

Target Enrichment of Illumina Libraries

The goal here is to adapt the SureSelect/Microarray enrichment kits for DNA libraries prepared using 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. In addition to making adjustments for adapters, we are adjusting the protocol for a kit composed of essentially 25X bait sets, rather than the “standard” 1X bait sets of probes. For instance, the Agilent kit contains 55,000 probes. We synthesized ~2,500 probes 25X. This essentially means that each probe is present 25X (or a similar concentration) in solution, so we need to dilute the SureSelect library to 1/25X. **Only dilute your probe mix if you have taken this approach to synthesis.** Although we are doing this, everything else relative to the hybridization is “normal”. Now, if you put the numbers together, you will realize that most capture kits do not provide sufficient hybridization solutions because we have many more units of enrichment that we can do (in the case of SureSelect, for a 10 individual kit, we now get $10 * 25X = 250$ individuals). Because everything about enrichments is the same, except for the library concentration, this means we’ll run out of all the other reagents we need. Not to fret – we know what they are from Gnirke et al. 2009 and Blumenstiel et al. 2010.

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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.

Materials

- DNA libraries at ~147 ng/uL
- SureSelect or MySelect or IDT bait library (stored at -80 C)
- SureSelect or MySelect hybridization reagents (Box 1 [-20 C] and Box 2 [Room Temperature]) or equivalent hybridization solutions (see below)
- 500 uM adapter oligo mix (IDT DNA) (SureSelect Block #3; see below)
- 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)

Assuming you need additional hybridization reagents:

- 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, your 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' - CAAGCAGAAGACGGCATACGAGATIIIIIIIIIGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT - 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' - AATGATACGGCGACCACCGAGATCTACACIIIIIIIIITCGTCGGCAGCGTCAGATGTGTATAAGAGACAG - 3'
5' - CAAGCAGAAGACGGCATACGAGATIIIIIIIIIGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG - 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

II. Probe Mix

Because most enrichment kits target more sites than we target, we generally dilute the resulting kit to enrich more samples than the standard amount in a given kit.

A. Agilent SureSelect

Depending on how you ordered your probes, they can vary in concentration. For birds, we have ordered the 2,200 probe set printed 25 times per “array” to fill out the array (55,000 spots / 2,200 probes). This means that every 1X aliquot of SureSelect probe mix contains 25X the probes that we actually want. When we target UCEs, we’ve discovered it’s best to use 2X probe mix with each sample or pool of samples. This is an increase from what we were originally using and is colloquially known as “2X” probe mix.

To enrich 1 sample:

1. Remove 0.5 uL of MySelect probe mix
2. Add this to 4.5 uL of RNase free ddH₂O

To enrich 12 samples:

1. Remove 6.0 uL of MySelect probe mix
2. Add this to 54.0 uL of RNase free ddH₂O

To enrich 24 samples:

1. Remove 12.0 uL of MySelect probe mix
2. Add this to 108.0 uL of RNase free ddH₂O

B. Mycoarray MySelect

Similarly, if you’ve printed ~5,000 probes to fill out a 55,000 probe array, then each probe is represented approximately 10 times, so you have a 10X solution of probe mix. For a single sample, you’ll then want to dilute each aliquot of probes by a factor of 5. This is an increase from what we were originally using and is colloquially known as “2X” probe mix.

To enrich 1 sample:

1. Remove 1.0 uL of MySelect probe mix
2. Add this to 4.0 uL of RNase free ddH₂O

To enrich 12 samples:

1. Remove 12.0 uL of MySelect probe mix
2. Add this to 48.0 uL of RNase free ddH₂O

To enrich 24 samples:

1. Remove 24.0 uL of MySelect probe mix
2. Add this to 96.0 uL of RNase free ddH₂O

III. Other buffers needed if you are diluting probe mix

C. Binding buffer

2. Assemble the following components in a 50 mL sterile conical tube (note units):

10	mL	5.0 M NaCl
500	uL	1.0 M Tris-HCl (pH 7.5)
100	uL	0.5 M EDTA
39.4	mL	ddH ₂ O
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50.0	mL	Total Volume

D. Wash Buffer #1

1. Assemble the following components in a 50 mL sterile conical tube (note units):

2.5	mL	20X SSC
0.5	mL	10% SDS
47	mL	ddH ₂ O
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50	mL	Total Volume

E. Wash Buffer #2

1. Assemble the following components in a 50 mL sterile conical tube (note units):

250	uL	20X SSC
500	uL	10% SDS
49.25	mL	ddH ₂ O
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50	mL	Total Volume

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 mats – this is far more secure than strip tubes. You may want to test in a similar fashion.
2. Previous day: If your library preps are < 147 ng/uL, dry down samples from library preparation using a vacuum concentrator on low heat (< 45 C) and reconstitute samples to 147 ng/uL. We generally quantify before lypholization using the Qubit, measure the volume, dry down, and re-hydrate to 147 ng/uL based on pre-lypholization volume and concentration values.
3. Set a water bath or incubator to 65 C.
4. Spin up a thermal cycler.
5. Mix the following at room temperature (includes excess for pipetting error). Do not place on ice. This is the **Hybridization Buffer**. You may need to incubate at 65 C to ensure all components are in solution.

Reagent	1X (uL)
Hyb1 (20X SSPE)	25
Hyb2 (0.5 M EDTA)	1
Hyb3 (50X Denhardt's)	10
Hyb4 (10% SDS)	13
Total	49

6. If precipitate forms, warm the buffer mix at 65 C in the water bath.
7. Pipet 40 uL of **Hybridization Buffer** into each well of a strip tube, up to the number of libraries you are preparing. Label this strip tube as “Hyb Buffer”.
8. Seal the wells of the tubes with caps and spin down. Keep at room temp.

9. Although we are capturing < 3.0 Mb, we've been following the instructions for capturing ≥ 3.0 Mb, because it keeps protocols aligned btw. SureSelect and MySelect¹. Mix the following in a 1.5 mL tube (includes excess) – this is the **RNase Block Mix**:

Reagent	1X (uL)
RNase Block (25%)	1
Nuclease-Free Water	3
Total	4

10. Place a strip tube (or a PCR plate that seals with strip caps) *on ice* and add 2 uL of **RNase Block Mix** to each well.
11. Add 5 uL of (2X) probe library to each well of the strip tube, up to the number of wells needed. See Solutions II.A or Solutions II.B for what constitutes “2X” probe mix.
12. Mix by pipetting.
13. Seal the wells of the tubes with caps, spin down, return to ice. This is the **Capture Library**.
14. In a 1.5 mL tube, prepare a master-mix of blocking reagents (includes excess) and mix by vortexing. The “Custom” Block #3 is equivalent to the blocking oligos listed in “Solutions” above. 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)
Block #1 (Green)	3
Block #2 (Blue)	3
Block #3 (Brown)/Custom	0.7
Total	6.7

15. In a strip tube you tested for 24-hour incubations, add 3.4 uL of each 147 ng/uL library preparation, up to the number of libraries you are enriching.
16. Add 5.6 uL of **Block Mix** to each well and mix by pipetting. This is the **Block Mix + Library**.
17. Seal the wells of the tube with caps.

¹ See add-ons for the steps mimicking the SureSelect protocol for capturing < 3.0 Mb, if interested.

18. Setup the following thermal profile on a thermal cycler:

95 C for 5 minutes	(Step #1: Block mix denature)
⇒ 65 C for 1 minutes	(Step #2: Hyb strip load)
⇒ 65 C for 5 minutes	(Step #3: Hyb strip warm)
⇒ 65 C for 1 minutes	(Step #4: Capture library load)
⇒ 65 C for 2 minutes	(Step #5: Capture library warm)
⇒ 65 C for 3 minutes	(Step #6: Xfer Block and Hyb to Capture Library)
⇒ 65 C for 24 hours	(Step #7: Hybridization)
⇒ 65 C hold	

19. Place the strip tube containing the **Block Mix + Library** into column 1 of the thermal cycler, and start the protocol above. Incubate the **Block Mix + Library** at 95 C for 5 minutes during Step #1.
20. During the 1 minute load time (Step #2), place the strip tube containing the **Hybridization Buffer** into column 2 of the thermal cycler. Allow the cycler to proceed through Step #3, incubating the **Hybridization Buffer** at 65 C for 5 minutes.
21. During the 1 minute load time (Step #4), place the strip tube containing the **Capture Library** into column 3 of the thermal cycler. Allow the cycler to proceed through Step #5.
22. At Step #6, open the lid of the thermal cycler, very carefully remove the caps from all 3 strip tubes.
23. Transfer 13 uL of **Hybridization Buffer** (column #2) to the **Capture Library** (column #3), using a multichannel pipet and filter tips (on all 8 or 12 spots) for > 2 samples.
24. Mix by pipetting.
25. Set a pipet on 10 uL and transfer the entire contents of the **Block Mix** (column #1) to the **Capture Library** (column #3).
26. Mix by pipetting.
27. Remove the strip tubes from columns #1 and #2.
28. Apply a *new* set of strip caps to column #3 taking care to seal the tubes extremely well. Alternatively, you can run the hybridizations in a 96-well plate with a rubber mat. We have had better success using plates with rubber mats for long hybridizations.
29. Allow the cycler to proceed through Step #7, incubating the **Block-Hyb-Probe-DNA Mix** for 24 hours at 65 C.

Steps (Day Two)

1. Prepare several 750 uL aliquots of **Wash Buffer #2** in 1.5 mL microtubes, 3 aliquots per sample and incubate these at 65 C in a water bath, heat block, or shaking heat block.

2. Resuspend Dynal beads vigorously until they resemble chocolate milk.
3. For each hybridization, aliquot 50 uL of Dynal beads into sterile 1.5 mL tubes.
4. Add 200 uL **Binding Buffer** to each 1.5 mL tube containing Dynal beads.
5. Vortex beads + buffer 5 seconds.
6. Place beads + buffer into magnetic separator.
7. Remove and discard supernatant.
8. Add 200 uL **Binding Buffer** to each 1.5 mL tube containing Dynal beads.
9. Vortex beads + buffer 5 seconds.
10. Place beads + buffer into magnetic separator.
11. Remove and discard supernatant.
12. Add 200 uL **Binding Buffer** to each 1.5 mL tube containing Dynal beads.
13. Vortex beads + buffer 5 seconds.
14. Place beads + buffer into magnetic separator.
15. Remove and discard supernatant.
16. Resuspend beads in 200 uL **Binding Buffer**.
17. Add contents of each strip-tube well to a *different* 1.5 mL tube containing washed Dynal beads.
18. Invert beads + enrichment mixture several times.
19. Incubate beads + enrichment mixture on a Nutator or equivalent for 30 minutes at room temperature. You can also shake these gently by hand.
20. Centrifuge briefly at < 500 RPM.
21. Place beads + buffer into magnetic separator.
22. Remove AMPure from the refrigerator, and allow to warm to room temperature.
23. Remove the supernatant from the beads in the separator.
24. Add 500 uL of **Wash Buffer #1** to each sample.
25. Vortex for 5 seconds.
26. Incubate for 15 minutes at room temperature, vortexing every 5 minutes.
27. Centrifuge briefly at < 500 RPM.
28. Place beads + buffer into magnetic separator.
29. Remove the supernatant.
30. Add 500 uL of pre-warmed (65 C) **Wash Buffer #2**.
31. Vortex for 5 seconds.

32. Incubate the sample at 65 C in a water bath/heat block/shaking bath/shaking heat block for 10 minutes, vortexing very 3-4 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**.
37. Vortex for 5 seconds.
38. Incubate the sample at 65 C in a water bath or on a heat block for 10 minutes, vortexing very 3-4 minutes.
39. Centrifuge briefly at < 500 RPM.
40. Place beads + buffer into magnetic separator.
41. Remove the supernatant.
42. Add 500 uL of pre-warmed (65 C) **Wash Buffer #2**.
43. Vortex for 5 seconds.
44. Incubate the sample at 65 C in a water bath or on a heat block for 10 minutes, vortexing very 3-4 minutes.
45. Centrifuge briefly at < 500 RPM.
46. Place beads + buffer into magnetic separator.
47. Remove the supernatant.
48. Ensure all **Wash Buffer #2** is removed and let beads dry in magnetic separator for 5-10 m.
49. Remove beads from magnetic separator.
50. Add 31 uL ddH₂O to the bead and mix well.
51. Proceed to the post-hybridization limited cycle PCR recovery (illumina-seqcap-post-hybridization document). You will use 15 uL of the liquid from step 50 in the post-enrichment amplification. It will be brown. That's okay.