

ChIP-seq library construction using TruSeq adapters
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Blunt DNA Ends (End Repair)

1. Mix 10 ng ChIP DNA
 5 µl NEB T4 DNA ligase buffer
 2 µl 10 mM dNTPs
 0.5 µl End Repair Enzyme Mix
 H₂O to make final volume 50 µl
2. Incubate at 20° C for 30 min in PCR machine.
3. Add 50 µl AMPure XP beads and 50 µl 30 % PEG₈₀₀₀ 1.25 M NaCl
4. Incubate at room temperature for 15 min.
5. Place on magnetic rack for 5 min.
6. Remove and discard supernatant. When removing supernatant do so very slowly with pipetman being careful not to take any beads.
7. Keep sample in magnetic rack and add 200 µl of freshly prepared 80% ethanol.
8. Incubate for 30 seconds. Remove and discard all supernatant.
9. Repeat steps 7 and 8 one more time.
10. Add 18 µl TE/10 and pipet up and down until beads are completely resuspended.
11. Incubate at room temperature for 2 min.
12. Place in magnetic rack for 5 min.
13. Transfer 16.5 µl of the supernatant to a new 0.2 ml PCR tube. Again it is important to remove the supernatant very slowly being careful not to take any beads. If you accidentally remove some beads, pipet sample back into same tube and wait for the beads to separate again.

Add 'A' Bases to the 3' Ends of the DNA fragments

14. Mix 16.5 µl End-repaired DNA (from step 14)
 2 µl 10X NEB Buffer #2
 1 µl 4 mM dATP
 0.5 µl 5 U/µl Klenow 3' to 5' exo minus (NEB)
15. Incubate at 37° C for 30 min.

Ligate Adapters to DNA fragments

16. Mix 20 µl 'A'-tailed DNA (from step 16)
 25 µl 2x Quick Ligase Buffer (NEB)
 1 µl Annealed TruSeq Adapters (0.25 µM) (see note #1)
 1 µl H₂O
 1.5 µl Quick Ligase (2,000 U/µl NEB)

17. Incubate for 20 minutes at room temperature.
18. Add 5 µl 0.5 M EDTA, pH 8.0
19. Add 50 µl AMPure XP beads and 50 µl 30 % PEG₈₀₀₀ 1.25 M NaCl
20. Incubate at room temperature for 20 min.
21. Place on magnetic rack for 5 min.
22. Remove and discard supernatant. When removing supernatant do so very slowly with pipetman being careful not to take any beads.
23. Keep sample in magnetic rack and add 200 µl of freshly prepared 80% ethanol.
24. Incubate for 30 seconds. Remove and discard all supernatant.
25. Repeat steps 24 and 25 one more time.
26. Add 15.5 µl TE/10 and pipet up and down until beads are completely resuspended.
27. Incubate at room temperature for 2 min.
28. Place in magnetic rack for 5 min.
29. Transfer 14 µl of the supernatant to a new 0.2 ml PCR tube. Again it is important to remove the supernatant very slowly being careful not to take any beads. If you accidentally remove some beads, pipet sample back into same tube and wait for the beads to separate again.

Convert Y-shaped adapters to dsDNA (see note #2)

30. On ice mix 14 µl Adapter-ligated DNA (from Step 31)
 1 µl TruSeq PCR primer cocktail (25 µM)
 15 µl 2X Kapa HiFi HotStart Ready Mix
31. Amplify with the following PCR protocol
 - a. 45 sec at 98° C
 - b. 5 cycles of:
 - 15 sec at 98° C
 - 30 sec at 63° C
 - 30 sec at 72° C
 - c. 1 min at 72° C
 - d. Hold at 4° C
32. Add 30 µl AMPure XP beads and 30 µl 30 % PEG₈₀₀₀ 1.25 M NaCl
33. Incubate at room temperature for 15 min.
34. Place on magnetic rack for 5 min.
35. Remove and discard supernatant. When removing supernatant do so very slowly with pipetman being careful not to take any beads.
36. Keep sample in magnetic rack and add 200 µl of freshly prepared 80% ethanol.
37. Incubate for 30 seconds. Remove and discard all supernatant.
38. Repeat steps 38 and 39 one more time.

39. Add 11.5 μ l TE/10 and pipet up and down until beads are completely resuspended.
40. Incubate at room temperature for 2 min.
41. Place in magnetic rack for 5 min.
42. Transfer 10 μ l of the supernatant to a new 1.5 ml tube. Again it is important to remove the supernatant very slowly being careful not to take any beads. If you accidentally remove some beads, pipet sample back into same tube and wait for the beads to separate again.

Size Select Library on Agarose Gel

43. Prepare a 2.5% MetaPhor/SeaKem LE (3:1 ratio) agarose 1X TAE gel with ethidium bromide using small tooth comb.
44. Load 200 ng of 100 bp ladder next to each sample leaving one lane empty between sample and ladder.
45. Add 4 μ l 5X loading dye (containing xylene cyanol and bromophenol blue) to sample.
46. Load sample(s) on gel with at least one lane between different samples and markers to avoid cross contamination.
47. Run gel at 120 V for 30 min.
48. Cut out slice of gel with clean scalpel that contains material between 250 and 450 bp and place in 1.5 ml tube (see note #3)
49. Determine volume of gel slice by zeroing scale with empty tube then weighing the tube with your gel slice (1 mg = 1 μ l).
50. Add 5 volumes of Qiagen Buffer QG (e.g. if you gel slice is 0.2 g, add 1000 μ l of QG)
51. Incubate at room temperature until gel slice has completely dissolved. Mix continuously by hand or in tube rotator (do not vortex).
52. Add 1 volume isopropanol.
53. Mix with pipetman.
54. Apply 650 μ l to Qiagen minElute column (see note #4).
55. Spin for 30 seconds in microfuge.
56. Remove liquid from collection.
57. Repeat steps 57 to 59 until you have passed entire sample through column.
58. Apply 500 μ l Qiagen Buffer QG to column.
59. Spin in microfuge for 1 min.
60. Remove liquid from collection tube.
61. Apply 750 μ l of Qiagen buffer PE to column
62. Let stand for 2 min
63. Spin in microfuge for 1 min
64. Remove liquid from collection tube
65. Spin in microfuge for 1 min
66. Transfer to new 1.5 ml tube
67. Add 10 μ l Qiagen buffer EB
68. Let stand for 5 min.

69. Spin in microfuge for 1 min.
70. Add an additional 10 µl Qiagen buffer EB, let stand for 5 min and spin for 1 min in microfuge (both elutions are collected into the same tube)
71. Transfer eluted DNA to 0.2 ml PCR tube

Amplify Library by PCR

72. On ice mix 19 µl Size-selected DNA (from Step 74)
 1 µl TruSeq PCR primer cocktail (25 µM)
 20 µl 2X Kapa HiFi HotStart Ready Mix
73. Amplify with the following PCR protocol
 - a. 45 sec at 98° C
 - b. 5 to 13 cycles of (see note #5):
 - 15 sec at 98° C
 - 30 sec at 63° C
 - 30 sec at 72° C
 - c. 1 min at 72° C
 - d. Hold at 4° C
74. Add 60 µl of well-mixed AMPure XP beads directly to 0.2 ml PCR tube and vortex.
75. Incubate at room temperature for 15 min.
76. Place on magnetic rack for 5 min.
77. Remove and discard supernatant. When removing supernatant do so very slowly with pipetman being careful not to take any beads.
78. Keep sample in magnetic rack and add 200 µl of freshly prepared 80% ethanol.
79. Incubate for 30 seconds. Remove and discard all supernatant.
80. Repeat steps 80 and 81 one more time.
81. Add 12 µl TE/10 and pipet up and down until beads are completely resuspended.
82. Incubate at room temperature for 2 min.
83. Place in magnetic rack for 5 min.
84. Transfer 10 µl of the supernatant to a new 1.5 ml tube. Again it is important to remove the supernatant very slowly being careful not to take any beads. If you accidentally remove some beads, pipet sample back into same tube and wait for the beads to separate again.

Analyze library by running 1 µl on Bioanalyzer (see note #6)

Quantitate Library using this protocol:

<http://ethanomics.wordpress.com/ngs-qpcr-library-quantitation-protocol/>

Notes:

1. Some sequencing experiments require the use of fewer than 12 index

sequences in a lane with a high cluster density. In such cases, select indexes carefully to ensure optimum base calling and demultiplexing by having different bases at each cycle of the index read. Illumina recommends the following sets of indexes for low-level pooling experiments.

Pool of 2 samples:

• Index #6 GCCAAT • Index #12 CTTGTA

Pool of 3 samples:

• Index #4 TGACCA • Index #6 GCCAAT • Index #12 CTTGTA

Pool of 6 samples: • Index #2 CGATGT • Index #4 TGACCA • Index #5 ACAGTG • Index #6 GCCAAT • Index #7 CAGATC • Index #12 CTTGTA

2. This step was added because the self-ligated Y-shaped adapter dimers appear to run very slow on a 2% agarose gel. I cut above 250 bp and they were still in the sample. This also brings into question where exactly your sample ligated to the Y-shaped adapters is running. Furthermore, the relative migration of funny-shaped DNA molecules changes with the percentage of the gel, type of agarose, etc. So to know exactly where everything is running, I assumed it would be better to convert the Y-shaped DNA to double-stranded DNA. Everything should be converted to double-stranded DNA after two PCR cycles, but since the recovery of DNA during the gel extraction is low, I thought it would be a good idea to add a couple extra cycles of PCR. Theoretically you could do the entire PCR enrichment at this step but then you would have to worry about some of the sample running too high because of the formation of 'bubbles' and 'daisy chains' when primers become limiting.

3. The double-stranded adapters add 121 bp to the DNA fragments. Calculate the size of your DNA fragments by running a portion of the reverse cross-linked input sample on a 2.2% agarose gel. Add 121 bp to that number and cut a gel slice containing your adaptor ligated DNA. Also be sure that your gel slice is well above 121 bp as that is where the self-ligated adapters run. Cut the minimal area possible to keep your gel slice under 200 mg.

4. I pass the dissolved gel slice through the column twice, i.e. I apply the dissolved gel slice to the column, spin in the microfuge for 1 minute and take the liquid that has passed through the column and apply it to the column again. I don't know if this improves recovery but I figure it couldn't hurt.

5. See this protocol to determine the correct number of cycles to do your PCR amplification:

<http://ethanomics.wordpress.com/ngs-pcr-cycle-quantitation-protocol/>

6. The image from the Bioanalyzer should be a smear the same size as the band you cut out in the size selection step. If you have larger products, this is most likely due to over-amplification. When primers become limiting you get hybrid DNA

molecules resulting from the adaptors ends of DNA molecules annealing. This creates bubble shaped molecules and daisy chains of molecules. Contrary to what is stated by Illumina, this should not be a problem, as your library will be denatured before attachment to the flow cell. On the other hand, if you have a sharp band around 121 bp, this is from self-ligated adaptors and is a problem.

7. All enzymes are purchased from NEB (except the Kapa polymerase).
8. The Kapa polymerase requires fewer cycles to achieve the same amplification as with Phusion polymerase.
9. I have reduced the amount of enzyme used and after first run through it seems to make no difference.

Adapter Annealing Protocol

1. Resuspend adapter oligonucleotides at a concentration of 100 μ M in:
10 mM Tris-HCl, pH 7.8
0.1 mM EDTA, pH 8.0
50 mM NaCl
2. Mix 25 μ l of the TruSeq Universal Adapter with 25 μ l of the indexed adapter.
3. Anneal oligos on a thermal cycler with the following program:
 - a. 2 min at 95° C
 - b. 140 cycles of
30 sec at 95° C (decrease temp 0.5° C every cycle)
 - c. Hold at 4° C
4. Dilute annealed adaptors 1:200 in H₂O

TruSeq PCR Primer Cocktail

1. Resuspend TruSeq PCR Primer 1 and TruSeq PCR Primer 2 in TE/10 (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) to a concentration of 100 μ M.
2. In new tube mix:
25 μ l TruSeq PCR Primer 1 (100 μ M)
25 μ l TruSeq PCR Primer 2 (100 μ M)
50 μ l H₂O

End Repair Enzyme Mix

30 μ l 3 U/ μ l T4 DNA polymerase (NEB)
6 μ l 5 U/ μ l Klenow Fragment (NEB)
30 μ l 10 U/ μ l T4 DNA Polynucleotide Kinase (NEB)

TE/10

10 mM Tris-HCl, pH 8.0
0.1 mM EDTA

Oligonucleotide Sequences

* = phosphorothioate bond

TruSeq Universal

AATGATACGGCGACCACCGAGATCTTACACTCTTTCCCTACACGACGCTCTTCCGATC*T

TruSeq Adapter, Index 1

/5Phos/GATCGGAAGAGCACACGTCTGAACTCCAGTCACATCAGCATCTCGTATGCCGTCTTCTGCTT*G

TruSeq Adapter, Index 2

/5Phos/GATCGGAAGAGCACACGTCTGAACTCCAGTCACCGATGTATCTCGTATGCCGTCTTCTGCTT*G

TruSeq Adapter, Index 3

/5Phos/GATCGGAAGAGCACACGTCTGAACTCCAGTCACTTAGGCATCTCGTATGCCGTCTTCTGCTT*G

TruSeq Adapter, Index 4

/5Phos/GATCGGAAGAGCACACGTCTGAACTCCAGTCACTGACCAATCTCGTATGCCGTCTTCTGCTT*G

TruSeq Adapter, Index 5

/5Phos/GATCGGAAGAGCACACGTCTGAACTCCAGTCACACAGTGATCTCGTATGCCGTCTTCTGCTT*G

TruSeq Adapter, Index 6

/5Phos/GATCGGAAGAGCACACGTCTGAACTCCAGTCACGCCAATATCTCGTATGCCGTCTTCTGCTT*G

TruSeq Adapter, Index 7

/5Phos/GATCGGAAGAGCACACGTCTGAACTCCAGTCACCAGATCATCTCGTATGCCGTCTTCTGCTT*G

TruSeq Adapter, Index 8

/5Phos/GATCGGAAGAGCACACGTCTGAACTCCAGTCACACTTGAATCTCGTATGCCGTCTTCTGCTT*G

TruSeq Adapter, Index 9

/5Phos/GATCGGAAGAGCACACGTCTGAACTCCAGTCACGATCAGATCTCGTATGCCGTCTTCTGCTT*G

TruSeq Adapter, Index 10

/5Phos/GATCGGAAGAGCACACGTCTGAACTCCAGTCACTAGCTTATCTCGTATGCCGTCTTCTGCTT*G

TruSeq Adapter, Index 11

/5Phos/GATCGGAAGAGCACACGTCTGAACTCCAGTCACGGCTACATCTCGTATGCCGTCTTCTGCTT*G

TruSeq Adapter, Index 12

/5Phos/GATCGGAAGAGCACACGTCTGAACTCCAGTCACCTTGTAATCTCGTATGCCGTCTTCTGCTT*G

TruSeq Adapter, Index 13

/5Phos/GATCGGAAGAGCACACGTCTGAACTCCAGTCACAGTCAAATCTCGTATGCCGTCTTCTGCTT*G

TruSeq Adapter, Index 14

/5Phos/GATCGGAAGAGCACACGTCTGAACTCCAGTCACAGTTCCATCTCGTATGCCGTCTTCTGCTT*G

TruSeq Adapter, Index 15

/5Phos/GATCGGAAGAGCACACGTCTGAACTCCAGTCACATGTCAATCTCGTATGCCGTCTTCTGCTT*G

TruSeq Adapter, Index 16

/5Phos/GATCGGAAGAGCACACGTCTGAACTCCAGTCACCCGTCCATCTCGTATGCCGTCTTCTGCTT*G

TruSeq Adapter, Index 18

/5Phos/GATCGGAAGAGCACACGTCTGAACTCCAGTCACGTCCGCATCTCGTATGCCGTCTTCTGCTT*G

TruSeq Adapter, Index 19

/5Phos/GATCGGAAGAGCACACGTCTGAACTCCAGTCACGTGAAAATCTCGTATGCCGTCTTCTGCTT*G

TruSeq PCR 1

AATGATACGGCGACCACCGA*G

TruSeq PCR 2

CAAGCAGAAGACGGCATACGA*G

Note: The adaptor sequences are from Illumina. The PCR primer sequences are my best guess as to what Illumina is using in their PCR Primer Cocktail. To use more barcodes, simply change the highlighted sequence