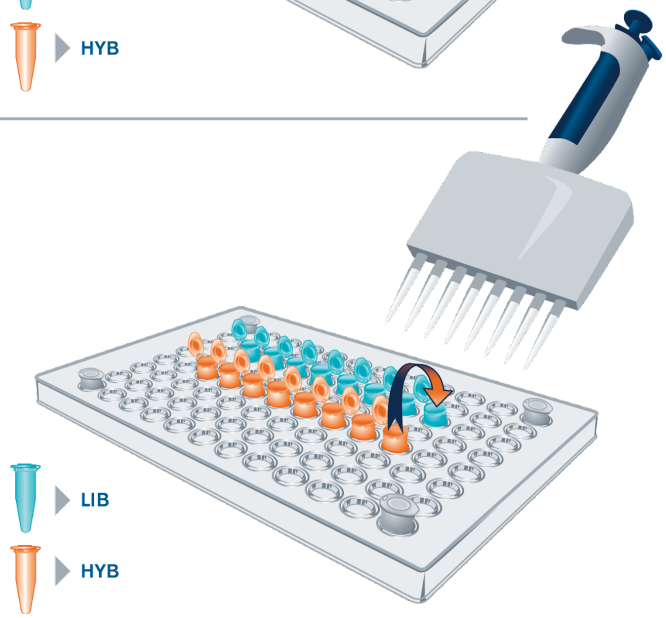



2. Once the cycler reaches the hybridization temperature during step 2, pause the program, put the **HYBs** in the thermal cycler, close the lid, and resume the program.




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 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.

## S2.1 Choose a wash temperature ($T_W$ - typically identical to $T_H$)

### 65°C

When bait-target sequence divergence is expected to be 10% or less

### 62°C

When bait-target sequence divergence is expected to be 10 to 15%

### 60°C

When bait-target sequence divergence is expected to be 15 to 25%

## S2.2 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 the $T_W$ (e.g., 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 the $T_W$ | Thermal cycler set to $T_W$ |
| Pipettors and tips for 20 – 500 µL | Pipettors and tips for 20 – 200 µL; **multichannel pipettor strongly recommended** |

## S2.3 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 $T_W$ in the water bath or oven for at least 30 minutes before use.

## S2.4 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. 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.*

## S2.5 Binding beads and hybrids

1. Heat the bead aliquots to the $T_W$ (e.g., 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 to keep the beads suspended, followed by briefly centrifuging.

## S2.6 Bead washing

1. Pellet the beads with the MPC until the solution is clear. Remove and discard the supernatant.
   ***TIP:*** *This supernatant can be optionally saved as a backup for re-capture with the same or different baits.*
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 the mixture.
3. Incubate for 5 minutes at the $T_W$ 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 and Amplification

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

## S3.1 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 |

## S3.2 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<br>3B. Immediately pellet the beads in the MPC and collect the supernatant containing the enriched libraries |

## S3.3 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.*

## S3.3 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 | 45 seconds | |
| 5 | 72°C | 5 minutes | |
| 6 | 8°C | ∞ | |

**Minimize cycles where possible. Cycles required to meet molarity requirements of sequencing platform may exceed 14.*

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.

# APPENDIX

## SA1: 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 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).

## SA2: myBaits Procedure Quick Guide - Standard 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 | 5.5 |
| **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: 0; Salmonids: 2.5
*Plants: 5.0; Salmonids: 0

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 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 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 5 minutes at the hybridization temperature, 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 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 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.**

# High Sensitivity Protocol

For high background contamination, ancient DNA, high bait-target divergence, and/or short insert fragments

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# REQUIREMENTS AND RECOMMENDATIONS

## Input library

**TYPE** Use myBaits with PCR-amplified and amplifiable NGS libraries, including Illumina TruSeq®-style, Illumina Nextera® Flex-style, Ion Torrent, or other libraries with universal adapter priming sites. 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. Dual-indexed libraries are strongly recommended to reduce the hazard of mis-indexing induced by PCR jumping events.

**VOLUME** Each myBaits target enrichment reaction has space for 7 µL total NGS library volume. Many pools will require concentration to 7 µL with vacuum centrifugation or other means. Perform this in advance of your myBaits experiments. Libraries should be suspended in nuclease-free buffer or water.

**MASS & POOLING** A wide range of total library mass can be successfully enriched with myBaits (nanograms to micrograms). Optimal pooling parameters (both in terms of number of libraries and total mass per library) will vary between library types and bait sets and may require trials to identify. However, many configurations should work well.

**Non-degraded, non-contaminated DNA libraries (e.g. libraries with bait-target divergence expected mean >25%):** To minimize variation in capture performance among pooled samples, only pool libraries of comparable anticipated bait-genome sequence identity (i.e, taxon), DNA quality, starting DNA amount, library insert length, and relative target constituent vs. background. Pool equal amounts of each library. When libraries derived from organisms with genome sizes smaller than 5 Gbp, pool eight libraries, 250 ng each, for a total of 2 µg per enrichment reaction. For genomes 5 Gbp or larger, pool eight libraries, 1 µg each for a total of 8 µg.

**Degraded and contaminated DNA libraries:** Pool as few libraries as possible, and input as much as possible to capture, up to a total of 12 µg. If pooling is required, make certain that libraries are balanced by endogenous content to ensure each sample contributes the same amount of target content. For example, if two libraries are to be pooled, and library A has 20% endogenous template and library B has 10% endogenous template, include twice the mass of library B as library A in the enrichment pool.

## 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.

## Special note on two-round enrichment protocols:

The High Sensitivity protocol includes two rounds of enrichment. This may require that you purchase additional myBaits Reagents (without Baits). For example, if you plan to use all 16 reactions of a myBaits kit in the High Sensitivity protocol, you will need to have both the 16 reaction kit as well as at least 16 additional reactions-worth of myBaits Reagents. These can be purchased in 16 or 48 reaction kits, catalog numbers 300016 and 300048, respectively.

## 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

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.

### H1.1 Choose a hybridization temperature ($T_H$)

**63°C**

For DNA libraries with low GC content or with expected bait-target divergence of less than 15%

**60°C**

For libraries with expected bait-target sequence divergence of 15 to 25%

**55°C**

For libraries with insert length distributions mostly shorter than the myBaits probe length, or expected bait-target sequence divergence higher than 25%

### H1.2 Prepare materials

| Reagents |
|---|
| Nuclease-free Water (“H2O”) |
| Hyb N and Hyb S from Box 1 |
| Hyb D and Hyb R from Box 2 |
| Block C, Block O, and Block X from Box 2 |
| Baits from Box 3 **KEEP ON ICE** |
| Libraries or library pools in 7 µL per reaction |

| Equipment |
|---|
| Nuclease-free microcentrifuge tubes (×2) |
| 0.2 mL strips with attached lids (×1 per 8 reactions) |
| Pipettors and tips; multichannel for 20 µL recommended |
| Vortex mixer and mini-centrifuge for above tube types |
| Heat block set to 60°C |
| 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** | $T_H$ | 5m |
| **3** | $T_H$ | ∞ |

### H1.3 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"***

## H1.4 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"***

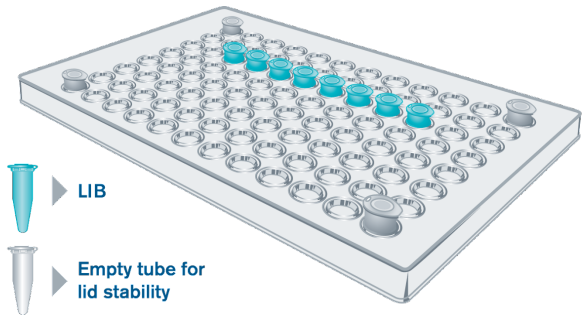
## H1.5 Reaction assembly

**Double-check the thermal program:**

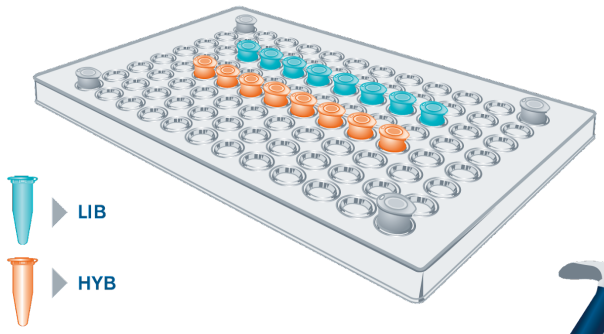
*Set lid temperature 5 to 10°C above each step temperature to minimize evaporation*

| Step | Temperature | Time |
|---|---|---|
| 1 | 95°C | 5m |
| 2 | $T_H$ | 5m |
| 3 | $T_H$ | ∞ |

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

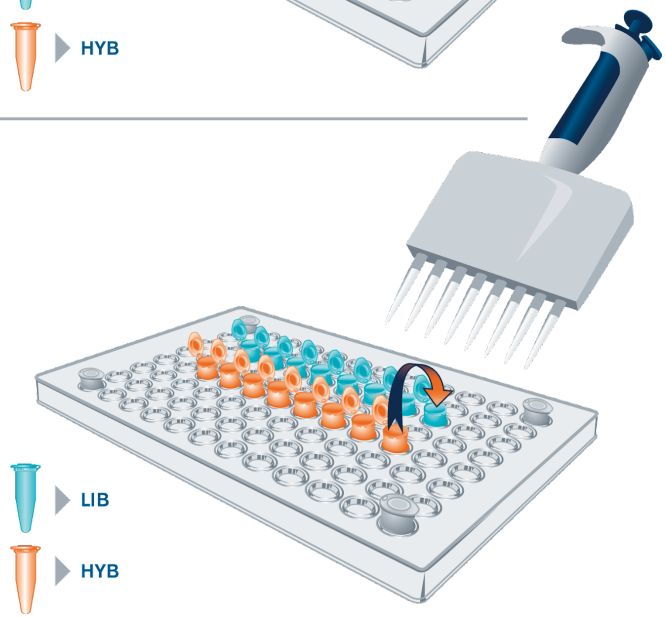



2. Once the cycler reaches the hybridization temperature during step 2, pause the program, put the **HYBs** in the thermal cycler, close the lid, and resume the program.




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 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.

## H2.1 Choose a wash temperature ($T_W$ - typically identical to $T_H$)

**63°C**

When bait-target sequence divergence is expected to be 15% or less

**60°C**

When bait-target sequence divergence is expected to be 15 to 25%

**55°C**

When bait-target sequence divergence is expected to be higher than 25%

## H2.2 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 the $T_W$ (e.g., 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 the $T_W$ | Thermal cycler set to $T_W$ |
| Pipettors and tips for 20 – 500 µL | Pipettors and tips for 20 – 200 µL; **multichannel pipettor strongly recommended** |

## H2.3 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 $T_W$ in the water bath or oven for at least 30 minutes before use.

## H2.4 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. 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.*

## H2.5 Binding beads and hybrids

1. Heat the bead aliquots to the $T_W$ (e.g., 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 to keep the beads suspended, followed by briefly centrifuging.

## H2.6 Bead washing

1. Pellet the beads with the MPC until the solution is clear. Remove and discard the supernatant.
   ***TIP:*** *This supernatant can be optionally saved as a backup for re-capture with the same or different baits.*
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 the $T_W$ 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.

## H3.1 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 |

## H3.2 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<br>3B. Immediately pellet the beads in the MPC and collect the supernatant containing the enriched libraries |

## H3.3 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 H3.3, 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.*

## H3.3 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 8 cycles* | *First round of enrichment: 14 Second round of enrichment: 8* |
| 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).

## H3.4 Perform a second round of enrichment

**If this is the end of the first time through step H3.3 (one of two):**

1. Combine both purified amplification reactions generated above and concentrate to 7 µL.
2. Repeat steps H1.1 through H3.3 using this once-enriched template as input.

**If this is the end of your second time through step H3.3 (two of two)**, the enriched libraries are now ready for quantification, quality-assessment, and sequencing.

# APPENDIX

## HA1: 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).

## HA2: myBaits Procedure Quick Guide - High Sensitivity 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: 0; Salmonids: 2.5
*Plants: 5.0; Salmonids: 0

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., 63°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 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 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 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 5 minutes at the hybridization temperature, 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 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 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.

# Long Insert Protocol

For target enrichment of NGS libraries containing inserts 1-10 kilobase pairs in length

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# REQUIREMENTS AND RECOMMENDATIONS

## Input library

**TYPE** Use myBaits with PCR-amplified and amplifiable NGS libraries with universal adapter priming sites. For long insert library preparation, we recommend the procedure described in Witek et al. 2016 (doi: 10.1038/protex.2016.027). 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. Dual-indexed libraries are strongly recommended to reduce the hazard of mis-indexing induced by PCR jumping events.

**VOLUME** Each myBaits target enrichment reaction has space for 7 µL total NGS library volume. Many pools will require concentration to 7 µL with vacuum centrifugation or other means. Complete this in advance of your myBaits experiments. Libraries should be suspended in nuclease-free buffer or water.

**MASS** For long insert capture, we recommend using 250 ng total library per enrichment reaction, as quantified with intercalating dye assay (e.g. Qubit).

**POOLING** Optimal pooling parameters (both in terms of number of libraries and total mass per library) will vary between library types and bait sets, and will require trials to identify. However, many configurations should work well. To minimize variation in capture performance among pooled samples, only pool libraries of comparable anticipated bait-genome sequence identity (i.e, taxon), starting DNA amount, library insert length, and relative target constituent vs. background. Pool equal amounts of each library. For long insert capture, by default we recommend pooling three libraries, 83 ng each, for a total of ~250 ng per enrichment reaction.

## 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.

## Special note for handling long-insert libraries

Use gentle repeated pipetting rather than vortexing to homogenize solutions that contain both beads (MyOne C1 or SPRI) and long insert library. This helps reduce potential mechanical shearing.

## 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

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.

### L1.1 Choose a hybridization temperature ($T_H$)

**65°C**

When bait-target sequence divergence is expected to be less than 10%

**62°C**

When bait-target sequence divergence is expected to be 10 to 15%

**60°C**

When bait-target sequence divergence is expected to be 15 to 25%

### L1.2 Prepare materials

| Reagents |
|---|
| Hyb N and Hyb S from Box 1 |
| Hyb D and Hyb R from Box 2 |
| Block C, Block O, and Block X from Box 2 |
| Baits from Box 3 **KEEP ON ICE** |
| Libraries or library pools in 7 µL per reaction |

| Equipment |
|---|
| Nuclease-free microcentrifuge tubes (×2) |
| 0.2 mL strips with attached lids (×1 per 8 reactions) |
| Pipettors and tips; multichannel for 20 µL recommended |
| Vortex mixer and mini-centrifuge for above tube types |
| Heat block set to 60°C |
| 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 | Parameters |
|---|---|
| **1** | 60°C, 10 minutes |
| **2** | 95°C, 10 minutes |
| **3** | Reduce 0.1°C per second to $T_H$ |
| **4** | Hold at $T_H$ |

## L1.3 Hybridization Reaction Setup

1. Once all 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 Capture Mix in a microcentrifuge (MC) tube combining the reagents in the order indicated. **The following volumes are already adjusted for pipetting error:**

| *MOST TAXA* | |
|---|---|
| **Component** | **µL / Reaction** |
| Hyb N | 9.25 |
| Hyb D | 3.5 |
| Hyb S* | 0.5 |
| Hyb R | 1.25 |
| Block O | 2.5 |
| Block C | 2.5 |
| Block X | 0.5 |
| NF Water | - |
| Baits | 5.5 |
| TOTAL | **25.5** |

| *PLANTS* | |
|---|---|
| **Component** | **µL / Reaction** |
| Hyb N | 9.25 |
| Hyb D | 3.5 |
| Hyb S* | 0.5 |
| Hyb R | 1.25 |
| Block O | 5.0 |
| Block C | - |
| Block X | 0.5 |
| NF Water | - |
| Baits | 5.5 |
| TOTAL | **25.5** |

| *SALMONIDS* | |
|---|---|
| **Component** | **µL / Reaction** |
| Hyb N | 9.25 |
| Hyb D | 3.5 |
| Hyb S* | 0.5 |
| Hyb R | 1.25 |
| Block O | - |
| Block C | 2.5 |
| Block X | 0.5 |
| NF Water | 2.5 |
| Baits | 5.5 |
| TOTAL | **25.5** |

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

   Briefly vortex and centrifuge to collect.

3. For each capture reaction, aliquot **23 µL** of Capture Mix to a 0.2 mL well/tube.
4. Add 7 µL of individual or pooled libraries to each Capture Mix aliquot and mix by gently pipetting.
5. Place the reactions in the thermal cycler and run the thermal program, incubating overnight.

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

| Step | Parameters |
|---|---|
| **1** | 60°C, 10 minutes |
| **2** | 95°C, 10 minutes |
| **3** | Reduce 0.1°C per second to $T_H$ |
| **4** | Hold at $T_H$ |

# 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.

*For long insert libraries, mix reactions using gentle pipetting or inversion rather than vortexing in order to minimize shearing effects*

## L2.1 Choose a wash temperature ($T_W$ - typically identical to $T_H$)

**65°C**

When bait-target sequence divergence is expected to be 10% or less

**62°C**

When bait-target sequence divergence is expected to be 10 to 15%

**60°C**

When bait-target sequence divergence is expected to be 15 to 25%

## L2.2 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 the $T_W$ (e.g., 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 the $T_W$ | Thermal cycler set to $T_W$ |
| Pipettors and tips for 20 – 500 µL | Pipettors and tips for 20 – 200 µL; **multichannel pipettor strongly recommended** |

## L2.3 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 $T_W$ in the water bath or oven for at least 30 minutes before use.

## L2.4 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. 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.*

## L2.5 Binding beads and hybrids

1. Heat the bead aliquots to the $T_W$ (e.g., 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 to keep the beads suspended, followed by briefly centrifuging.

## L2.6 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 mix by pipetting. Briefly centrifuge to collect.
3. Incubate for 5 minutes at the $T_W$ in the heat block or thermal cycler. Agitate at the 2.5 minute mark by gentle pipetting. Briefly centrifuge to collect the mixture.
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 and Amplification

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

## L3.1 Prepare materials

| Reagents |
|---|
| Buffer E (Box 2) |
| PCR primers for amplifying libraries (e.g., P5 and P7) |
| PCR reagents for post-capture amplification |
| PCR purification system, e.g., silica columns or SPRI beads |

| Equipment |
|---|
| Tubes appropriate for PCR master mix assembly |
| Tubes or strips for 50 µL PCR amplification |
| Pipettors and tips capable of 5 – 100 µL volumes |
| Vortex mixer and mini-centrifuge for above tube types |
| Thermal cycler |

## S3.2 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 L3.3 using this bead resuspension as template in amplification | 2B. Incubate the suspension at 95°C for 5 minutes<br>3B. Immediately pellet the beads in the MPC and collect the supernatant containing the enriched libraries |

## L3.3 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) | - | 10 * | - |
| | **TOTAL** | **50** | **-** |

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

## L3.3 Library amplification (continued)

2. Cycle the reactions with the following thermal program:

| Step | Temperature | Time | |
|---|---|---|---|
| 1 | 98°C | 3 minutes | |
| 2 | 95°C | 30 seconds | ×25 cycles* |
| 3 | 62°C | 20 seconds | |
| 4 | 68°C | 10 minutes | |
| 6 | 8°C | ∞ | |

**Minimize cycles where possible. Cycles required to meet molarity requirements of sequencing platform may exceed 25.*

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, additional size-selection (if required), and then platform-specific library preparation and sequencing.

   **If insufficient total mass was acquired from a single amplification reaction for e.g. PacBio or Oxford Nanopore library preparation, perform additional amplifications using the remaining non-amplified enriched library.**

# APPENDIX

## LA1: 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).

## LA2: myBaits Procedure Quick Guide - Long Insert Protocol

1. For each reaction, build in the following Capture Mix appropriate to your target taxon; pipetting error is built in:

| *MOST TAXA* | |
|---|---|
| **Component** | **µL / Reaction** |
| Hyb N | 9.25 |
| Hyb D | 3.5 |
| Hyb S | 0.5 |
| Hyb R | 1.25 |
| Block O | 2.5 |
| Block C | 2.5 |
| Block X | 0.5 |
| NF Water | - |
| Baits | 5.5 |
| TOTAL | **25.5** |

| *PLANTS* | |
|---|---|
| **Component** | **µL / Reaction** |
| Hyb N | 9.25 |
| Hyb D | 3.5 |
| Hyb S | 0.5 |
| Hyb R | 1.25 |
| Block O | 5.0 |
| Block C | - |
| Block X | 0.5 |
| NF Water | - |
| Baits | 5.5 |
| TOTAL | **25.5** |

| *SALMONIDS* | |
|---|---|
| **Component** | **µL / Reaction** |
| Hyb N | 9.25 |
| Hyb D | 3.5 |
| Hyb S | 0.5 |
| Hyb R | 1.25 |
| Block O | - |
| Block C | 2.5 |
| Block X | 0.5 |
| NF Water | 2.5 |
| Baits | 5.5 |
| TOTAL | **25.5** |

2. For each enrichment reaction, aliquot **23 µL** of Capture Mix to their own tubes.
3. To each Capture Mix aliquot, add **7 µL** of each library or library pool.
4. Incubate the reactions in the thermal cycler for 10 minutes @ 60°C, then 10 minutes @ 95°C, and then drop to the hybridization temperature (e.g., 65°C) at a rate of 0.1°C per second. **Be sure to use a heated lid.** Incubate at the hybridization temperature for 16-24 hours.
5. **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 the hybridization temperature for at least 45 minutes.
6. 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.
7. Combine the warmed beads with the hybridization reactions and incubate for 5 minutes at the hybridization temperature, agitating at 2.5 minutes to keep beads suspended.
8. 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 the hybridization temperature. Wash four times with 180 µL washes if using a 96-well magnetic particle concentrator and 0.2 mL strips/tubes.
9. Resuspend the beads in 30 µL Buffer E and then use 10 µL of this in a 50 µL library 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 10 µL of the supernatant in a 50 µL amplification reaction.
10. 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.** The enriched libraries are now ready for quantification, quality-assessment, and sequencing.