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v 1.6

Target Enrichment of Illumina Libraries

The goal here is to adapt the Mycroarray Mybaits enrichment kits for DNA libraries prepared using iTru (or TruSeq/TruSeq-style/Illumina Nextera) adapters and common library preparation kits. We deviate from these standard protocols by incorporating custom adapter blockers to the enrichment process. Although we are doing this, everything else relative to the hybridization is "normal." However, Mycroarray can now synthesizes these blockers for you.

License

Bibliography

Agilent Technologies, Inc.: **Sure Select Target Enrichment Protocol v1.0**. 2009:1-42.

Blumenstiel B, Cibulskis K, Fisher S, DeFelice M, Barry A, Fennell T, Abreu J, Minie B, Costello M, Young G, Maquire J, Kernytsky A, Melnikov A, Rogov P, Gnirke A, Gabriel S: **Targeted exon sequencing by in-solution hybrid selection.** Curr Protoc Hum Genet 2010, **Chapter 18**:Unit 18.4.

Mycroarray: **Mybaits In-Solution Sequence Capture for Targeted High-Throughput Sequencing User Manual v3.0**. 2015:1-15.

Tewhey R, Nakano M, Wang X, Pabón-Peña C, Novak B, Giuffre A, Lin E, Happe S, Roberts DN, LeProust EM, Topol EJ, Harismendy O, Frazer KA: **Enrichment of sequencing targets from the human genome by solution hybridization**. Genome Biol 2009, **10**:R116.

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Versions

- 1.0 Original
- 1.1 Change blocking adapters => 2 pairs of TruSeq primers versus 4. The new blockers incorporate inosine to bind to a 10 nt index sequence (Nov. 19, 2011).
- 1.2 Cleanup to standardize and clarify (Mar. 6, 2012)
- 1.3 Change probe concentration to 2X of what we originally used. Update block mix with statement on using custom Cot-1 (e.g. chicken when working with birds). Title blocker section for TruSeq adapters. Add an Illumina Nextera blocker section (Sep. 11, 2012)

. 1.4 Update Nextera blockers to give full-length blocking sequence with Inosines as universal blocker. Recommend final AMPure cleanup at 1.0X versus 1.8X. This produces larger (on average) contigs following assembly (March 28, 2013).
. 1.5 Added on-bead PCR changes for post-enrichment amplification.
. 1.6 changed for undiluted Mybaits kits (Mycroarray) and iTru tags

Materials

- **DNA libraries at ~72 ng/uL**
- **SureSelect or MYbaits or IDT bait library (stored at -80 C)**
- **SureSelect or MYbaits hybridization reagents (Box 1 [-20 C] and Box 2 [Room Temperature]) or equivalent hybridization solutions (see below)**
- **500 uM adapter oligo mix (iTru blockers) (Mycroarray can customize this as Block #3)**
- **Strip tubes and caps or plates and rubber mats**
- **AMPure XP or Serapure substitute (home-brew AMPure)**
- **Life Technologies Dynabeads MyOne Streptavidin C1 (Life Technologies 65001)**

IF you need additional hybridization reagents (no longer necessary with MYbaits kits):

- **20 X SSPE (Life Technologies AM9767) (SureSelect Hyb #1)**
- **0.5 M EDTA (Life Technologies AM9261) (SureSelect Hyb #2)**
- **50 X Denhardt's Solution (Life Technologies 750018) (SureSelect Hyb #3)**
- **10 % SDS (Life Technologies AM9822) (SureSelect Hyb #4)**
- **Human Cot-1 DNA (Life Technologies 15279-101) (SureSelect Block #1)**
- **Salmon sperm (Life Technologies 15632-011) (SureSelect Block #2)**
- **Superase-IN (Life Technologies AM2694) (SureSelect RNAse Blocker)**
- **1 M Tris HCl (Amresco E199) (SureSelect neutralization buffer)**
- **0.1 N sodium hydroxide (Fisher AC12419-0010) (SureSelect elution buffer)**
- **20 X SSC (Life Technologies AM9770) (for wash buffer)**
- **5 M NaCl (Amresco E529-500) (for binding buffer)**

I. Blocking Mix

The blocking mix is one of the most critical components during the enrichment. Without it, you run the risk of adapter-ligated DNA hybridizing together end-to-end. You pull out what you want, but lots of other stuff you don't want comes along in a big daisy chain. Thus, the purpose of Adapter Blocking Mix is to hybridize to the ends of adapter ligated DNA **before** you add your probe mix. As such, the blocking mix should match the adapters you've added to your libraries.

A. 10 nucleotide TruSeq library blocker (500 uM each primer, final concentration)

If you are working with standard Illumina barcodes, you kit may contain the correct blockers for the sequencing adapters. If you are using longer indexes (e.g. 10 nt),

you will need custom blockers. **The blockers below assume 10 nt indexes. Adjust the number of Inosines (I) to reflect your index length.**

1. You need the following oligos (250 nM synthesis) 5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT - 3' 5'-CAAGCAGAAGACGGCATACGAGATIIIIIIIIIIGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT - 3'
2. Hydrate the above with ddH2O or TLE to 1000 uM (1 times the number of nMol)
3. Combine 50 uL of each blocker in a 1.5 mL tube
4. This is now equivalent to Block #3

B. 8 nucleotide Nextera library blocker (500 uM each primer, final concentration)
If you are enriching libraries prepared using either of Illumina's Nextera Kits (Illumina Nextera or Illumina Nextera XT), then you need to block indexes on both ends of the library fragments. Nextera indexes are 8 bp long, each. **The blockers below assume 8 nt indexes (the standard Nextera index length).**

1. You need the following oligos (250 nM synthesis)
   5' – - 3' 5' – - 3'
2. Hydrate the above with ddH2O or TLE to 1000 uM (1 times the number of nMol)
3. Combine 50 uL of each blocker in a 1.5 mL tube
4. This is now equivalent to Block #3

AATGATACGGCGACCACCGAGATCTACACIIIIIIIITCGTCGGCAGCGTCAGATGTGTATAAGAGACAG

CAAGCAGAAGACGGCATACGAGATIIIIIIIIGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG

C. 8-nucleotide iTru outward blockers – ***WE ARE CURRENTLY USING THIS SYSTEM***
You will need the following oligos (250nM synthesis – OR Mycroarray will custom synthesize these blockers for you in the kit, although they are inward facing)

iTru_R1i8P5-2_outward_blocker
5'GATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTIIIIIIIIGTGTAGATCTCGGTGGTCGCCGTATCAT 3'
iTru_R2i8P7_ outward_blocker
5'AGATCGGAAGAGCACACGTCTGAACTCCAGTCACIIIIIIIIATCTCGTATGCCGTCTTCTGCTTG 3'

1. Hydrate the above with ddH2O or TLE to 1000 uM (1 times the number of nMol)
2. Combine 50 uL of each blocker in a 1.5 mL tube
3. This is now equivalent to Block #3 (if you ordered these from Mycroarray as part of the kit, it will just be Block #3)

II. Probe Mix

With the Mycroarray MYbaits kits, there is no longer a need to dilute, so we use the kit as-is. The RNA probes are in Box #3 and stored at -80C. Always work with the probes on **ICE.**

Steps (Day One)

1. Previous day: If using strip tubes, you may want to add 27 uL ddH2O to a set of strip tubes, and ensure you can incubate for 24 h at 65 C with minimal evaporation. Evaporation should not be more than about 15%. Assuming you have tubes that meet these standards, you are ready to rock. I recommend using PCR plates and sealing with rubber/silicone mats – this is far more secure than strip tubes. You may want to test in a similar fashion.
2. Previous day: Your sheared, amplified, and tagged library preps at 72 ng/uL are your **SOLID GOLD**.
3. Set a water bath or incubator to 65 C.
4. Spin up a thermal cycler.
5. Mix the following at room temperature. Do not place on ice. This is the **Hybridization Buffer**. Thoroughly vortex Hyb1 before use and bring Hyb4 to room temperature before use. You may need to incubate at 65 C to ensure all components are in solution. Calculate an appropriate master mix (the volumes already account for pipetting error).

Reagent	**1X (uL per reaction)**
Hyb1	9.0
Hyb2	0.5
Hyb3	3.5
Hyb4	0.5
Total	**13.5**

6. If precipitate forms, warm the buffer mix at 65 C in the water bath or incubator.
7. Pipet 12.5 uL of **Hybridization Buffer** into wells of a 96-well plate, up to the number of libraries you are preparing. Designate each well with the **Hybridization Buffer (HYB)** mixture using a colored sharpie. See appendix for plate map suggestions.
8. Seal the wells of the tubes with a sealing mat and tap down. Keep aside at room temp.
9. Mix the following mastermix in a 1.5 mL tube (calculate the appropriate size based on your number of libraries). This is the **Capture library (keep on ICE)**:

Reagent	**1X (uL per reaction)**
RNase Block	1.0
Bait Library	5.5
Total	**6.5**

10. Place this mixture (still in the master mix tube) back on **ICE!**

11. In a 1.5 mL tube, prepare a master-mix of blocking reagents (calculate the appropriate size based on your number of libraries, already includes pipetting error) and mix by vortexing. Block #3 is your tube of library specific blocking oligos. If you have custom Cot-1 DNA for your species, then substitute that Cot-1 for Block #1 below. This mixture is the **Block Mix**:

Reagent	**1X (uL per reaction)**
Block #1	2.5
Block #2	2.5

****REPLACE Block#2** with ddH20 for parrotfish or anything closely related to Salmonidae**

Block #3 Custom	0.5
Total	**5.5**

12. Add 5.0 uL of **Block Mix** to wells in a different 96-well plate up to the number of libraries you are enriching. You will need two run two thermalcyclers for the pre-incubation steps if you have more than 48 samples you are preparing. Mark the locations of the Block Mix with a second colored sharpie.
13. Add 7.0 uL of each 72 ng/uL library preparation (**SOLID GOLD**) to the **Block Mix**-filled wells, up to the number of libraries you are enriching. Mix by pipetting up and down.
14. This is the **Block Mix + DNA Library (LIB)** and should total 12uL. Seal and set aside.
15. Add 6.0uL of the **Capture Library** (Baits + RNA mastermix block still sitting on ice) to each **HYB** (Hybridization Buffer wells) and mix by pipetting up and down. Those wells should now total 18.5uL each. Tap the contents to the bottom if needed.
15. Seal the plates with a rubber/silicone mats.
16. Setup the following thermal profile on a thermal cycler (lid at 105C):

95C	5 minutes
65C	5 minutes (hybridization temp)
65C	24 hours (hybridization temp)

17. Place the **LIB** plate in a preheated (95C) thermalcycler. Start the above cycling profile.
18. After the cycler progresses to step 2 (65C) hit pause, open the cycler and remove the sealing mat carefully.
19. Transfer the **HYB** aliquots to unoccupied wells in the plate in the thermalcycler using a multichannel pipettor. If you have more than 48 libraries, the whole **HYB** plate would go on a second thermalcycler set to 65C.
20. Unpause the machine and allow the 5-minute step to complete.
21. Allow the cycler to proceed through Step #3, then hit pause.
22. Open the cycler again, and remove the sealing mat carefully.

23. Using a multichannel pipettor, transfer the contents of the **HYB** wells (18uL) into the **LIB** wells.
24. Unpause the machine and allow the program to proceed, incubating the **HYB+LIB Mix** for 24 hours at 65 C.

Steps (Day Two)

1. Prepare **Wash Buffer #2.2** in a 50mL Falcon tube(s). Scale up or down appropriately for number of capture reactions you've run. Bring Hyb4 to room temperature before use. Wash Buffer#2.2 can be stored at 4C for 1 month. For 33 reactions:

Reagent	
Hyb #4	400 uL
ddH20 water	39.6 mL
Wash Buffer 2	10 mL
Total	50 mL

2. Heat Wash Buffer 2.2 to the hybridization temperature in a water bath for at least **45 minutes** before use
3. Resuspend Dynal beads vigorously until they resemble chocolate milk.
4. For each hybridization, aliquot 30 uL of Dynal beads into sterile 1.5 mL tubes.
5. Place each tube onto the rare-earth magnet stand and allow the beads to separate until the liquid is clear (~3 min).
6. Pipette off and discard supernatant.
7. Add 200 uL **Binding Buffer** to each 1.5 mL tube containing Dynal beads.
8. Vortex beads + buffer 5 seconds.
9. Place beads + buffer into magnetic separator.
10. Remove and discard supernatant.
11. Add 200 uL **Binding Buffer** to each 1.5 mL tube containing Dynal beads.
12. Vortex beads + buffer 5 seconds.
13. Place beads + buffer into magnetic separator.
14. Remove and discard supernatant.
15. Add 200 uL **Binding Buffer** to each 1.5 mL tube containing Dynal beads.
16. Vortex beads + buffer 5 seconds.
17. Place beads + buffer into magnetic separator.
18. Remove and discard supernatant.
19. Resuspend beads in 70 uL **Binding Buffer**.

NOTE: These wash steps can be done at larger volumes up to 210uL of beads at a time in 1.5mL tubes. Use 1400 uL volumes for the wash, resuspend in 490 uL of Binding Buffer, and aliquot out the 70uL for each capture reaction at the end of the three washes

20. Heat the 70uL aliquot tubes of Dynal beads to hybridization temperature (65C) for 2 minutes.
21. Add contents of each capture reaction from Day 1 to corresponding 1.5 mL tubes containing washed Dynal beads. Mix by pipetting.
22. Incubate beads + enrichment mixture on a Nutator or shaking incubator for 30 minutes at room temperature. You can also shake these gently by hand every 5 minutes.

23. Tap contents displaced by agitation down to the bottom of the tubes. Use a VERY slow, short spin if necessary.
24. Place beads + buffer into magnetic separator and let sit for 2-3 min.
25. Remove Serapure (AMPure replacement) from the refrigerator, and allow to warm to room temperature.
26. Remove the supernatant from the beads in the separator.
27. Add 500 uL of warmed **Wash Buffer #2.2** to each sample.
28. Vortex for 5 seconds, briefly spin down in a centrifuge.
29. Incubate for 10 minutes at hybridization temperature (65C) in a shaking incubator or mix by agitating by hand every 5 minutes.
30. Centrifuge briefly at < 500 RPM.
31. Place beads + buffer into magnetic separator.
32. Remove the supernatant.
33. Add 500 uL of pre-warmed (65 C) **Wash Buffer #2.2**.
34. Vortex for 5 seconds.
32. Incubate for 10 minutes at hybridization temperature (65C) in a shaking incubator or mix by agitating by hand every 5 minutes.
33. Centrifuge briefly at < 500 RPM.
34. Place beads + buffer into magnetic separator.
35. Remove the supernatant.
36. Add 500 uL of pre-warmed (65 C) **Wash Buffer #2.2**.
37. Vortex for 5 seconds.
38. Incubate for 10 minutes at hybridization temperature (65C) in a shaking incubator or mix by agitating by hand every 5 minutes.
39. Centrifuge briefly at < 500 RPM.
40. Place beads + buffer into magnetic separator.
41. Remove the supernatant.
42. Ensure all **Wash Buffer #2.2** is removed (without touching the pellet) and let beads dry in magnetic separator for 5 minutes.
43. Remove beads from magnetic separator.
44. resuspend beads with 30uL Tris/TWEEN solution (10mM Tris CL 0.05% TWEEN20 pH 8) and mix well. This product is **SUPER-DUPER SOLID GOLD**.

******ALTERNATIVELY*** the bead bind and wash steps can be done in capped strip tubes on a 96-well magnetic plate. All volumes remain the same – incubations can take place in a thermalcycler (lid temp at least +10C of incubation temp) and agitations done by hand at 10-minute intervals. Strips should be spun down briefly/gently using a strip tube centrifuge.***

45. Proceed to the post-hybridization limited cycle PCR recovery (Illumina-iTru-post-hybridization document). You will use 15 uL of the liquid from step 44 in the post- enrichment amplification. It will be brown. That's okay.

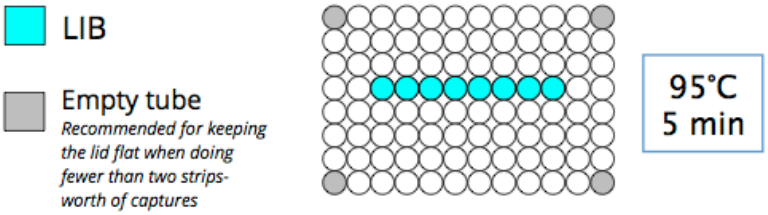
APPENDIX:

Suggested Plate Orientations (from MYbaits Protocol)

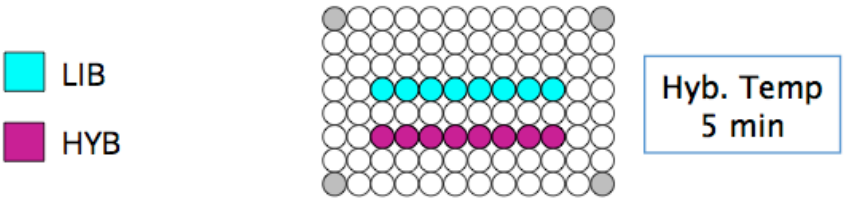
1.3 Reaction Assembly

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

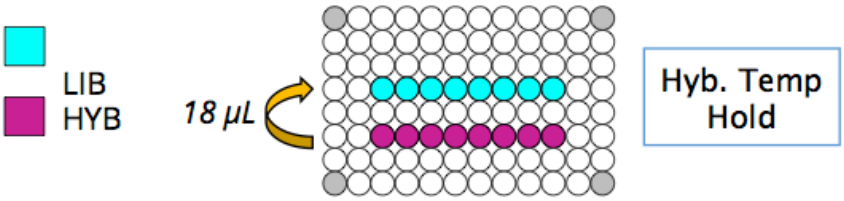
   Double-check that the lid temperature is programmed to stay at 105°C, or at least 10°C above each step temperature, to keep evaporation to a minimum.



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).