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V2.5

Post-hybridization Amplification Using Illumina Libraries

The goal here is to use a limited-cycle PCR to amplify the relatively small amount of enriched product (the "**super-duper-solid-gold**" that we get out of the target enrichment process) into a reasonable amount (ideally about 2 ng/uL). We use master mixes here because you want to get this right, the first time.

License

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Materials

- NEB Phusion Taq (master mix) or **Kapa HiFi HotStart Taq ReadyMix** (Kapa Library Amp Kit KK2612)

- Amplification primers for Illumina TruSeq and iTru adapters P5 and P7 (includes phosphorothioate bonds) in mix of 5 uM each of TruSeq Forward and Reverse primers Upper5'–AATGATACGGCGACCACCGAGA*T-3' Lower 5' – CAAGCAGAAGAC GGCATACGAGA*T- 3'

- AMPure XP beads or Serapure substitute

- Rare-earth magnet stand (Ambion AM10055 or NEB S1506S) or plate (Agencourt SPRIPlate 96R - Ring Super Magnet Plate Beckman Coulter A32782)

Changelog

- v2.0 (6/7/2012) - Removed all the other amplification mixes – settled on 2

- v2.0 (6/7/2012) - Slightly modified AMPure steps to be in line with other protocols

- v2.1 (9/11/2012) – Change suggested cycle number to 18

- v2.2 (10/19/2012) – Make primer concentrations the same for Phusion
- v2.4 (11/3/2014) - Reduce PCR cycle number and range of values
- v2.5 (02/09/2015) – adjust for iTru library prep and new Kapa kits

Steps

It is possible to use different polymerases/master mixes/kits. You may want to set up several reactions – a test reaction or two at 25 uL total volume to test amplification and a "final" large 50 uL amplification that will be the template you carry over for sequencing.

At Bowdoin, we are using the Kapa Library Amp kit (HiFi HotStart ReadyMix)

Kapa HiFi HotStart Master Mix (50 uL reaction)

1. Setup the following PCR reactions in plate or strip-tube format. You **can** master mix these for multiple tubes/reactions:

Reagent	**1X (uL per reaction)**
ddH2O NF	5.0
2X Kapa HiFi HotStart ReadyMix	25.0
P5 primer at 10uM	2.5
P7 primer at 10uM	2.5
master mix Total	35.0
on-bead enriched library (SDSG)	15.0
Total	50.0

2. Cycle using the following. You may need to adjust or optimize cycle number (might consider 12-16 cycles. Do not go over 18-20 cycles if possible.):

iTruXL profile name
98 C for 2 minutes =>
12-16 cycles of:
98 C for 20 seconds
60 C for 30 seconds
72 C for 60 seconds =>
72 C for 5 minutes =>
10 C

Post-PCR purification

3. Allow Serapure beads to warm to room temperature for 30 minutes. Vortex or shake Serapure beads until they are homogenous and resemble well-mixed

chocolate milk.

4. Prepare sufficient, **fresh** 70% EtOH for each of the samples. You need about 1000 uL each sample. Freshness of EtOH is critical to Serapure success.

5. Transfer your PCR mix (which should be brown because it contains streptavidin beads) to a clean 1.5 mL tube.

6. Place 1.5 mL tube on magnetic separator until solution is clear.

7. Transfer supernatant to a new 1.5 mL tube. You can throw away the tube that contains the remaining streptavidin beads.

8. In a 1.5 mL tube, combine PCR product and 90 uLSerapure beads (1.8X). Mix by pipetting or vortexing.

9. Incubate the mixture for 5 minutes at room temperature.

10. Move 1.5 mL tubes to a rare-earth magnet stand.

11. Let sit for about 3 minutes or until solution is clear.

12. Using a separate filter-tip for each 1.5 mL tube, aspirate liquid from tubes and discard.

13. Add 200 uL 70% EtOH to each tube in the stand.

14. Incubate 30 seconds.

15. Using a separate filter-tip for each 1.5 mL tube, aspirate EtOH from tubes and discard.

16. Add 200 uL 70% EtOH to each tube in the stand.

17. Incubate 30 seconds.

18. Using a separate filter-tip for each 1.5 mL tube, aspirate EtOH from tubes and discard.

19. Allow the tubes containing the Serapure beads to dry for approximately 5 minutes or until there is no longer a smell of EtOH. You do not want the Serapure beads to appear “cracked” or “crusty”. In my experience, it takes about 7 minutes for tubes to air-dry in a low humidity environment. Do not

dry on a heat block. Use sterile toothpicks or pipette tips to remove EtOH blobs.

20. Remove the 1.5 mL tube from the rare-earth magnet stand and add 22 uL ddH2O.

21. Mix beads and water by pipetting up and down.

22. Gently and slowly (< 2000 RPM) spin tubes for 2-3 seconds.

24. Place tubes back on magnet stand, allow beads to separate, and **aspirate liquid into clean 1.5 mL microtubes**. Because we're using Serapure, we'll get out almost or exactly what we put in, so set your pipet on 31-32 uL.

25. Discard leftover tubes containing Serapure beads.

26. Quantify a 1-2 uL aliquot of the enriched, cleaned library using the Qubit.

27. [Optional] You may wish to run an additional PCR to ensure that you actually enriched your target loci relative to the unenriched "controls." BCF has primers available.

28. [Optional] Quantify libraries using qPCR (Kapa Library Quantification kit).

29. Combine libraries at equimolar ratios dependent on your needs or those of the sequencing center. If you are using the 5k locus set and a HiSeq 2000 with v3 sequencing chemistry, then you can successfully pool up to 96 libraries.

****ALTERNATIVELY, you can do the speed bead (Serapure) cleanup using a magnetic 96-well plate in place of 1.5mL tubes. Work with capped strip tubes so you can mix and spin down. Make sure EtOH volumes are less than 200uL.****