MYcroarray

Attention all MYbaits® users:

The following MYbaits protocol has
been replaced with a **newer version,**
which can be accessed from:

http://www.mycroarray.com/mybaits/manuals.html

The following manual is made available for legacy purposes only.

Please consult your MYbaits contact if you are unsure which version
to use with your kit, or if you have any other questions.

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MYcroarray

MYbaits

Sequence Enrichment for Targeted Sequencing

User Manual

Version 2.3.1 - 5/22/2014

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Get the latest version at http://www.mycroarray.com/pdf/MYbaits/manuals.html

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I. Introduction

MYbaits is a fully customizable liquid-phase DNA capture system for targeted sequencing or any other applications requiring sequence enrichment. Each kit is custom made to target your sequences of interest.

What does the bait library contain?

Each MYbaits kit contains a custom library of biotinylated single stranded RNA baits designed per your recommendation. Each library can contain up to 100,000 different bait sequences. We first synthesize a library of DNA oligonucleotides using our proprietary parallel DNA synthesis technology. The DNA library is then converted into biotinylated RNA baits by *in vitro* transcription. Each sequence from the bait library is present in the pool at an average concentration of 50 pM. Depending on the number of baits in your library, a capture experiment will use from 0.25 to 0.5 fmole of each bait. This represents 1.5 to 3 x 10^8 molecules per baits. As a comparison, 1 microgram of human genomic DNA library contains 3 x 10^5 copies of the haploid genome.

How does it work?

Our approach is based on the work of Gnirke et *al*. (Solution Hybrid Selection with Ultra-long Oligonucleotides for Massively Parallel Targeted Sequences. 2009. Nature Biotechnology 27(2):182-189).

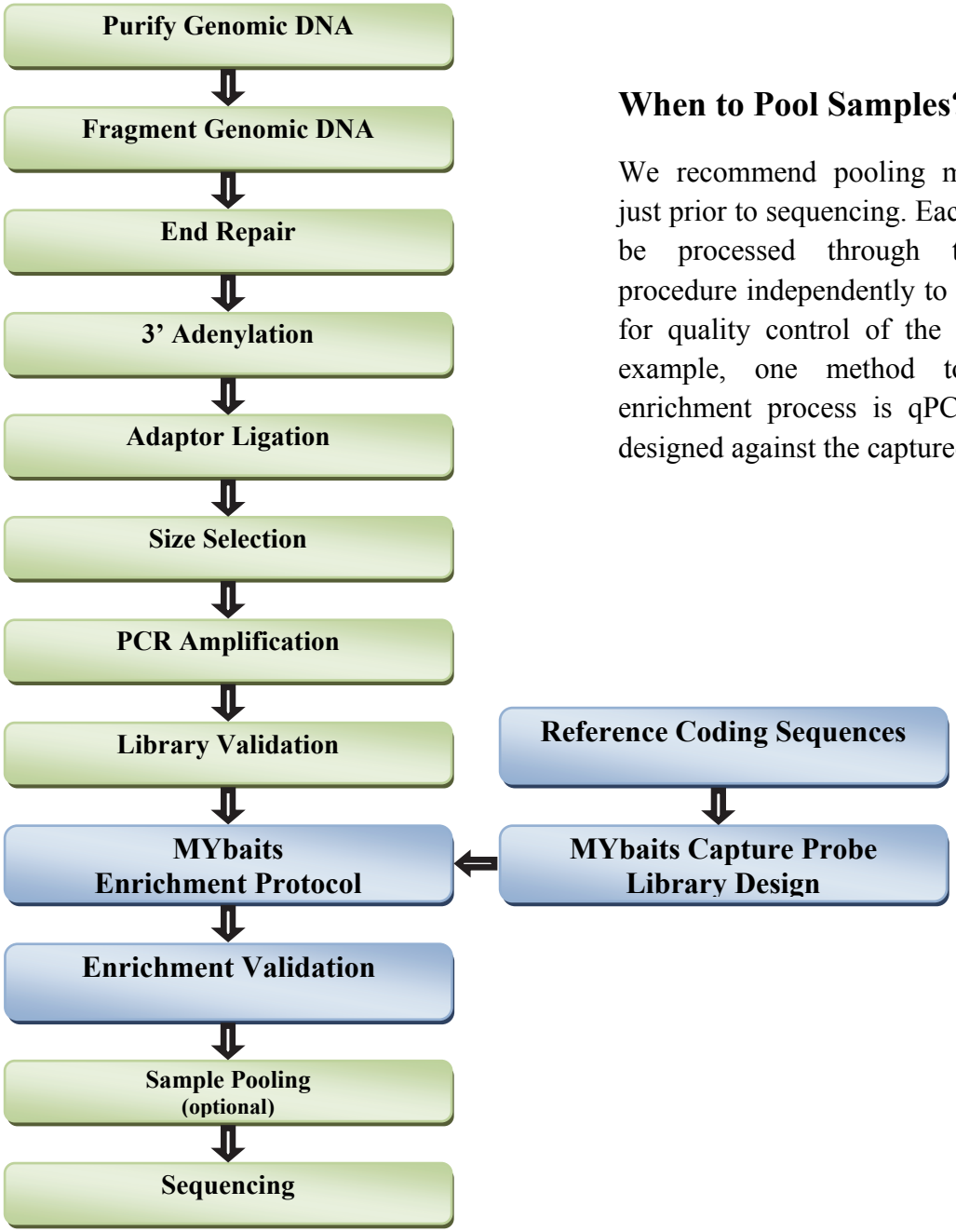
The genomic DNA library is heat-denatured and hybridized to the RNA baits in stringent conditions for 36 hours. This gives enough time for a bait to hybridize to a complementary target sequence. After hybridization, the biotinylated baits hybridized to captured material are pulled out of the solution with streptavidin-coated magnetic beads. Any DNA molecule that may have bound non-specifically to the magnetic beads are washed away and the captured genomic DNA is released by chemical degradation of the RNA baits.

Depending on the total length of the targeted sequences, it may be necessary to perform a limited PCR amplification post-capture to have enough material for sequencing. For example, when targeting 3 Mb of human sequence (1/1000th of human genome) and starting from 5 micrograms of genomic library, the theoretical amount of recoverable material is 5 nanograms. But in practice, the recovered amount will be lower due to inevitable loss of material at various steps.

When to perform the capture during sequencing library preparation?

We strongly recommend performing the capture on fully prepared and validated sequencing library. Your genomic DNA should be fragmented, size purified and the sequencing adaptor ligated. The library preparation process is depicted in Figure 1. This kit has been tested with 100 – 500 ng of genomic library. Working with lower or larger amounts of starting material may require some optimization. Figure 1 shows an outline

Figure 1. Library Preparation Workflow



When to Pool Samples?

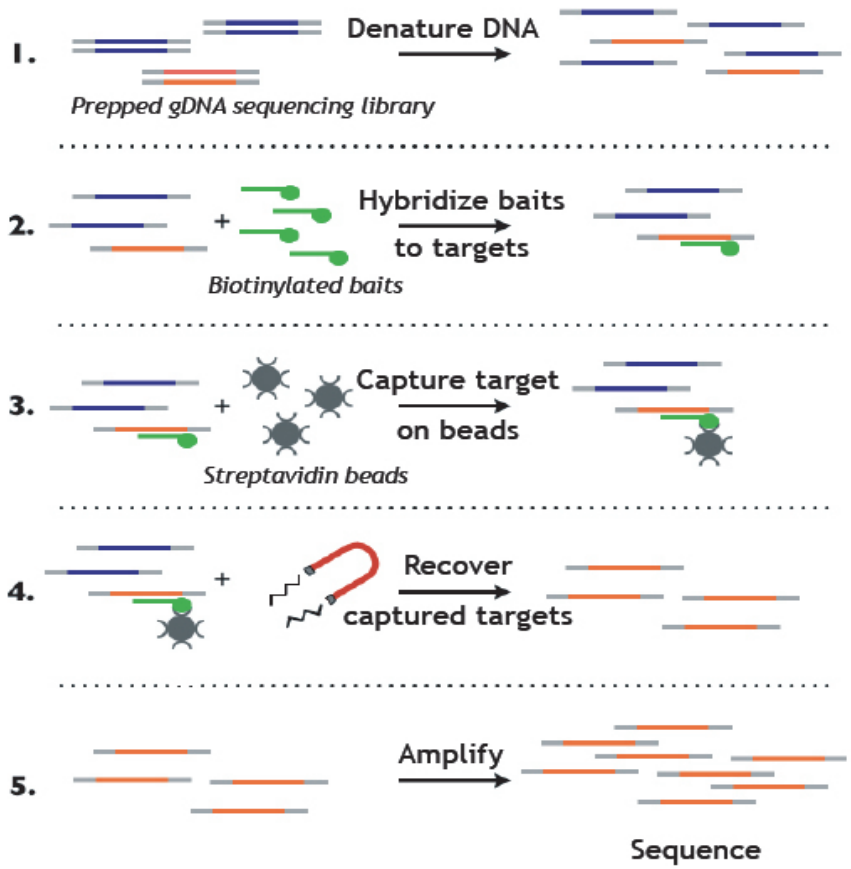
We recommend pooling multiple samples just prior to sequencing. Each sample should be processed through the enrichment procedure independently to allow the ability for quality control of the enrichment. For example, one method to validate the enrichment process is qPCR with primers designed against the captured material.

Procedure Overview

The MYbaits enrichment process is depicted in Figure 2.

1. ***Denature DNA***: The sequencing prepared, adaptor ligated genomic DNA library is denatured to generate single stranded DNA molecules.
2. ***Hybridize baits to targets***: The biotinylated RNA baits are hybridized to the denatured DNA library under optimal conditions.
3. ***Capture target on beads***: The hybridized DNA:RNA complexes are bound to magnetic streptavidivn beads.
4. ***Recover captured targets***: The DNA-RNA complexes are then purified magnetically from the unbound genomic DNA and subsequently enriched following release of the RNA baits.
5. ***Amplify***: The captured genomic DNA is amplified and sequenced.

Figure 2. MYbaits Targeted Enrichment Overview



II. Important Considerations for Successful Enrichment

Due to the customization of each application, it is necessary to evaluate the effect each supplied reagent may have on your capture efficiency. Below are some points to take into consideration prior to starting the enrichment process.

- Human Cot-1 (Block #1) is used to specifically bind repetitive sequences found in the human genome. Replacing Block #1 with a Cot-1 specific for the species in which the baits were designed against may increase efficacy of the enrichment.

- Salmon Sperm DNA (Block #2) is used as a non-specific block. It may be necessary to exclude Block #2 (by replacing the volume with Molecular Biology Grade Water) from the hybridization solution if the genomic DNA library used is closely related to the Salmon species. This will prevent possible cross-reactivity of Block #2 to the customized baits.

- If quantitative PCR is used to monitor enrichment, primer pairs should be designed such that the amplicon length is longer than the bait length or such that both primers cannot bind to a particular bait sequence. The RNA baits may contain trace amounts of DNA template used during production that could potentially be amplified with primers directed against a unique bait sequence.

- Platform specific primers for Post-Capture Amplification: We recommend using the primers from the kit used to prepare the genomic DNA library.

  Suggested Primers for:

  454 Sequencing Platform (Titanium and Rapid):
  For the A-adaptor: `5′-CCATCTCATCCCTGCGTGTC-3′`
  For the B-adaptor: `5′-CCTATCCCCTGTGTGCCTTG-3′`

  Ion Torrent Sequencing Platform:
  Forward primer (Primer A-key): `5′-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3′`
  Reverse primer (Primer P1-key): `5′-CCTCTCTATGGGCAGTCGGTGAT-3′`

  SOLiD Sequencing Platform:
  Primer 1: `5′-CCACTACGCCTCCGCTTTCCTCTCTATG-3′`
  Primer 2: `5′-CTGCCCCGGGTTCCTCATTCT-3′`

  Illumnia Sequencing Platform: refer to library preparation method for primer sequences

III. Materials

Reagents provided in MYbaits kits and storage conditions.

Box #1: Store at 4°C

Product	Amount*	Components	Cap Color
HYB #1	1.5 ml	20X SSPE	Orange
HYB #2	60 µl	500 mM EDTA	Red
HYB #4	1 ml	10% Sodium Dodecyl Sulfate	Teal¥
Binding Buffer	45 ml	1 M NaCl; 10 mM Tris-HCl, pH 7.5; 1 mM EDTA	
Wash Buffer 1∂	30 ml	1X SSC, 0.1% SDS	
Wash Buffer 2+	80 ml	0.1X SSC, 0.1% SDS	
Neutralization Buffer∂	3.75 ml	1 M Tris-HCl, pH 7	

While the tube label may read "Room Temperature", we now recommend storage at 4°C.

* Amounts shown here are based on a 50 reactions kit size.

¥ Formerly distributed with a PINK cap color.

∂ This version of the protocol does not use this buffer.

+ This version of the protocol uses a diluted version of Wash Buffer 2 prepared in section V.

Box #2: Store at -20°C in a non-frost-free freezer

Product	Amount*	Components	Cap Color
HYB #3	700 µl	50 X Denhardt's Solution	Yellow
BLOCK #1	125 µl	1 µg/µl Human Cot-1 DNA	Green
BLOCK #2	125 µl	1 µg/µl Salmon Sperm DNA	Blue
BLOCK #3	30 µl	Proprietary Blocking Agent	Gold¥
RNase Block	70 µl	SUPERase-In (20 U/µl)	Purple

* Amounts shown here are based on a 50 reactions kit size.

¥ Formerly distributed with a BROWN cap color

Box #3: Store at -80°C

Product	Amount	Components	Cap Color
Capture Probe Library+	variable	Biotinylated RNA Baits (probes)	White

+ The Capture Probe Library is sensitive to freeze-thaw cycles. If performing a small number of captures at a time, it is recommended to aliquot the library to decrease its susceptibility to degradation.

Reagents to be provided by user

- Dynabeads® MyOne™ Streptavidin C1 (Life Technologies™, # 65001)
- QIAquick PCR Purification Kit (Qiagen, #28104)
- Molecular Biology Grade Water
- PCR primers compatible with the sequencing platform to be used (see Appendix A)
- KAPA HiFi HotStart ReadyMix (KAPA BIOSYSTEMS, #KK2601)
- SDS, 10% solution (Life Technologies™, # AM9822) if a more stringent wash buffer is to be prepared.

Required equipment and supplies

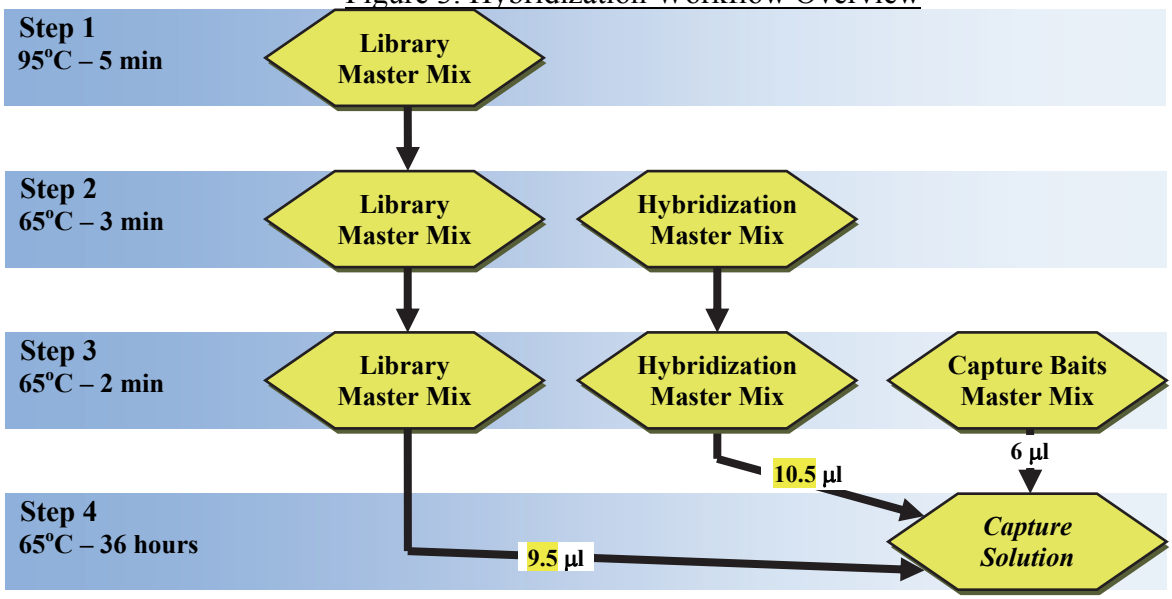
- Vacuum Centrifuge Concentrator
- Vortex Mixer
- Microcentrifuge
- RNase-free Pipettors and Tips
- BioRad C1000 Thermocycler or compatible thermocycler (see Appendix C)
- 0.2 ml tubes (BioRad, #TFI-0201): Thermocycler-compatible, thin-wall, nuclease-free
- 1.5 ml low adhesion microcentrifuge tubes (VWR, #20170-650): Nuclease-free
- Magnetic Particle Stand (Life Technologies™, #123-21D, DynaMag™-2)
- Tube Rotator (Fisher Scientific, #400110Q)
- Circulating Water Bath (set at 65°C)
- Spectrophotometer (Nanodrop) or Fluorometer (QuantiFluor)

IV. Hybridization

This step involves denaturing and hybridizing the sequencing library to a pool of custom complementary RNA baits. MYbaits kit has been tested with 100 – 500 ng of input genomic library. Smaller or larger amounts may require optimization.

Overview

Figure 3. Hybridization Workflow Overview



Before starting

- Evaluate the effect ***Block #1*** and ***Block #2*** will have on your capture experiment. (See tips in the "Important Considerations for Successful Enrichment" section).
- Equilibrate the ***HYB #4*** tube at room temperature to fully dissolve the SDS that may have precipitated during storage at 4°C.
- Remove ***Wash Buffer 1*** and ***Wash Buffer 2*** from 4°C when starting the hybridization and leave at room temperature until use in section V to **fully dissolve the SDS** that may have precipitated during storage at 4°C and to ensure proper temperature equilibration.

Recommendations

- Set hot-lid temperature to 10°C above block temperature for hybridization to decrease the occurrence of evaporation.
- Place 0.2 ml tubes in the thermocycler block corners to ensure the hot lid is making proper contact with the tubes.

1. Set the following program on a thermocycler. (See **Appendix C** for recommended thermocyclers. Performing the validation procedure is highly recommended prior to continuing).

Step	Temperature	Time (minutes)
1	95°C	5
2	65°C	3
3	65°C	2
4	65°C	∞

2. Prepare a ***Library Master Mix*** in a 0.2 ml nuclease-free tube at room temperature and mix by vortexing. Set aside until step 5.

   Note: Prior to preparing the Library Master Mix, it may be necessary to concentrate the sequencing library by using a vacuum centrifuge concentrator in order to have 100 – 500 ng of library DNA in 5.9 µl.

Component	Amount (11.5 µl)
Block #1 (Green)	2.5
Block #2 (Blue)§	2.5
Block #3 (Brown)§	0.6
Sequencing library (100 – 500 ng)	5.9

   § Before starting, see tips in the **"Important Considerations for Successful Enrichment"** section.

3. Prepare a ***Hybridization Master Mix*** in a 0.2 ml nuclease-free tube at room temperature and mix by vortexing. Set aside until step 6.

Component	Amount (29.6 µl)*
HYB #1 (Orange)	20
HYB #2 (Red)	0.8
HYB #3 (Yellow)	8
HYB #4 (Pink)	0.8

   * The master mix volume is greater than needed but is formulated to enable accurate pipetting of HYB #2 and HYB #4.

4. Prepare a ***Capture Baits Master Mix*** in a 0.2 ml nuclease-free tube on ice and mix by pipetting. Keep on ice until step 7.

Component	Amount (6 µl)
Capture Probe (White)+	5
RNase Block (Purple)	1

+ The Capture Probe Library is sensitive to freeze-thaw cycles. If performing a small number of captures at a time, it is recommended to aliquot the library to decrease its susceptibility to degradation.

5. Transfer the tube containing the *Library Master Mix* to the thermocycler and start the program set in step 1. This will denature the DNA library for 5 minutes at 95°C.

6. Once the thermocycler program reaches step 2, transfer the prepared *Hybridization Master Mix* to the thermocycler. Leave the Library Master Mix in the thermocycler. This will pre-warm the Hybridization Master Mix for 3 minutes at 65°C.

7. Once the thermocycler program reaches step 3, transfer the prepared *Capture Baits Master Mix* to the thermocycler. Leave all other tubes in the thermocycler. This will pre-warm the Capture Baits Master Mix for 2 minutes at 65°C.

8. While keeping the tubes at 65°C, perform the following steps quickly. Carefully pipette 9.5 µl of the *Library Master Mix* up and down three times then transfer to the *Capture Baits Master Mix*. Next, carefully pipette 10.5 µl of the *Hybridization Master Mix* up and down three times and transfer to the *Capture Baits Master Mix*. Mix by pipetting.

9. Hybridize the solution at 65°C for 16 hours. Depending on the application, hybridization time may need some optimization between 12 and 48 hours.

V. Recovery of Captured Targets

This step consists of recovering the captured targets from the hybridization solution. Targeted DNA sequences are hybridized to biotinylated RNA baits. RNA baits, either hybridized to a complementary DNA molecule or free, are pulled out of the hybridization solution by the means of streptavidin-coated magnetic beads. Beads are then washed to remove any non-specific carry over of DNA molecules.

Before starting

- **If higher wash stringency is required**, prepare a five fold dilution of Wash Buffer 2 by adding 340 µl Wash Buffer 2 to 1360 µl 0.1% SDS solution (**not included**). Prepare the 0.1% SDS solution from a 10% stock solution by adding 15 µl 10% SDS (HYB #4) to 1485 µl nuclease-free water.

- Preheat an aliquot (~1.7 mL per capture) of Wash Buffer 2 at 65°C in a water bath for **at least 1 hour**.

1. Transfer 50 µl of Dynabeads® MyOne™ Streptavidin C1 magnetic beads to a new 1.7 ml tube.

2. Pellet beads using a magnetic particle stand and discard the supernatant.

3. Wash the beads by adding 200 µl ***Binding Buffer*** and vortexing the tube for 5-10 seconds. Briefly centrifuge for ~5 sec and place on magnetic particle stand for two minutes to pellet the beads. Remove and discard supernatant.

4. Repeat step 3 twice for a total of three washes.

5. Resuspend the beads in 20 µl ***Binding Buffer*** and transfer the beads to a 0.2 ml tube. Transfer the tube to the thermocycler containing the hybridization solution set at 65°C and incubate for two minutes.

6. Transfer the entire volume of Binding Buffer/Beads to the hybridization solution from step 9 of Section III. Incubate for 45 minutes at 65°C in the thermocycler, mixing the solution every 10-15 minutes by pipetting up and down.

7. Quickly transfer the entire volume of Binding Buffer/Beads/hybridization solution to a 1.5 ml tube and immediately pellet the beads with the magnetic particle stand for two minutes and remove supernatant.

8. Add 500 µl of the (optionally diluted) ***Wash Buffer 2*** from 65°C to the beads and mix via pipetting. Incubate for **5** minutes at 65°C in a circulating water bath. Pellet beads with magnetic particle stand for two minutes and remove supernatant.

9. Repeat step 8 twice for a total of three 65°C washes. After the third wash make sure all additional buffer is removed by giving the tube a quick spin after the supernatant has been removed and re-pelleting the beads with the magnetic particle stand.

10. Resuspend the beads with 30 µl Molecular Biology Grade Water. Elution of the captured sample off the beads is not necessary.

Expected amounts of recovered material are very small and cannot be detected by spectrophotometry.

The sample can be stored at -20°C after this step, if necessary.

VI. Post-Capture Amplification

This step consists of amplifying the small amount of captured DNA recovered in the previous step in order to have enough material for sequencing. It is important to limit the number of cycles to get just enough material while minimizing PCR amplification bias. We recommend using the KAPA HiFi DNA Polymerase, which compare favorably to other DNA polymerases (Quail, et al., Nature 9:10-11 (2012)). **Other enzymes have not been tested for direct on-beads amplification.**

1. Prepare a 50 µl PCR reaction on ice in a 0.2 ml nuclease-free tube and mix by pipetting.

Component	Amount	Final Concentration
Nuclease-free water	18.5	
2X KAPA HiFi HotStart ReadyMix	25	1X
PCR primers mix (10 µM each)	1.5	0.3 µM
Captured Library$^{\Delta}$	5*	

Δ Pipet up and down to fully resuspend beads prior to transferring to amplification mix.

* The recommended capture library input amount is between 5 and 15 µl. Adjust the volume of water according to capture library input.

2. Place the tubes in a thermocycler and run the following program:

Step	Temperature	Time
1	98°C	30 seconds
2	98°C	20 seconds
3	See Appendix A	30 seconds
4	72°C	See *
5	Repeat step 2 through 4 for 8 to 14 cycles**	
6	72°C	5 minutes
7	4 °C	∞

* Extension time (Step 4) will depend on the genomic library average fragment size. Use 30 seconds for fragments shorter than 500 bp, 45 seconds for fragments with size between 500 and 700 bp and 1 minute for fragment sizes ranging from 700 bp to 1 Kb.

** Number of cycles should be adjusted to get the desired amount of material for sequencing. It is recommended to minimize PCR amplification.

3. Purify the PCR product using the QIAquick PCR Purification kit following the manufacturer's instructions.

   - Use 30 µl of Buffer EB (or Molecular Biology Grade Water) for the final elution step.

4. Measure the DNA concentration with a spectrophotometer or fluorometer.

VII. Appendix

A. Post-capture PCR Amplification Primers

The PCR primers to use for the post-capture amplification depend on the sequencing platform to be used. The sequence of the PCR primers should be obtained from your sequencer's representative.

The annealing temperature during the PCR amplification should be set 5°C below the lowest melting temperature (Tm) of the primers.

B. Thermocyclers

Recommendation: We have successfully tested and recommend the **BioRad C1000** and **S1000** thermocyclers with dual 48 blocks. They show minimal evaporation over a period of 72 hours compared to competitors.

Validation: Before performing the first hybridization, it is highly recommended to validate that the combination of thermocycler and tubes you are using will not allow more than 15% evaporation over the planned duration of the hybridization. This can be accomplished by incubating water (27 µl) in the thermocycler for the intended capture duration (24 - 48 hours) then measuring the loss of volume (want no greater than 4 µl loss).

Suggestions:

- A thermocycler with a heated lid to prevent condensation on the tube cap **MUST** be used for capture hybridization. We recommend adjusting the lid temperature to 10°C above hybridization temperature (75°C).
- It is important for the lid to be seated properly on the tube. Our recommendation for this is to place four tubes identical to the ones being used for capture hybridization in the corners of the thermocycler block.

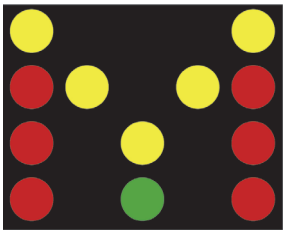
C. MSDS

To obtain an MSDS for a particular box, click on the relevant link below:

MYbaits Box #1, 50 reactions
http://www.mycroarray.com/msds/MYbaits-Box1-MSDS.pdf

MYbaits Box #2, 50 reactions
http://www.mycroarray.com/msds/MYbaits-Box2-MSDS.pdf

MYbaits Box #3, 8 reactions
http://www.mycroarray.com/msds/MYbaits-Box3-MSDS.pdf



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