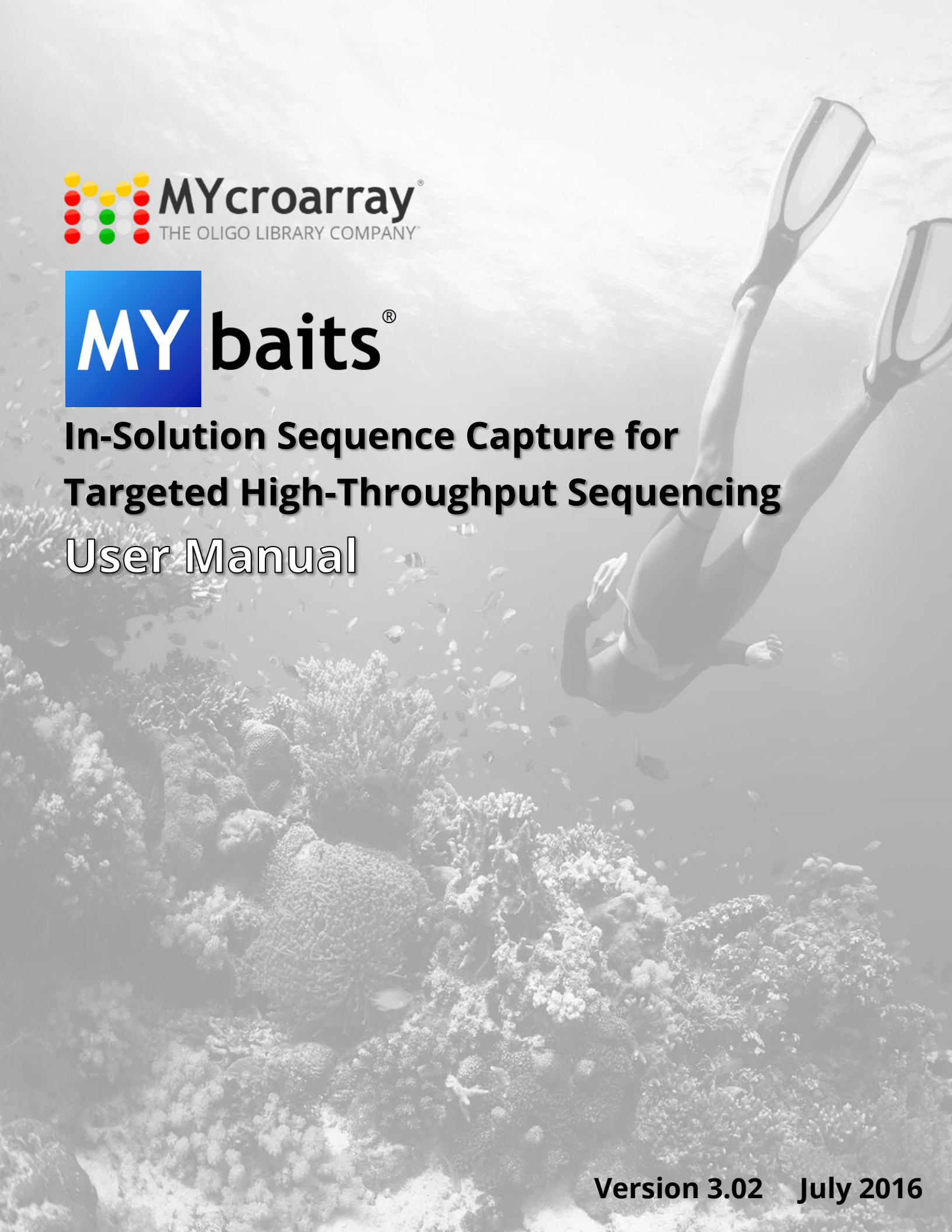

MYcroarray®
THE OLIGO LIBRARY COMPANY®

MYbaits®

In-Solution Sequence Capture for Targeted High-Throughput Sequencing

User Manual

Version 3.02 July 2016

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Get the latest manual at http://www.mycroarray.com/mybaits/manuals.html

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CONTENTS

INTRODUCTION

MYbaits® is a fully customizable in-solution DNA capture (targeted enrichment) system. We use our versatile DNA synthesis technology to make oligonucleotides complementary to your specific sequence targets of interest. We then transcribe these oligos into biotinylated RNAs, generating "baits." The MYbaits® kit procedure is similar to Gnirke *et al.* 2009 (doi: 10.1038/nbt.1523) and can be divided into six main steps:

1) DNA sequencing library is heat-denatured in the presence of adapter-specific blocking oligonucleotides

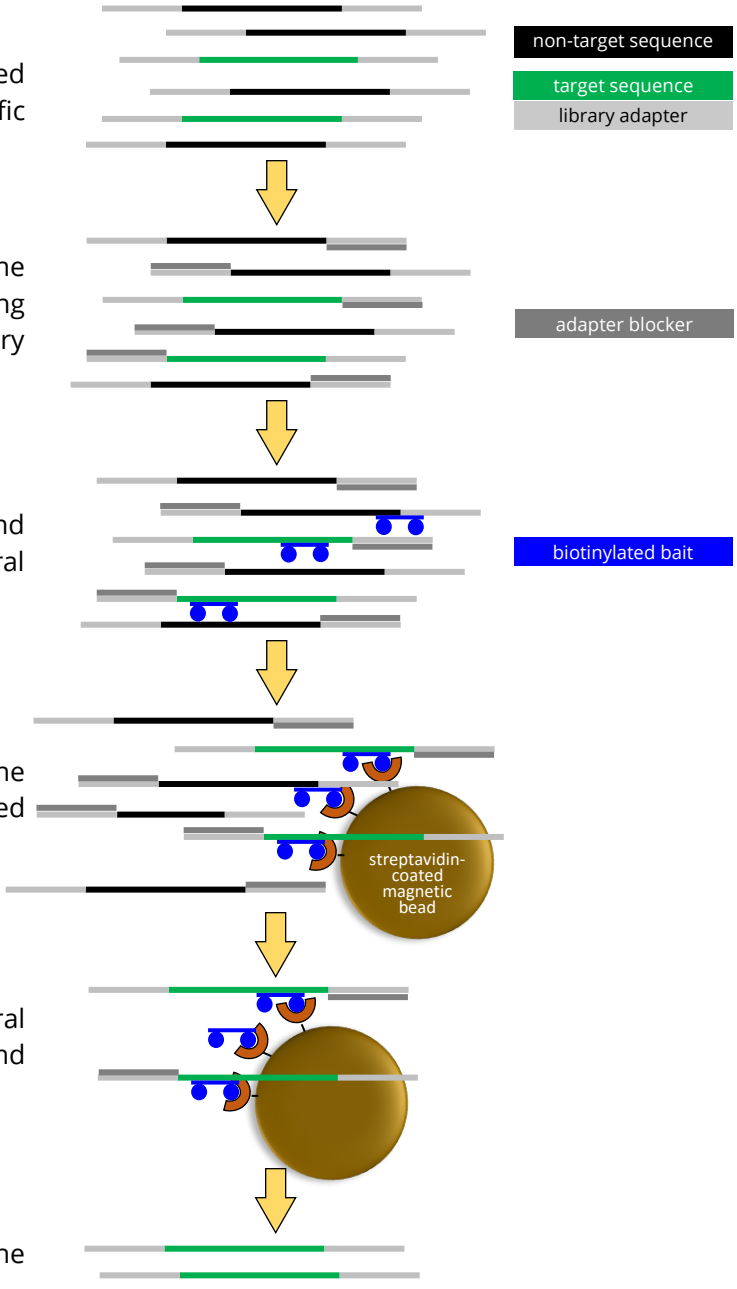
2) Library and blockers are dropped to the hybridization temperature, allowing blockers to hybridize to the library adapters

3) Biotinylated RNA baits are introduced and allowed to hybridize to targets for several hours

4) Bait-target hybrids are pulled out of the solution with streptavidin-coated magnetic beads

5) Beads are stringently washed several times to remove non-hybridized and nonspecifically-hybridized molecules

6) Captured DNA library is released from the beads and amplified



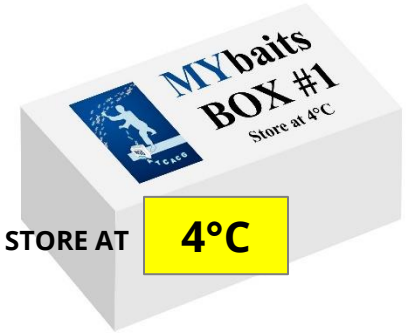
PROCEDURE CHANGES SINCE THE PREVIOUS MANUAL

The most recent manual prior to this was Version 2.3.1. **All kit components remain backwards-compatible with that version.** The current procedure differs in the following ways:

- Protocol recommendations for enriching libraries with very low target amounts (e.g., from ancient, forensic, archival, or environmental DNA) are now included (p. 4).
- We now provide two separate capture Hybrid Bind & Wash protocols, one using a magnetic particle collector for ~1.5 mL tubes (Part 2A, pages 9 & 10), and another other using a 96-well magnetic particle collector (Part 2B, pages 11 & 12).
- Capture reactions are now a total of 30 µL instead of 26 µL (p. 7).
- Reagent volumes used in capture reaction setup now accommodate some pipetting error, with built-in excess (p. 7),
- Total library input volume is now 7 µL instead of 5.9 µL (p. 7).
- Capture reaction setups now use two mixes that are combined on the thermal cycler in one pipetting step, instead of three mixes combined in two steps (p. 8).
- Hybrid Bind & Wash uses 30 µL of streptavidin-coated magnetic beads instead of 50 µL (p. 10 & 12)
- Beads are resuspended in 70 µL Binding Buffer per capture instead of 20 µL (p. 10 & 12)
- Beads are now incubated with captured library for 30 minutes instead of 45 minutes (p. 10 & 12)
- Washing with Wash Buffer 1 and Wash Buffer 2 is no longer performed. Instead, washing with Wash Buffer 2.2 (a diluted version of provided Wash Buffer 2) is now standard procedure (p. 10 & 12).
- Eluting the captured library from the beads with NaOH is no longer recommended. Bead-bound library is now taken directly to amplification, or is eluted off the beads with heat instead (p. 13).
- S*INCE VERSION 3.0*: Typos in shipped volumes and in Bead Preparation are fixed.
- *SINCE VERSION 3.01*: Mention of Wash buffer 1 and Neutralization buffer removed from most sections

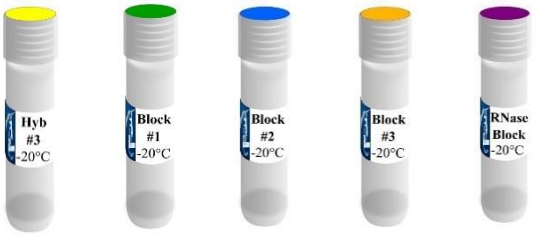
Wash Buffer 1 and Neutralization Buffer are no longer used in the standard procedure

KIT COMPONENTS

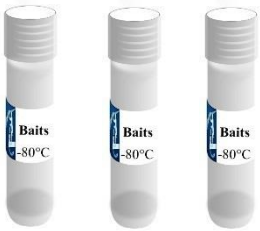


HYB #1 | HYB #2 | HYB #4 | Binding Buffer | Wash Buffer 2

Wash Buffer 1 and Neutralization Buffer are no longer used in the MYbaits® procedure. Contact us if you have need of these reagents and/or the protocol that uses them



HYB #3 | BLOCK #1 | BLOCK #2 | BLOCK #3 | RNase Block



Baits Tubes

Box #1 (4°C)	Volume
HYB #1	1.5 mL
HYB #2	60 µL
HYB #4 [a]	800 µL to 1 mL
Binding Buffer	45 mL
Wash Buffer 2 [b]	80 mL

Box #2 (-20°C)	Volume
HYB #3	700 µL
BLOCK #1	Varies [c]
BLOCK #2	Varies [c]
BLOCK #3	30 µL
RNase Block [e]	Varies [d]

Box #3 (-80°C)	Volume
Baits [e]	5.5 µL per reaction

[a] New formula, see Appendix

[b] For Wash Buffer 2.2 (p.10 & 12)

[c] 12 reaction kit: 40 µL
24 reaction kit: 70 µL
48 reaction kit: 125 µL

[d] 12-24 reaction kit: 40 µL
48 reaction kit: 70 µL

[e] 12 reaction kit: 33 µL per tube
24+ reaction kit: 44 µL per tube

See the Appendix (p. 14) for kit reagent formulae

RECOMMENDATIONS

Capture template

Use MYbaits® with **Illumina®, Ion Torrent®,** and **454®** sequencing libraries. Blockers specific to your library type and index configuration are included in your kit as **Block #3**.

Consult with us before using MYbaits® with libraries prepared with Illumina Nextera®

For most applications, we recommend using between 100ng and 500ng of library for capture (7 µL at 14-72 ng/µL). MYbaits® can be used with as few as 1 ng and as many as 2 µg of library. For libraries with a substantial non-target component (e.g., ancient, forensic, or environmental samples), maximize the target component in each capture by using as much library as possible up to 2 µg, and consider two rounds of capture for higher percentage of reads on-target.

Pooling libraries

Capturing individual libraries produces the best per-sample results. However, libraries can be pooled into single capture reactions. We recommend trial captures with different pooling schemes to determine what works best for your particular samples and bait set. When pooling libraries that vary in relative target content (e.g., ancient, forensic, or environmental samples), try to equilibrate by observed or expected *target* molarity, rather than by total library molarity.

Only dual-indexed libraries should be pooled, in order to avoid index dissociation via jumping PCR during post-capture library amplification (see Kircher* et al. *2012; doi: 10.1093/nar/gkr771; also see Rohland & Reich 2012; doi: 10.1101/gr.128124.111 for an alternative approach)

Temperatures

For most applications, we recommend 65°C for hybridization, bead-bait binding, and wash temperatures. For samples where a majority of targets are shorter than the baits (i.e., from degraded DNA sources), we recommend **55°C** for all three steps for improved captured target complexity, or **65°C** if higher on-target percentage is the priority. Temperatures for optimal sensitivity and specificity vary by bait set and library, but these have consistently performed well for a broad spectrum of bait sets and samples.

Hybridization time

For most applications, hybridize for 16 to 24 hours. For very rare targets (e.g., those in ancient, forensic, or environmental samples) hybridize for 24 to 40 hours. Shorter and longer times can be tolerated, though will require trials to identify optimal performance.

Ensure that the chosen combination of tubes and thermal cycler allows no more than 15% volume evaporation (4.5 of 30 µL) over the chosen time and temperature.

REQUIRED MATERIALS

Equipment

- Nuclease-free 50 mL, 1.7 mL low-bind and 0.2 mL low-bind tubes. For low-bind, we recommend Axygen MAXYmum Recovery™ tubes.
- For 96-well Hybrid Bind & Wash procedure (Part 2B), 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 (e.g., BioRad C1000) 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 15% volume evaporation over the chosen time and temperature

- Magnetic particle collector for ~1.5 mL tubes (e.g., Life Technologies DynaMag™-2, #123-210) AND/OR 96-well magnetic particle collector (e.g., Alpaqua 96R Ring Magnet Plate, #A001219 or similar)
- Vortex mixer
- Microcentrifuge
- Water bath set to chosen hybridization and wash temperature
- Dry bath / heat block

Reagents

- Nuclease-free molecular biology-grade ("**NF**") water (~150 mL)
- Dynabeads® MyOne™ Streptavidin C1 magnetic beads (Invitrogen, #650-01) (30 µL per capture)
- 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)
- 10mM Tris-Cl, 0.05% TWEEN®-20 solution (pH 8.0-8.5)

Recommended

- Multi-channel pipettor capable of pipetting up to 20 µL
- For 96-well Hybrid Bind & Wash procedure (Part 2B), a multi-channel pipettor for up to 200 µL

PROCEDURE

Part 1: Hybridization

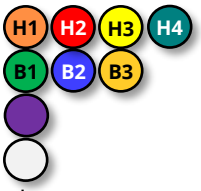
The following corresponds to **page 1, steps 1 through 3**. Here, sequencing libraries are denatured, their adapters are blocked by adapter blocker, and baits are allowed to encounter and then hybridize to their targets.

1.1 Getting Started

Gather these components:

Reagents:

- HYB reagents (Box 1 & 2)
- BLOCK reagents (Box 2)
- RNase Block (Box 2) ***Keep on ice***
- Baits (Box 3) ***Keep on ice***
- Sequencing libraries to be enriched



Thoroughly vortex HYB #1 (H1) ***before use, and bring HYB #4*** (H4) ***to room temperature to fully dissolve its SDS before use***

Equipment:

- 1.7 mL (×2) and 50 mL (×1) nuclease-free tubes
- Low-bind 0.2mL tubes with individual caps (×2 per reaction)
- Pipettors & tips; **multichannel pipettor for pipetting up to 20 µL recommended**
- Vortex mixer
- Thermal cycler

Program the thermal cycler:

Program the lid temperature to stay at 105°C, or at least 10°C above each step temperature, to keep evaporation to a minimum.

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

1.2 Mix Setups

1. Assemble the **Hybridization Mix**, briefly vortex and centrifuge:

Component	µL per Reaction
H1 **HYB #1**	9
H2 **HYB #2**	0.5
H3 **HYB #3**	3.5
H4 **HYB #4**	0.5
RNase Block	1
Baits	5.5
TOTAL	**20**

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

   These are now referred to as "HYBs"

3. Assemble the **Blockers Mix** and briefly vortex:

   Replace BLOCK #2 with nuclease-free molecular biology-grade water if targeting a species closely related to salmon (family Salmonidae).

Component	µL per Reaction
B1 **BLOCK #1**	2.5
B2 **BLOCK #2**	2.5
B3 **BLOCK #3**	0.5
TOTAL	**5.5**

4. For each capture reaction, aliquot **5 µL** Blockers Mix to a low-bind 0.2mL tube

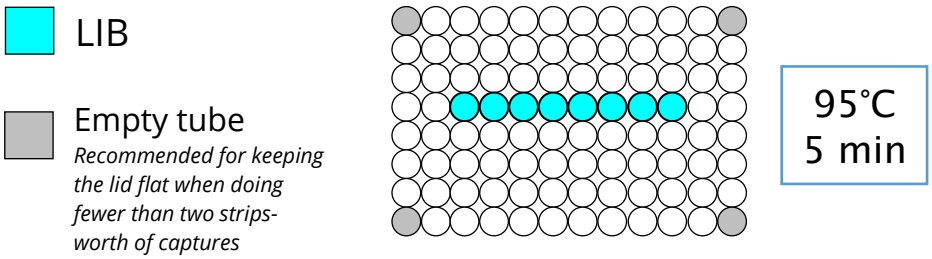
5. Add **7 µL** DNA library (100 – 500ng) to each Blockers Mix aliquot and homogenize by pipetting.

   These are now referred to as "LIBs" total volume: 12 µL

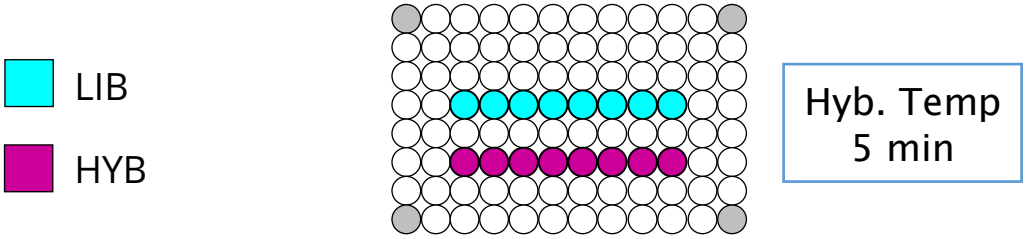
1.3 Reaction Assembly

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

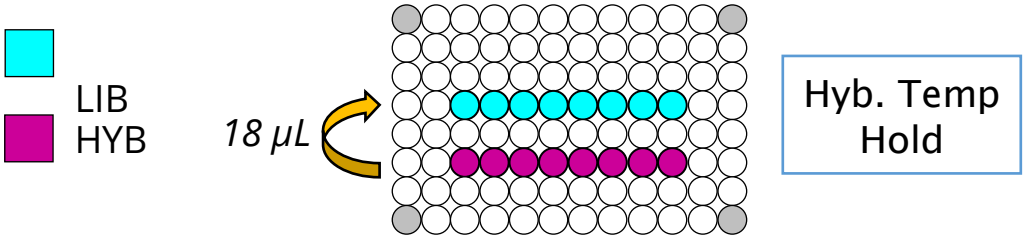
   Double-check that the lid temperature is programmed to stay at 105°C, or at least 10°C above each step temperature, to keep evaporation to a minimum.



2. Once the cycler reaches step 2 of the program (the hybridization temperature), 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. Close the lid of the thermal cycler and allow the reactions to incubate at your chosen hybridization temperature for your chosen time (e.g., 16 hours).

PROCEDURE

Part 2A: Hybrid Bind & Wash using a 1.5 mL Magnetic Particle Collector

The following corresponds to **page 1, steps 4 and 5.** Here, bait-target hybrids are bound to streptavidin-coated magnetic beads, and then non-hybridized and non-specifically hybridized DNA are removed with a series of wash steps.

Follow this version of Hybrid Capture & Wash if using exclusively a magnetic particle collector that fits ~1.5 mL tubes. If you have a 96-well magnetic particle collector, we suggest following "*Part 2B: Hybrid Bind & Wash using a 96-well Magnetic Particle Collector*" instead, starting on page 11.

2A.1 Getting Started

Start ~90 minutes before intended hybridization stop-time

Gather these components:

Reagents:

- HYB #4 (H4)
- Binding Buffer
- Wash Buffer 2
- Dynabeads® MyOne™ Streptavidin C1 Beads (30 µL per reaction)
- Nuclease-free molecular biology-grade ("**NF**") water (~150 mL)
- 10mM Tris-Cl, 0.05% TWEEN®-20 solution (pH 8.0-8.5).

Bring HYB #4 and Wash Buffer 2 to room temperature to dissolve SDS prior to use

Equipment:

- Nuclease-free 1.7 mL low-bind tubes (1 per capture reaction)
- Nuclease-free 50 mL tubes (1 per 33 capture reactions)
- Pipettors and tips for 20 µL – 500 µL volumes
- Magnetic particle collector for ~1.5 mL tubes
- Water bath set to hybridization temperature
- Vortex mixer
- Minicentrifuge with adapters for 1.7 mL and 0.2 mL tubes

2A.2 Wash Buffer 2.2 Preparation

The following procedure makes enough Wash Buffer 2.2 for washing **33 capture reactions**; scale up if needed. Wash Buffer 2.2 can be stored at 4°C for 1 month.

1. Combine 400 µL HYB #4, 39.6 mL NF water and 10 mL Wash Buffer 2 in a 50 mL tube. Vortex thoroughly, **label "Wash Buffer 2.2"**
2. Heat the Wash Buffer 2.2 to the hybridization temperature in the water bath **for at least 45 minutes before use.**

2A.3 Bead Preparation

1. For each capture reaction, aliquot 30 µL Dynabeads® MyOne™ Streptavidin C1 beads to a 1.7 mL low-bind tube.
2. Pellet the beads in the magnetic particle collector (**"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 3 seconds 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.

TIP: *Beads can be prepared in larger batches, up to 8 reactions-worth (240 µL) at a time in a 1.7 mL tube. When doing so, multiply the wash and resuspension volumes by the number of reactions in the batch. For example, for eight reactions-worth, wash three times with 1.6 mL and resuspend in 560 µL Binding Buffer, then aliquot 70 µL suspension to individual tubes for each binding reaction.*

2A.4 Bead-Bait Binding

1. Heat the bead aliquots to the hybridization temperature (e.g., 65°C) for at least 2 minutes in the water bath.
2. Transfer each capture reaction to the heated bead aliquots. Mix by pipetting.
3. Incubate the libraries + beads in the water bath at the hybridization temperature for 30 minutes. Agitate every 5 minutes by flicking the tube to keep the beads in suspension.

2A.5 Bead Washing

1. Pellet the beads in the MPC for 2 minutes and remove the supernatant.
2. Add 500 µL heated Wash Buffer 2.2 to the beads, briefly vortex, and briefly centrifuge.
3. Incubate for 10 minutes at the hybridization temperature in the water bath with occasional agitation by flicking and centrifuging the tube. Pellet in the MPC and remove the supernatant.
4. Repeat step 3 two times for a total of three washes. After the third wash and pelleting, remove as much fluid as possible without touching the bead pellet.
5. **Continue to Part 3, page 13.**

Part 2B: Hybrid Bind & Wash using a 96-well Magnetic Particle Collector

The following corresponds to **page 1, steps 4 and 5.** Here, bait-target hybrids are bound to streptavidin-coated magnetic beads, and then non-hybridized and non-specifically hybridized DNA are removed with a series of wash steps.

Follow this protocol if you have a 96-well magnetic particle collector. It can be used with any number of captures. For binding and washing steps, use PCR strips with individually-attached caps to enable vortexing and minimize cross-contamination. Work with up to six 8-well strips on a single 96-well particle collector at a time to enable opening the lids.

2B.1 Getting Started

Start ~90 minutes before intended hybridization stop-time

Gather these components:

Reagents:

- HYB #4 (H4)
- Binding Buffer
- Wash Buffer 2
- Dynabeads® MyOne™ Streptavidin C1 Beads (30 µL per reaction)
- Nuclease-free molecular biology-grade ("**NF**") water (~50 mL)
- 10mM Tris-Cl, 0.05% TWEEN®-20 solution (pH 8.0-8.5).

Bring HYB #4 and Wash Buffer 2 to room temperature to dissolve SDS prior to use

Equipment:

- 0.2 mL standard-profile PCR strips with individually-attached caps (one well per reaction)
- Thermal cycler with heated lid
- Nuclease-free 50 mL tubes (1 per 68 capture reactions)
- Pipettors and tips for 20 µL – 500 µL volumes
- **STRONGLY RECOMMENDED:** Multichannel pipettor for 20-200 µL
- 96-well magnetic particle collector
- Water bath set to hybridization temperature
- Vortex mixer
- Minicentrifuge with adapters for 0.2 mL tubes/strips

2B.2 Wash Buffer 2.2 Preparation

The following procedure makes enough Wash Buffer 2.2 for washing **68 capture reactions**; scale up if needed. Wash Buffer 2.2 can be stored at 4°C for 1 month.

1. Combine 400 µL HYB #4, 39.6 mL NF water and 10 mL Wash Buffer 2 in a 50 mL tube. Vortex thoroughly, **label "Wash Buffer 2.2"**
2. Heat the Wash Buffer 2.2 to the hybridization temperature in the water bath **for at least 45 minutes before use.**

2B.3 Bead Preparation

1. For each capture reaction, aliquot 30 µL Dynabeads® MyOne™ Streptavidin C1 beads to a 0.2 mL tube of a PCR strip with individually-attached caps.
2. Pellet the beads in the magnetic particle collector (**"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 3 seconds and centrifuge briefly. Pellet in the MPC for 2 minutes, 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.

TIP: *With a MPC for ~1.5mL tubes, beads can be prepared in batches of up to 8 reactions-worth (240 µL) at a time in a 1.7 mL tube. When doing so, multiply the wash and resuspension volumes by the number of reactions in the batch. For example, for eight reactions-worth, wash three times with 1.6 mL and resuspend in 560 uL Binding Buffer, then aliquot 70 µL suspension to 0.2 mL wells for each binding reaction.*

2B.4 Bead-Bait Binding

1. Heat the bead aliquots to your chosen hybridization temperature (e.g., 65°C) for 2 minutes in the thermal cycler. Set the lid temperature at least 10°C higher than the block.
2. Transfer each capture reaction to the heated bead aliquots. Mix by pipetting.
3. Incubate the libraries + beads in the thermal cycler at the hybridization temperature for 30 minutes. Agitate every 10 minutes by flicking the tubes and briefly centrifuging.

2B.5 Bead Washing

1. Pellet the beads in the MPC for 2 minutes and remove the supernatant.
2. Add 180 µL heated Wash Buffer 2.2 to the beads, briefly vortex, and briefly centrifuge.
3. Incubate for 10 minutes at the hybridization temperature in the thermal cycler; agitate every 3 minutes by briefly vortexing then centrifuging the strip. Pellet and remove the supernatant.
4. Repeat step 3 three times for a total of four washes. After the final wash and pelleting, remove as much fluid as possible without touching the bead pellet.

PROCEDURE

Part 3: Library Elution & Amplification

The following corresponds to **page 1, step 6.** Here, the bead-bound enriched library is released from the RNA bait via heat denaturation, and then amplified in preparation for sequencing.

3.1 Enriched Library Elution

1. Add 30 µL of 10mM Tris-Cl, 0.05% TWEEN®-20 solution (pH 8.0 – 8.5) to the washed beads and thoroughly resuspend by pipetting.

If you are using KAPA® HiFi polymerase for amplification, skip steps 2 and 3, and use this bead suspension directly in amplification. For other polymerases, continue to step 2.

2. Incubate the suspension at 95°C for 5 minutes.
3. Pellet the beads in the MPC, and remove the supernatant, which contains the enriched library.

3.2 Suggested Amplification Setup

Prior to sequencing, we recommend amplifying the enriched library 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).

Suggested post-capture PCR reaction composition and thermal program

Component	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**

Using bead-bound library as template in PCR works well with KAPA® HiFi polymerase, but is likely possible with other polymerases. We have strong evidence that **Phusion® HiFi** (Thermo Fisher Scientific) **does *not* couple well with bead-bound library as template.**

Step	Temperature	Time	
Activation	98°C	2 minutes	
Denaturation	98°C	20 seconds	× 8 to 14 cycles†
Annealing	(primers-specific)	30 seconds	
Extension	72°C	length-dependent*	
Final Extension	72°C	5 minutes	
End	8°C	∞	

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

† Use as few cycles as necessary to obtain sufficient molarity for sequencing

Following amplification, purify the product with your preferred PCR cleanup method.

If beads were used in the amplification, pellet the beads first in the MPC and remove and purify only the supernatant.

APPENDIX

A1. MYbaits® Kit Reagents Formulae & MSDS

BOX 1: STORE AT 4°C

Component	Volume	Composition	Lid Color
HYB #1	1.5 mL	20X SSPE	Orange
HYB #2	60 µL	0.5M EDTA, pH 8.0	Red
HYB #4	800 µL to 1 mL	10% Sodium Dodecyl Sulfate (SDS)	Teal
Binding Buffer	45 mL	1M NaCl; 10mM Tris-HCl, pH 7.5; 1mM EDTA	-
Wash Buffer 2	80 mL	0.1X SSC, 0.1% SDS	-

BOX 2 (12, 24, and 48 reaction kit sizes): STORE AT -20°C (non-frost-free)

Component	Volume	Composition	Lid Color
HYB #3	700 µL	50X Denhardt's Solution	Yellow
BLOCK #1	40, 70, or 125 µL	1 µg/µL human C_0t-1 DNA	Green
BLOCK #2	40, 70, or 125 µL	1 µg/µL salmon sperm DNA	Blue
BLOCK #3	30 µL	Library-specific adapter blockers	Gold
RNase Block	40 or 70 µL	SUPERase-In (20 U/µL)	Purple

BOX 3: STORE AT -80°C

Component	Volume	Composition	Lid Color
Baits	5.5 µL per reaction	Your custom biotinylated RNA oligonucleotides	White

MSDS:

MYbaits® Box 1: http://www.mycroarray.com/msds/MYbaits-Box1-MSDS.pdf

MYbaits® Box 2: http://www.mycroarray.com/msds/MYbaits-Box2-MSDS.pdf

MYbaits® Box 3: http://www.mycroarray.com/msds/MYbaits-Box3-MSDS.pdf

A2. MYbaits® Procedure Quick Guide

1. For each reaction, build the following solutions:

Hybridization Mix	
Component	µL per Reaction
H1 **HYB #1**	9
H2 **HYB #2**	0.5
H3 **HYB #3**	3.5
H4 **HYB #4**	0.5
RNase Block	1
Baits	5.5
TOTAL	**20**

Blockers Mix	
Component	Volume per Reaction
B1 **BLOCK #1**	2.5
B2 **BLOCK #2**	2.5
B3 **BLOCK #3**	0.5
TOTAL	**5.5**

2. For each reaction, aliquot **5 µL** of Blockers Mix and then add **7 µL** each library – now **"LIBs"**
3. For each reaction, aliquot **18.5 µL** of Hybridization Mix to their own tubes – now **"HYBs"**
4. Incubate the LIBs in the thermal cycler for 5 minutes @ 95°C and then drop to the hybridization temperature (e.g., 65°C). **Set the lid temperature to at least 10°C above each step temperature.**
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 2.2 by combining 400 µL HYB #4, 39.6 mL nuclease-free molecular biology-grade water and 10 mL Wash Buffer 2 in a 50 mL tube. Vortex thoroughly and warm to the hybridization temperature in the water bath for at least 45 minutes.
8. Prepare 30 µL of magnetic 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 with occasional agitation.
10. Pellet the beads and remove the supernatant, and then wash the beads three times with 500 µL warmed Wash Buffer 2.2, keeping the washes at the hybridization temperature. Wash four times with 180 µL washes if using a 96-well magnetic particle collector and 0.2 mL strips/tubes.
11. Resuspend the beads in 30 µL of 10mM 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.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