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 1.3.8 - 06/14/2013

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Get the latest version at http://www.mycroarray.com/pdf/MYbaits-manual.pdf

For research use ONLY. Not intended for diagnostic use.

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. Then the DNA library is 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 genome.

How does it work?

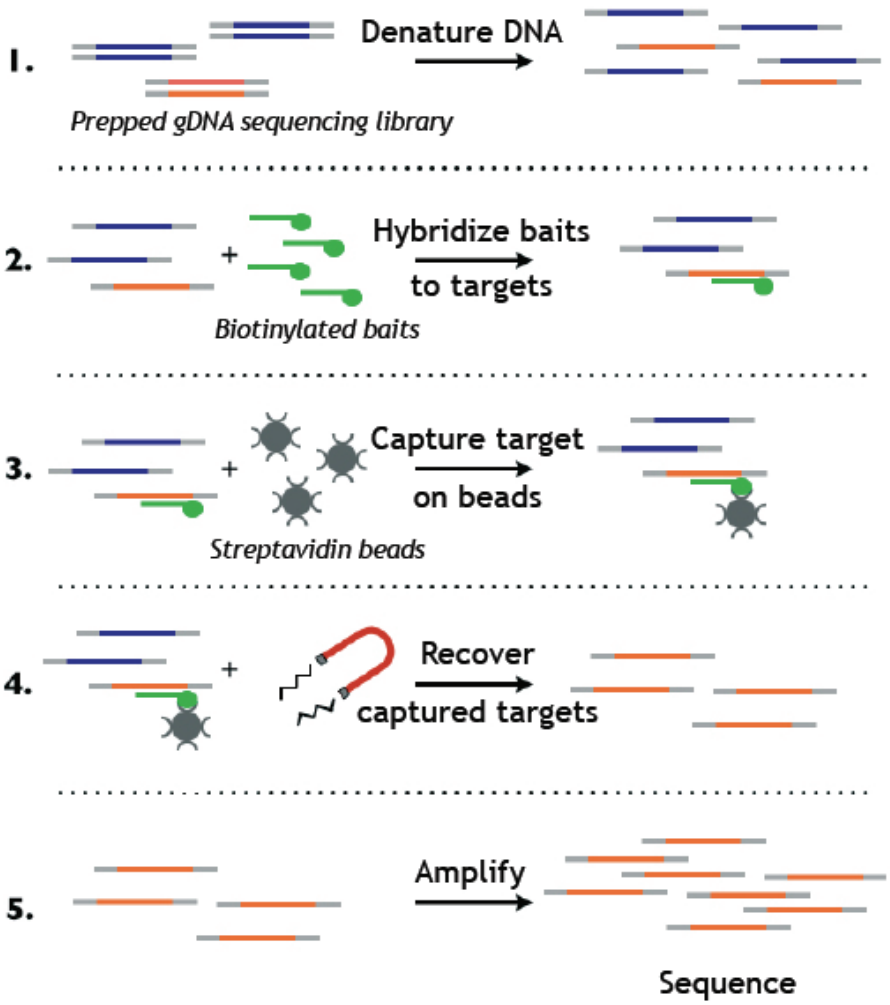
Our approach is based on the work of Gnirke et *al*. (Solution Hybrid Selection with Ultra-long Oligonucleotides for Massively Parallel Targeted Sequences. 2009. Nature Biotechnology 27(2):182-189).

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 have been fragmented, size purified and the sequencing adaptor ligated. 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.



II. 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	750 µl	1% 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.

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

- PCR primers compatible with the sequencing platform to be used (see Appendix A)
- Elution Buffer (see Appendix B)
- Nuclease-free Water
- Dynabeads® MyOne™ Streptavidin C1 (Invitrogen, # 650-01)
- Herculase II Fusion DNA Polymerase (Stratagene, #600677)
- QIAquick PCR purification Kit (Qiagen, #28704)

Required equipment and supplies

- BioRad C1000 Thermocycler or compatible thermocycler (see Appendix C)
- Non-stick, sterile, nuclease-free tubes compatible with the thermocycler
- Magnetic particle stand (Life Technologies™, #123-210, DynaMag™-2)
- Vortex mixer
- Water bath set at 65 °C
- Lab rotator (Thermo Scientific, #400110Q)

III. 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.

Before starting, equilibrate HYB #4 tube at room temperature to **fully dissolve SDS** that may have precipitated during storage at 4°C.

1. Set the following program on a thermocycler. (See Appendix C for recommended thermocyclers and validation procedure).

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

2. Prepare Library Master Mix in a nuclease-free tube and mix by vortexing. Set aside until step 5.
   Note: It may be necessary to concentrate the genomic library by reducing the volume using a SpeedVac in order to have 100 – 500 ng of library DNA in 3.4 µl before preparing the Library Master Mix.

Component	Amount (9 µl)
Block #1	2.5
Block #2	2.5
Block #3	0.6
Sequencing library (100 – 500 ng)	3.4

3. Prepare Hybridization Master Mix in a nuclease-free tube and mix by vortexing. Set aside until step 6.

Component	Amount (36.8 µl)
Hyb #1	20
Hyb #2	0.8
Hyb #3	8
Hyb #4	8

4. Prepare Capture Baits Master Mix in a nuclease-free tube and mix by pipetting. Set aside until step 7.

Component	Amount (6 µl)
Capture Probe (baits)	5
RNase Block	1

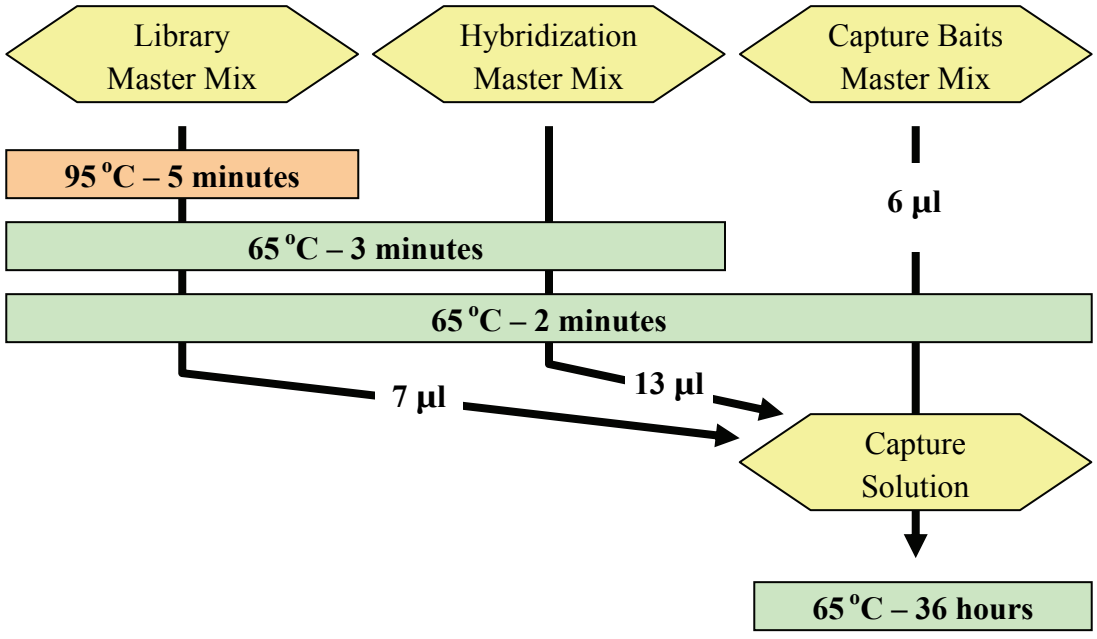
5. Transfer the tube containing the Library Master Mix to the thermocycler and start the program set in step 1. This will denature the DNA library for 5 minutes at 95°C.

6. Once the thermocycler program reaches step 2 temperature (65 °C), transfer the tube containing the 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 temperature (65 °C), transfer the tube containing the 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 tubes at 65°C, transfer 7 µl of Library Master Mix and 13 µl of Hybridization Master Mix to Capture Baits Master Mix and mix via pipetting.

9. Hybridize solution at 65°C for 36 hours. Depending the application, hybridization time may need some optimization between 24 and 48 hours.



IV. 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, equilibrate Wash Buffer 1 bottle at room temperature to **fully dissolve SDS** that may have precipitated during storage at 4°C and preheat Wash Buffer 2 at 65°C in a water bath for **at least 1 hour**.

1. Transfer 50 µl of MyOne Streptavidin C1 magnetic beads to a new 1.5 ml tube.
2. Pellet beads using a magnetic particle stand and discard the supernatant.
3. Add 200 µl Binding Buffer to beads to wash. Vortex tube for 5-10 seconds, place on magnetic particle stand for two minutes to pellet the beads and remove and discard supernatant.
4. Repeat step 3 twice for a total of three washes.
5. Resuspend the beads in 200 µl Binding Buffer.
6. Transfer the hybridization solution to the Binding Buffer/Beads and incubate 30 minutes at room temperature on a rotator. Pellet beads with magnetic particle stand for two minutes and remove supernatant.
7. Add 500 µl Wash Buffer 1 to the beads and briefly vortex to resuspend. Incubate 15 minutes at room temperature. Pellet beads with magnetic particle stand for two minutes and remove supernatant.
8. Add 500 µl 65°C Wash Buffer 2 to the beads and briefly vortex to mix. Incubate for 10 minutes at 65°C. 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 third wash make sure all additional buffer is removed.

V. Elution of Enriched Library

This step consists of releasing the captured DNA target molecules from the RNA baits. This is achieved by specifically degrading the RNA molecules by an alkaline treatment that will leave DNA molecules unaffected. All of the steps in this section are performed at room temperature.

The Elution Buffer is not provided with this kit. **Fresh** Elution Buffer should be prepared according to Appendix B.

1. Add 50 µl **freshly prepared** Elution Buffer to beads from step 9 of Section IV.
2. Vortex for 5-10 seconds to mix.
3. Incubate 10 minutes at room temperature.
4. Pellet the beads and transfer supernatant to a tube containing 70 µl Neutralization Buffer.

VI. Enriched Library Cleanup

This step consists of removing extra salts added during the release of the captured DNA molecules. It also permits the captured DNA to be concentrated.

1. Concentrate and desalt the solution using a QIAquick PCR Purification column following manufacturer's manual. The binding buffer should contain the pH indicator and pH should be adjusted if necessary. Elute with 30 µl buffer EB (or Molecular Biology grade water).

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

Note: The sample can be stored at -20 ^{o}C after this step if necessary.

VII. 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 Herculase II Fusion DNA Polymerase, which compare favorably to other DNA polymerases (Dabney and Meyer, BioTechniques 52:87-94 (February 2012)).

1. Prepare PCR Master Mix on ice in a nuclease-free tube and mix by pipetting.

Component	Amount (50 µl)
Nuclease-free water	32.5
5x Herculase II Buffer	10
dNTP mix (25mM each)	0.5
PCR primers mix (10 µM each)	1
Herculase II Fusion DNA Polymerase	1
Captured Library	5

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 14 times	
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.

3. Purify the PCR product using 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.

VIII. Appendix

A. Post-capture PCR Amplification Primers

PCR primers to use for the post-capture amplification depend on 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 Tm of the primers.

B. Elution Buffer

The recommended Elution Buffer is a solution of sodium hydroxide (100 mM). Due to the limited shelf life of diluted NaOH solutions, we do not include it in our kit. We recommend preparing fresh Elution Buffer just prior to usage.

Prepare a 10M NaOH stock solution in RNAse/DNAse free water using NaOH pellets (high purity or molecular biology grade). **This solution should be discarded after 1 week.**

Prepare the Elution Buffer (100 mM NaOH) from the stock solution using RNAse/DNAse free water. **This solution should be discarded after 1 day.**

C. Thermocyclers

A thermocycler with a heated lid to prevent condensation on the tube cap **MUST** be used for capture hybridization. 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.

Before performing the first hybridization please 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.

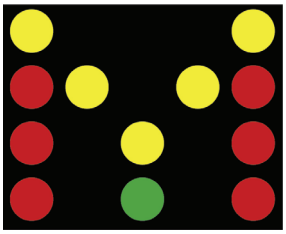
D. MSDS

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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