Illumina library prep protocol

Release 2.1

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Contents

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CHAPTER 1

Suggested Reading / References

CHAPTER 2

Summary

Prepare DNA libraries having unique sequence tagged adapters ligated to DNA fragments on a per-library basis.

This protocol incorporates the with-bead AMPure cleanup protocol, initially described in [Fish2011], which conducts all of the library preparation steps in the presence of SPRI/AMPure/SeraPure beads.

CHAPTER 3

Protocol

3.1 Materials

- Covaris/Bioruptor/etc. for shearing samples
- SPRI/AMPure/SeraPure beads for sample purification
- PEG solution mixture (lacking beads; e.g. 20% PEG, 2.5M NaCl from [Fisher2011])
- Rare-earth magnet stand (e.g. Ambion AM10055 or NEB S1506S)
- Kapa Biosystems Library Prep kit (KK8230 & KK8232)
- **Amplification primers for Illumina adapters (includes phosphorothioate bonds)**
  - Upper 5' – AAT GAT ACG GCG ACC ACC GAG A*T- 3'
  - Lower 5' – CAA GCA GAA GAC GGC ATA CGA GA*T- 3'
- Illumina TruSeq adapters, BFIDT adapters, iTrue adapters (see *Adapter Preparation*)
- Thermal cycler or ThermoMixer with digital temperature control that will accept 1.5 mL tubes.
- SpeedVac (if you need to dry down libraries)

3.2 Adapter Preparation

3.2.1 TruSeq v3-style adapters

If you are using custom TruSeq-style sequencing adapters (i.e., not the standard Illumina TruSeq adapters and something similar to those adapters in [Fair2012]) and you have ordered both indexed oligos and a universal oligo from IDT, you need make indexed adapters from the indexed oligo + universal oligo. This means combining one indexed oligo with the universal oligo, and annealing those to make double- stranded adapters. You need to do this for all adapters you will use, and it is best/easiest to do this in a PCR plate.

1. Assemble the following components to make Oligo Buffer:

1X TE (10 mM Tris pH 8.0, 1 mM EDTA)	9.9 mL
5 M NaCl	0.1 mL
Total	10.0 mL

2. Using the Oligo Buffer from above, assemble the following components to create the adapters:

Oligo Buffer	50.0 µL
100 uM TruSeq Universal Adapter	25.0 µL
100 uM TruSeq Indexed Adapter	25.0 µL
Total	100.0 µL

3. Anneal:
    - 95 C for 1 minute
    - -0.1 C/sec for each of 800 seconds (~13 minutes)
    - 14 C hold
4. Store adapters at -20 C.

3.3 Library preparation

1. Equilibrate DNA samples to ~ 20 ng/uL in 100 uL.
2. Shear DNA to a size distribution peaking around 400-600 bp using a Diagenode BioRuptor or Covaris.
3. Check size distribution on agarose gel (1.5% for 1.5 h at 100 V). If some samples are too large, shear again for 1-2 cycles.
4. Re-quantify using QuBit. Transfer 0.5 ug to 1 ug sheared DNA to a new tube.
5. Add 3.0X volume of AMPure XP beads to the digested product from Step 4 in a 1.5 mL microtube. Remember, we are altering the standard AMPure protocol by leaving the DNA bound to the beads while we end-repair, +A, and ligate adapters to DNA fragments. Mix by pipetting or gently vortexing.
6. Incubate the mixture for 5 minutes at room temperature.
7. Move 1.5 mL tubes to a rare-earth magnet stand.
8. Let sit for about 3 minutes or until solution is clear.
9. Using a separate filter-tip for each 1.5 mL tube, aspirate liquid from tubes and discard.
10. Add 500 uL 80% EtOH to each tube in the stand.
11. Incubate 30 seconds.
12. Using a separate filter-tip for each 1.5 mL tube, aspirate EtOH from tubes and discard.
13. Add 500 uL 80% 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. Allow the tubes containing the AMPure beads to dry for approximately 5 minutes or until there is no longer a smell of EtOH. You do not want the AMPure 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 to remove EtOH blobs.
17. Add 58 uL ddH20 to beads and mix gently by inverting or pipetting.
18. Assemble the end-repair reaction:

End Repair Buffer	7 µL
End Repair Mix	5 µL
Total	12 µL

19. Add 12 uL end-repair reaction mix to tube containing beads and DNA in 58 uL ddH20 (resulting in 70 uL total volume).
20. Pipette up and down to mix.
21. Incubate for 30 m at 20 C.
22. Add 120 uL PEG (1.7X) solution mixture to reaction with beads. Mix by pipetting or gently vortexing.
23. Incubate the mixture for 5 minutes at room temperature.
24. Move 1.5 mL tubes to a rare-earth magnet stand.
25. Let sit for about 3 minutes or until solution is clear.
26. Using a separate filter-tip for each 1.5 mL tube, aspirate liquid from tubes and discard.
27. Add 400 uL 80% EtOH to each tube in the stand.
28. Incubate 30 seconds.
29. Using a separate filter-tip for each 1.5 mL tube, aspirate EtOH from tubes and discard.
30. Add 400 uL 80% EtOH to each tube in the stand.
31. Incubate 30 seconds.
32. Using a separate filter-tip for each 1.5 mL tube, aspirate EtOH from tubes and discard.
33. Allow the tubes containing the AMPure beads to dry for approximately 5 minutes or until there is no longer a smell of EtOH. You do not want the AMPure 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 to remove EtOH blobs.
34. Add 42 uL ddH20 to beads and mix gently by inverting or pipetting.
35. Assemble the +A reaction:

10X A-tailing Buffer	5 µL
A-tailing Enzyme	3 µL
Total	8 µL

36. Add 8 uL +A reaction mix to the tube containing beads and DNA in 42 uL ddH20 (resulting in total volume of 50 uL).
37. Incubate for 30 m at 30 C.
38. Add 90 uL PEG solution (1.8X) to reaction with beads. Mix by pipetting or gently vortexing.
39. Incubate the mixture for 5 minutes at room temperature.
40. Move 1.5 mL tubes to a rare-earth magnet stand.
41. Let sit for about 3 minutes or until solution is clear.
42. Using a separate filter-tip for each 1.5 mL tube, aspirate liquid from tubes and discard.
43. Add 400 uL 80% EtOH to each tube in the stand.
44. Incubate 30 seconds.
45. Using a separate filter-tip for each 1.5 mL tube, aspirate EtOH from tubes and discard.
46. Add 400 uL 80% EtOH to each tube in the stand.
47. Incubate 30 seconds.
48. Using a separate filter-tip for each 1.5 mL tube, aspirate EtOH from tubes and discard.

49. Allow the tubes containing the AMPure beads to dry for approximately 5 minutes or until there is no longer a smell of EtOH. You do not want the AMPure 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 to remove EtOH blobs.
50. Add 33 uL ddH20 to beads and mix gently by inverting or pipetting.
51. Using separate pipette tips for each sample, add 2 uL of the sample-specific adapter mix (25 uM) to each tube. Remember that adapters are sample- specific.
52. Assemble the adapter ligation reaction:

5X Ligation Buffer	10 µL
DNA Ligase	5 µL
Total	15 µL

53. Add 15 uL ligation reaction mix to the tube containing beads and DNA in 35 uL ddH20 and adapters (to yield total volume of 50 uL).
54. Incubate for 15 m at 20 C (same for NEB).
55. Add 50 uL PEG solution (1.0X) mixture to reaction with beads. Mix by pipetting or gently vortexing. We're using less PEG solution at this step to help us remove very short fragments.
56. Incubate the mixture for 5 minutes at room temperature.
57. Move 1.5 mL tubes to a rare-earth magnet stand.
58. Let sit for about 3 minutes or until solution is clear.
59. Using a separate filter-tip for each 1.5 mL tube, aspirate liquid from tubes and discard.
60. Add 400 uL 80% EtOH to each tube in the stand.
61. Incubate 30 seconds.
62. Using a separate filter-tip for each 1.5 mL tube, aspirate EtOH from tubes and discard.
63. Add 400 uL 80% EtOH to each tube in the stand.
64. Incubate 30 seconds.
65. Using a separate filter-tip for each 1.5 mL tube, aspirate EtOH from tubes and discard.
66. Allow the tubes containing the AMPure beads to dry for approximately 5 minutes or until there is no longer a smell of EtOH. You do not want the AMPure 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 to remove EtOH blobs.
67. Add 50 uL ddH20 to beads+template. We will clean these again.
68. Add 50 uL PEG solution (1.0X) mixture to reaction with beads. Mix by pipetting or gently vortexing. We're using less PEG solution at this step to help us remove very short fragments.
69. Incubate the mixture for 5 minutes at room temperature.
70. Move 1.5 mL tubes to a rare-earth magnet stand.
71. Let sit for about 3 minutes or until solution is clear.
72. Using a separate filter-tip for each 1.5 mL tube, aspirate liquid from tubes and discard.
73. Add 400 uL 80% EtOH to each tube in the stand.
74. Incubate 30 seconds.
75. Using a separate filter-tip for each 1.5 mL tube, aspirate EtOH from tubes and discard.

76. Add 400 uL 80% EtOH to each tube in the stand.
77. Incubate 30 seconds.
78. Using a separate filter-tip for each 1.5 mL tube, aspirate EtOH from tubes and discard.
79. Allow the tubes containing the AMPure beads to dry for approximately 5 minutes or until there is no longer a smell of EtOH. You do not want the AMPure 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 to remove EtOH blobs.
80. Add 33 uL ddH20 to beads and mix gently by inverting or pipetting. You may wish to size select following this step but before PCR.
81. Incubate 2-5 minutes at room temp.
82. Move 1.5 mL tubes to a rare-earth magnet stand.
83. Let sit for about 3 minutes or until solution is clear.
84. Using a separate filter-tip for each 1.5 mL tube, aspirate liquid from tubes and transfer to a new, clean tube.
85. Quantify 2.0 uL using QuBit. If you used 500 ng to 1 ug of input DNA, the amount of total DNA you will have in solution should be 50-75% of what you started with.
86. Setup the following PCR reactions in separate 0.2 mL tubes. You can master mix these for multiple tubes/reactions:

2X Kapa HiFi HS RM Primer Mix (2.5 uM each of TruSeq Forward and Reverse primers) ddH2O	25 µL 5 µL 5 µL
Adapter-ligated DNA	15 µL

87. Cycle using the following. You may need to adjust or optimize cycle number:
    - 98 C for 45 seconds
    - 6-12 cycles of:
        - 98 C for 15 seconds
        - 60 C for 30 seconds
        - 72 C for 60 seconds
    - 72 C for 5 minutes
    - 4 C hold
88. In a 1.5 mL tube, combine PCR product and 90 uL AMPure XP beads (1.8X). Mix by pipetting or vortexing. You may wish to go as low as 50 uL AMPure XP (1.0X) to remove small fragments (< 300 bp).
89. Incubate the mixture for 5 minutes at room temperature.
90. Move 1.5 mL tubes to a rare-earth magnet stand.
91. Let sit for about 3 minutes or until solution is clear.
92. Using a separate filter-tip for each 1.5 mL tube, aspirate liquid from tubes and discard.
93. Add 200 uL 80% EtOH to each tube in the stand.
94. Incubate 30 seconds.
95. Using a separate filter-tip for each 1.5 mL tube, aspirate EtOH from tubes and discard.
96. Add 200 uL 80% EtOH to each tube in the stand.
97. Incubate 30 seconds.

98. Using a separate filter-tip for each 1.5 mL tube, aspirate EtOH from tubes and discard.
99. Allow the tubes containing the AMPure beads to dry for approximately 5 minutes or until there is no longer a smell of EtOH. You do not want the AMPure 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 to remove EtOH blobs.
100. Add 23 uL ddH20 to beads and mix gently by inverting or pipetting.
101. Incubate 2-5 minutes at room temp.
102. Move 1.5 mL tubes to a rare-earth magnet stand.
103. Let sit for about 3 minutes or until solution is clear.
104. Using a separate filter-tip for each 1.5 mL tube, aspirate liquid from tubes and transfer to a new, clean tube.
105. Quantify 2.0 µL using QuBit. You may wish to size select at this step to tighten the fragment distribution to your desired range. You need approximately 3.5 µL of 147 ng/µL library (~ 500 ng library) to go into the enrichment, if you are enriching samples on an individual (non-pooled) basis.
106. If you are combining libraries for pooled enrichment, combine 8 libraries together at equimolar ratios to produce 3.4 µL of pooled library at 147 ng/µL. This means that you need to add 62.5 ng of each library (total 8 libraries) to a strip tube and ensure that the volume is 3.4 µL (by adding water if < 3.4 µL or drying down the pooled libraries slightly and re- hydrating in 3.4 µL).

CHAPTER 4

Protocol info

4.1 Citing

4.2 License

4.3 Changelog

4.3.1 v2.1

- move to read-the-docs.org

4.3.2 v2.0

- Update for new Kapa kits.

4.3.3 v1.10

- Remove Fragmentase info. Change end-repair temp. to 20 C.

4.3.4 v1.9

- Update instructions for adapters to indicate when you would do this (September 24, 2012).

4.3.5 v1.8

- Add in NEB incubation temps, which are slightly different.

4.3.6 v1.7

- Fix incorrect total volumes for mixes.

4.3.7 v1.6

- Minor wording changes for clarity. Change template volume to 15 uL for Phusion PCR alternative.

4.3.8 v1.5

- Add adapter annealing section.

4.3.9 v1.4

- Add step telling users to aspirate ddH2O containing DNA after steps 64 and 84.

4.3.10 v1.3

- Remove all mention of using heat block to dry EtOH from samples.

4.3.11 v1.2

- Alter PEG solution mix to Fisher's original; Change ddH2O volumes for each step so we don't have to add water twice.

4.3.12 v1.1

- Change volumes of PEG Solution used during cleanup steps to increase post-shear to 3X and others to 2.2X. Final is 0.9X, which may be a little bit low.

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