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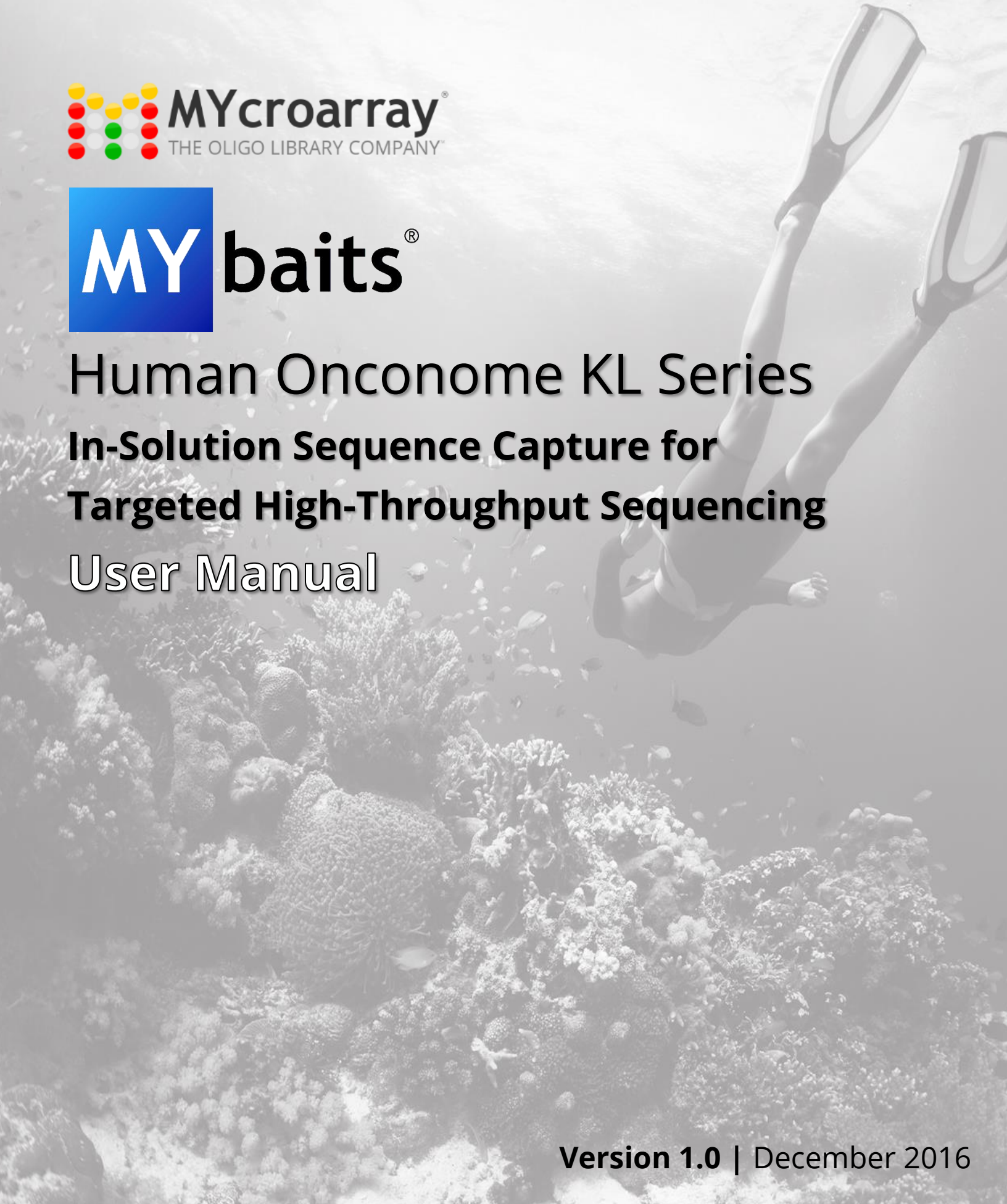
MYbaits®

Human Onconome KL Series

In-Solution Sequence Capture for Targeted High-Throughput Sequencing

User Manual



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INTRODUCTION

The MYbaits® Onconome KL series of human cancer targeted exome kits enrich thousands of exons in hundreds of cancer-related genes described in two major studies:

> Kandoth et al. 2013. "Mutational landscape and significance across 12 major cancer types". Nature 502:333-339, doi:10.1038/nature12634
>
> Lawrence et al. 2014. "Discovery and saturation analysis of cancer genes across 21 tumour types". Nature 505:495-501, doi: 10.1038/nature12912

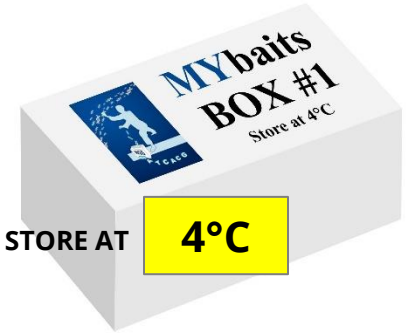
Both the KL v1.0 and KL v1.5 panels were developed through multiple design iterations in order to improve coverage uniformity and filter out repetitive genomic loci.

The laboratory procedure for MYbaits® Onconome KL target enrichment is similar to Gnirke *et al.* 2009 (doi: 10.1038/nbt.1523). It involves hybridizing baits to targets in DNA sequencing libraries, sequestering those hybrids on streptavidin-coated magnetic beads, washing away nonspecifically-bound library molecules, and amplifying the enriched library prior to high-throughput sequencing.

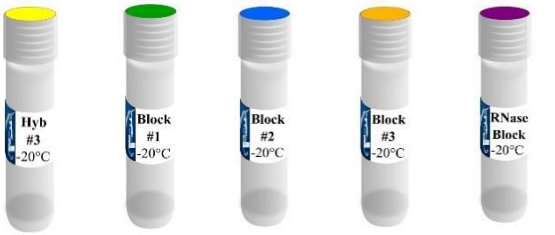
Kit components and procedure for both KL v1.0 and KL v1.5 are nearly identical to standard MYbaits®, except that **Wash Buffer 2 is used in lieu of Wash Buffer 2.2, numbers of washes are reduced, and two rounds of enrichment is standard.**

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

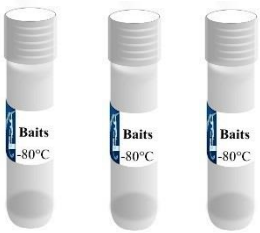
KIT COMPONENTS



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



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	800 µL to 1 mL
Binding Buffer	45 mL
Wash Buffer 2	80 mL

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

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

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

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

[c] 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 enrichment reaction)
- PCR primers for amplifying your sequencing libraries after capture, e.g., the "reamp" primers described in Meyer & Kircher 2010 (doi:10.1101:pdb.prot5448) for Illumina® libraries.
- PCR reagents (e.g., KAPA® HiFi HotStart ReadyMix, Kapa Biosystems) including universal library amplification primers for three 50 µL library amplifications per library or pool of libraries to be enriched. For example, if ten libraries will be enriched individually, a total of 30 amplifications will be performed.
- 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

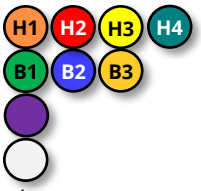
The following corresponds to **page 1, steps 1 through 3**. Here, sequencing libraries are denatured, their adapters are blocked by adapter blocker, and baits are allowed to encounter and then hybridize to their targets.

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

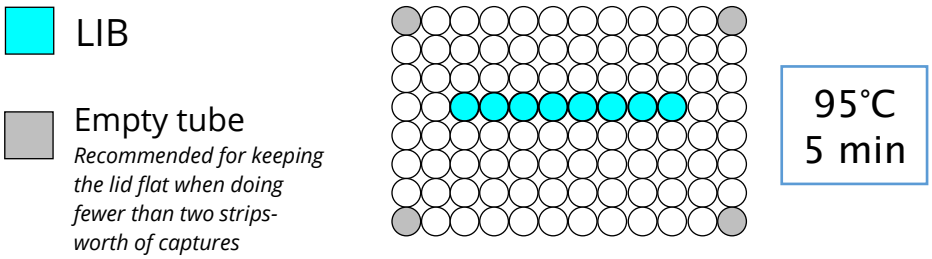
5. Add **7 µL** DNA library (100 – 500ng) to each Blockers Mix aliquot and homogenize by pipetting.

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

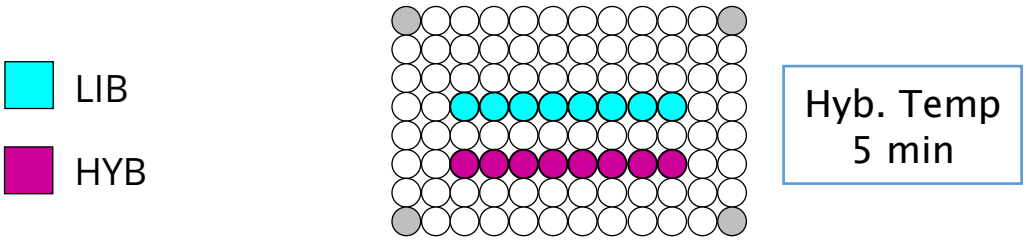
1.3 Reaction Assembly

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

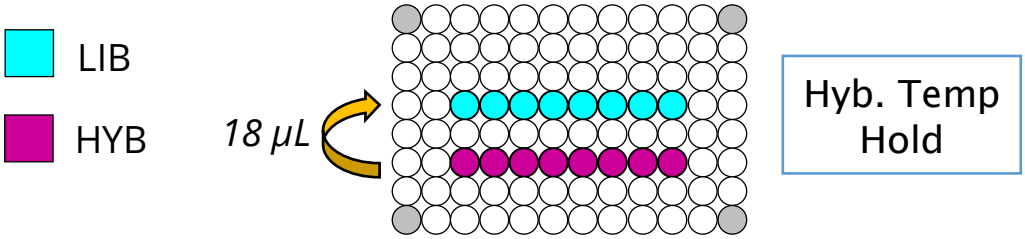
   Double-check that the lid temperature is programmed to stay at 105°C, or at least 10°C above each step temperature, to keep evaporation to a minimum.



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. When enriching libraries built from fresh human genomic DNA, first-round hybridization times can be as short as 2 hours, though second rounds should be at least overnight (16-24 hours) regardless of library type.

PROCEDURE

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

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.

2A.1 Getting Started

Start ~90 minutes before intended hybridization stop-time

Gather these components:

Reagents:

- 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).
- PCR reagents sufficient for one or two library amplifications per enrichment reaction

Equipment:

- Nuclease-free 1.7 mL low-bind tubes (1 per capture reaction)
- 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 Preparation

1. Heat Wash Buffer 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 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.
   If this is the second round of enrichment of these libraries*, repeat step 3 once, totaling 2 washes*
4. Remove as much fluid as possible without touching the bead pellet.
5. **Continue to Part 3.**

PROCEDURE

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

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:

- 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).
- PCR reagents sufficient for one or two library amplifications per enrichment reaction

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

1. Heat the Wash Buffer 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.
   If this is the second round of enrichment of these libraries*, repeat step 3 once, totaling 2 washes*
4. Remove as much fluid as possible without touching the bead pellet.
5. Continue to Part 3.

PROCEDURE

Part 3: Library Elution & Amplification

Here, the bead-bound enriched library is released from the RNA baits via heat denaturation either before or during PCR, and then amplified in preparation for a second round of enrichment, or if this is the end of the second round of enrichment, 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, now proceed directly to section 3.2, using this bead suspension in amplification. For other polymerases, proceed instead 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, amplify the enriched library using KAPA® HiFi (Kapa Biosystems). If this is the first round of enrichment, perform two amplifications per capture reaction. Suggested setup:

Component	Final Conc.	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 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

† After a second round of enrichment, 10-12 cycles may be necessary

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.

PROCEDURE

Part 4: Perform a second round of enrichment, or take to sequencing

If only one round of enrichment has been performed:

1. Combine the two purified amplification products produced in Part 3.
2. Concentrate this combination to 7 µL using vacuum centrifugation or other drying technology. If you would like to keep once-enriched library for later use, concentrate to 10 µL instead.
3. Enrich 7 µL of the concentrated library again by repeating Parts 1 through 3.

If the library has now been enriched twice:

1. Quantify the library using library quantitative PCR prior to sequencing.

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. Warm the Wash Buffer 2 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 once with 500 µL warmed Wash Buffer 2, keeping the washes at the hybridization temperature. Wash once with 180 µL washes if using a 96-well magnetic particle collector and 0.2 mL strips/tubes. If this is the second round of enrichment, wash an additional time for a total of two washes.
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 two (first enrichment round) or one (second enrichment round) 50 µL amplification reaction(s) 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 amplifications.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