

Prepare Genomic DNA

1. Harvest cells ($<5 \times 10^6$ cells) by trypsinization.
2. Spin down at 3,000 rpm for 5 min.
3. Remove growth media.
4. Resuspend cell pellet in 200 μ l PBS.
5. Add 20 μ l PureLink Proteinase K.
6. Add 20 μ l PureLink RNase A.
7. Mix well by brief vortexing.
8. Incubate at room temperature for 2 minutes.
9. Add 200 μ l PureLink Genomic Lysis/Binding Buffer and mix well by vortexing.
10. Incubate at 55°C for 10 minutes.
11. Add 200 μ l 100% ethanol. Mix well by vortexing for 5 seconds.
12. Transfer cell lysate to PureLink Spin Column
13. Centrifuge at 10,000 x g for 1 minutes.
14. Transfer column to new collection tube.
15. Add 500 μ l PureLink Wash Buffer 1.
16. Centrifuge at 10,000 x g for 1 minutes.
17. Transfer column to new collection tube.
18. Add 500 μ l PureLink Wash Buffer 2
19. Centrifuge at maximum speed for 3 minutes.
20. Place column in new 1.5 ml tube and add 70 μ l PureLink Genomic elution buffer.
21. Incubate at room temperature for 1 minute.
22. Centrifuge at maximum speed for 1 minute.
23. Add an additional 70 μ l PureLink Genomic elution buffer.
24. Incubate at room temperature for 1 minute.
25. Centrifuge at maximum speed for 1 minute.
26. Transfer DNA to new 1.5 ml tube.
27. Measure DNA concentration with NanoDrop.

Shear DNA with Covaris

28. Transfer <4 μ g DNA to Covaris microTube with AFA fiber.
29. Add H₂O to a final volume of 130 μ l.
30. Sonicate in Covaris S2 with the following settings:

Time	7 min
Duty Cycle	10%
Intensity	5
Cycles per Burst	200
Temperature	4°C
Power mode	Sweeping
Degassing mode	Continuous
AFA Intensifier	Yes
Water Level	14

31. Transfer DNA from microTube to 1.5 ml tube.
32. 130 µl of well-mixed Ampure XP beads and 130 µl 30 % PEG₈₀₀₀
1.25 M NaCl
33. Mix by pipetting up and down several times (See Note 9).
34. Incubate at room temperature for 15 min.
35. Place on magnetic rack for 5 min.
36. Remove and discard the supernatant. When removing supernatant do so very slowly with pipetman being careful not to take any beads.
37. Keep sample in magnetic rack and add 200 µl of freshly prepared 80% ethanol.
38. Incubate for 30 seconds. Remove and discard ALL the supernatant.
39. Repeat steps 37 and 38 one more time.
40. Let the beads dry at room temperature for 2 minutes.
41. Add 42.5 µl TE/10 and pipet up and down several times until pellet beads are completely resuspended.
42. Incubate at room temperature for 2 minutes.
43. Place in magnetic rack for 5 minutes.
44. Transfer 50 µl of the supernatant to a 0.2 ml PCR tube.
45. Quantitate DNA with nanoDrop.
46. Remove 2 µl and add 39 µl H₂O to new tube for input control

Prepare SssI CpG methylated DNA control

47. Mix: 1 µl 32 mM SAM (see Note 10)
19 µl H₂O
48. Mix: 10 µl 10X NEB buffer #2
10 µl 1.6 mM SAM (freshly diluted from step 46)
7 µl DNA from step #45
5 µl SssI methyltransferase (add last)
H₂O to a final volume of 100 µl
49. Incubate at 37°C for 1 hour.
50. 100 µl of well-mixed Ampure XP beads and 100 µl 30 % PEG₈₀₀₀
1.25 M NaCl
51. Mix by pipetting up and down several times (See Note 9).
52. Incubate at room temperature for 15 min.
53. Place on magnetic rack for 5 min.
54. Remove and discard the supernatant. When removing supernatant do so very slowly with pipetman being careful not to take any beads.
55. Keep sample in magnetic rack and add 200 µl of freshly prepared 80% ethanol.
56. Incubate for 30 seconds. Remove and discard ALL the supernatant.
57. Repeat steps 37 and 38 one more time.
58. Let the beads dry at room temperature for 2 minutes.
59. Add 42.5 µl TE/10 and pipet up and down several times until pellet beads are completely resuspended.
60. Incubate at room temperature for 2 minutes.
61. Place in magnetic rack for 5 minutes.
62. Transfer 41 µl of the supernatant to a 0.2 ml PCR tube.

Blunt DNA Ends (End Repair)

63. Perform the following steps of the protocol with three sets of reactions:
 - a) Fully CpG methylated DNA control
 - b) Input DNA
 - c) Sheared genomic DNA for MeDIP
64. Mix: 41 µl genomic DNA from step #45, 41 µl Input DNA from step #46 or 41 µl SssI methylated DNA from step #61.
 5 µl NEB T4 DNA ligase buffer
 2 µl 10 mM dNTPs
 2 µl End Repair Enzyme Mix
 H₂O to a final volume of 50 µl
65. Incubate at 20° C for 30 min in PCR machine
66. 50 µl of well-mixed Ampure XP beads and 50 µl 20 % PEG₈₀₀₀ 1.25 M NaCl
67. Mix by pipetting up and down several times.
68. Incubate at room temperature for 15 min.
69. Place on magnetic rack for 5 min.
70. Remove and discard the supernatant. When removing supernatant do so very slowly with pipetman being careful not to take any beads.
71. Keep sample in magnetic rack and add 200 µl of freshly prepared 80% ethanol.
72. Incubate for 30 seconds. Remove and discard ALL the supernatant.
73. Repeat steps 53 and 54 one more time.
74. Let the beads dry at room temperature for 2 minutes.
75. Add 17.5 µl TE/10 and pipet up and down several times until pellet beads are completely resuspended.
76. Incubate at room temperature for 2 minutes.
77. Place in magnetic rack for 5 minutes.
78. Transfer 16 µl of the supernatant to a 0.2 ml PCR tube. Transfer eluted DNA to new 1.5 ml tube

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

79. Mix: 16 µl End-repaired DNA (from step 60)
 2 µl 10X NEB Buffer #2
 1 µl 4 mM dATP
 1 µl 5 U/µl Klenow 3' to 5' exo minus
80. Incubate at 37° C for 30 min.

Ligate Adapters to DNA fragments

81. Mix: 20 µl 'A'-tailed DNA (from step 62)
 25 µl 2x Quick Ligase Buffer (NEB)
 2 µl Annealed TruSeq Adapters (50 µM for genomic DNA – 5 µM for fully methylated DNA control and input control)
 2 µl Quick Ligase (2,000 U/µl)
82. Incubate at room temp for 15 min.
83. Add 5 µl 0.5 M EDTA
84. Add 50 µl of well-mixed Ampure XP beads and 50 µl 20 % PEG₈₀₀₀ 1.25 M NaCl

85. Mix by pipetting up and down several times.
86. Incubate at room temperature for 15 min.
87. Place on magnetic rack for 5 min.
88. Remove and discard the supernatant. When removing supernatant do so very slowly with pipetman being careful not to take any beads.
89. Keep sample in magnetic rack and add 200 µl of freshly prepared 80% ethanol.
90. Incubate for 30 seconds. Remove and discard ALL the supernatant.
91. Repeat steps 53 and 54 one more time.
92. Let the beads dry at room temperature for 2 minutes.
93. Add 27 µl TE/10 and pipet up and down several times until pellet beads are completely resuspended.
94. Incubate at room temperature for 2 minutes.
95. Place in magnetic rack for 5 minutes.
96. Transfer 25 µl of the supernatant to a 0.2 ml PCR tube.
97. Transfer 2.5 µl of DNA to a new 1.5 ml tube, add 47.5 µl Proteinase K Digestion Buffer and put aside for input control.

Perform anti-5-methylcytosine immunoprecipitation

98. Add 20 µl MeDIP-seq blocking oligo (100 pmols/µl) to adapter ligated DNA from step 96.
99. Denature DNA for 10 min at 95° C in thermocycler.
100. Transfer tubes directly from 95° C to ice water bath.
101. Keep on ice for 10 min.
102. Mix: 42.5 µl denatured adapter-ligated DNA
0.5 µl 1 µg/µl anti-5meC antibody (33D3 Diagenode)
500 µl MeDIP Wash Buffer (ice cold)
103. Incubate overnight at 4°C.
104. Equilibrate beads. Prepare one 1.5 ml tube for each for each immunoprecipitation with 1 ml 1x IP Buffer and 10 µl of Sheep Anti-mouse M-280 Dynabeads to each tube. Note: The beads are usually a significant source of background and the minimum amount should be used. Invitrogen states that the binding capacity of the beads is 6.5-65 ng IgG/µl beads (yeah, that's a huge spread but that is what the product brochure obliquely says).
105. Transfer IP to the tube with the Dynabeads.
106. Incubate 2 hours at 4° C on tube rotator.
107. Spin briefly in microfuge at 3k RPM. Place in magnetic rack and remove liquid.
108. Add 1 ml IP Buffer, resuspend beads, spin briefly at 3.5k rpm, place in magnetic rack, and remove supernatant.
109. Repeat step 94 five more times for a total of six washes and change tubes two times during washes.
110. Resuspend beads in: 48 µl Proteinase K digestion buffer
2 µl 10 mg/ml Proteinase K
111. Process input and IP samples in parallel in the following steps.
112. Incubate 30 minutes at 50° C.
113. Place tubes in magnetic rack for 3 minutes.
114. Transfer supernatant to new 0.2 ml PCR tube.

115. Add 50 µl of well-mixed Ampure XP beads and 50 µl 20 % PEG₈₀₀₀ 1.25 M NaCl
116. Mix by pipetting up and down several times.
117. Incubate at room temperature for 15 min.
118. Place on magnetic rack for 5 min.
119. Remove and discard the supernatant. When removing supernatant do so very slowly with pipetman being careful not to take any beads.
120. Keep sample in magnetic rack and add 200 µl of freshly prepared 80% ethanol.
121. Incubate for 30 seconds. Remove and discard ALL the supernatant.
122. Repeat steps 102 and 103 one more time.
123. Let the beads dry at room temperature for 2 minutes.
124. Add 15.5 µl TE/10 and pipet up and down several times until pellet beads are completely resuspended.
125. Incubate at room temperature for 2 minutes.
126. Place in magnetic rack for 5 minutes.
127. Transfer 14 µl of the supernatant to a 0.2 ml PCR tube.

Convert ssDNA to dsDNA (see note #2)

128. On ice mix 14 µl Adapter-ligated DNA Input DNA (from step 83)
 or 14 µl immunoprecipitated DNA (from step 111)
 1 µl TruSeq PCR primer cocktail (25 µM)
 15 µl 2X Kapa HiFi HotStart Ready Mix
129. Amplify with the following PCR protocol
 - a. 45 sec at 98° C
 - b. 4 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
130. Add 30 µl of well-mixed Ampure XP beads and 30 µl 20 % PEG₈₀₀₀ 1.25 M NaCl
131. Mix by pipetting up and down several times (See Note 9).
132. Incubate at room temperature for 15 min.
133. Place on magnetic rack for 5 min.
134. Remove and discard the supernatant. When removing supernatant do so very slowly with pipetman being careful not to take any beads.
135. Keep sample in magnetic rack and add 200 µl of freshly prepared 80% ethanol.
136. Incubate for 30 seconds. Remove and discard ALL the supernatant.
137. Repeat steps 121 and 122 one more time.
138. Let the beads dry at room temperature for 2 minutes.
139. Add 15.5 µl TE/10 and pipet up and down several times until pellet beads are completely resuspended.
140. Incubate at room temperature for 2 minutes.
141. Place in magnetic rack for 5 minutes.

142. Transfer 14 µl of the supernatant to a 0.2 ml PCR tube.

Size Select Library on Agarose Gel

143. Prepare a 2.5% MetaPhor/SeaKem LE (3:1 ratio) agarose 1X TAE gel with ethidium bromide.
144. Load 200 ng of 100 bp ladder in two lanes at either side of gel.
145. Add 4 µl 5X loading dye (containing xylene cyanol and bromophenol blue) to sample.
146. Load sample(s) on gel with at least one lane between different samples and markers to avoid cross contamination.
147. Run gel at 120 V for 35 min.
148. Cut out slice of gel with clean scalpel that contains material between 200 and 400 bp and place in 1.5 ml tube (see note #3)
149. Determine volume of gel slice by zeroing scale with empty tube then weighing the tube with your gel slice (1 mg = 1 µl).
150. Add 5 volumes of Qiagen Buffer QG (e.g. if you gel slice is 0.2 g, add 1 ml of QG)
151. Incubate at room temperature until gel slice has completely dissolved. Mix continuously by hand or in tube rotator.
152. Add 1 volume isopropanol and mix by inverting several times.
153. Apply 650 µl to Qiagen MinElute column.
154. Spin for 30 seconds in microfuge.
155. Remove liquid from collection.
156. Repeat steps 139 to 141 until you have passed entire sample through column
157. Apply 500 µl Qiagen Buffer QG to column.
158. Spin in microfuge for 1 min.
159. Remove liquid from collection tube.
160. Apply 750 µl of Qiagen buffer PE to column
161. Let stand for 2 min
162. Spin in microfuge for 1 min
163. Remove liquid from collection tube
164. Spin in microfuge for 1 min
165. Transfer to new 1.5 ml tube
166. Add 15 µl Qiagen buffer EB
167. Let stand for 5 min
168. Spin in microfuge for 1 min
169. Transfer eluted DNA to 0.2 ml PCR tube

Amplify Library by PCR

170. On ice mix :
 - 14 µl Size-selected DNA (from Step 154)
 - 1 µl TruSeq PCR primer cocktail (25 µM)
 - 15 µl 2X Kapa HiFi HotStart Ready Mix
171. Amplify with the following PCR protocol
 - a. 45 sec at 98° C
 - b. 5 to 14 cycles of (see note #4):
 - 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
172. Add 54 µl of well-mixed AMPure XP beads.
 173. Incubate at room temperature for 15 min with occasional vortexing.
 174. Spin tube at 4,000 rpm for 5 seconds.
 175. Place on magnetic rack for at least 5 min.
 176. Remove and discard 105 µl of the supernatant.
 177. Keep sample in magnetic rack and add 200 µl of freshly prepared 80% ethanol.
 178. Incubate for 30 seconds. Remove and discard all supernatant.
 179. Repeat steps 163 and 164 one more time.
 180. Let the beads dry at room temperature for 2 minutes.
 181. Add 12.5 µl Resuspension Buffer and pipet up and down 10 times.
 182. Incubate at room temperature for 2 min.
 183. Place in magnetic rack for 5 min.
 184. Transfer 10 µl of the supernatant to a new 1.5 ml tube.

Analyze library by running 1 µl on Bioanalyzer (see note #5)
Quantitate Library with Kapa Biosystems Library Quantification Kit

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. However, I have stopped following this advice.

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 2.5% 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 sheared DNA from step 46 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.

4. Before PCR amplification take 1 µl of your size-selected DNA and mix it with 1 µl of TruSeq PCR Primer Cocktail, 8 µl H₂O and 10 µl 2X Kapa SYBR FAST Master Mix. Run on qPCR machine with same parameters as used for library amplification with 25 cycles. Determine the number of cycles it took to get to 50% of amplification (late log phase) and use that number of cycles minus 2.

5. 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 adapters 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 adapters and is a problem.

6. All enzymes are purchased from NEB (except the Kapa polymerase).

7. The Kapa polymerase requires fewer cycles to achieve the same amplification as with Phusion polymerase.

8. Possible improvements:

b. Kapa makes their library amplification PCR mix with SYBR green. Perhaps you can monitor the library amplification in real-time and remove it from the machine as it becomes amplified but before it is over-amplified.

9. AMPure XP guidelines:

a) Make sure that you achieve a homogenous solution of the beads and PEG by pipetting up and down a sufficient number of times.

b) When removing the supernatant after the binding step, remove all but 5 µl with a P200 pipetman. Then go back, using a P20, and carefully remove as much of the last 5 µl as you can without taking any beads.

c) When washing with 80% ethanol the beads stick to the wall of the tube better so you can remove the supernatant more quickly. However, I make sure I remove all traces of ethanol by going through each tube a second time with a new pipet tip.

d) Illumina recommends drying the beads, not only is this a waste of time but it probably reduces yield.

e) At the elution step, when transferring the eluted DNA to a new tube, it is important not to accidentally carryover any beads. I carefully look inside the tube as I am pipetting up the eluted DNA to make sure I don't accidentally take up any beads. Then I look at the eluted DNA in the pipet tip to see if I can see any beads. If there are, simply pipet the beads back into the tube you took them from and wait a few minutes for the sample to separate again in the magnetic rack. At this step it is important to leave at least 1 μ l behind, as it is not possible to remove all the liquid and not take any beads.

10. Three significant changes were made in this protocol from existing published protocols:

a. One issue with MeDIP-seq is that it requires the DNA to be single stranded, which is a potential problem since the Illumina adapters have base-pairing sequences. Thus, potentially every single stranded DNA molecule that is immunoprecipitated will bring down additional DNA molecules base-paired in the adapter region. This would substantially increase background. To circumvent this potential problem I add in a 50-fold excess of a 16 bp oligo that binds to the homologous region in the adapter, in order to block the adapter ligated DNA molecules from base pairing. The low melting temperature of the blocking oligo should prevent it from priming during subsequent PCR steps.

b. Four cycles of PCR are added before size selection by agarose gel electrophoresis, to convert the Y-shaped Illumina adapters into double stranded DNA. I have found that the Y-shaped adapters run much higher than the corresponding length of double-stranded DNA, thus making the identification of the correct size to excise from the gel difficult.

c. If there was a third thing, I forget what it was.

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

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

Oligonucleotide Sequences

* = phosphorothioate bond

TruSeq Universal

AATGATACGGCGACCAACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC*T

TruSeq Adaptor, Index 2

/5Phos/GATCGGAAGAGCACACGTCTGAACTCCAGTCACCGATGTATCTCGTATGCCGTCTTCTGCTT
*G

TruSeq Adaptor, Index 4

/5Phos/GATCGGAAGAGCACACGTCTGAACTCCAGTCACTGACCAATCTCGTATGCCGTCTTCTGCTT
*G

TruSeq Adaptor, Index 5

/5Phos/GATCGGAAGAGCACACGTCTGAACTCCAGTCACACAGTGATCTCGTATGCCGTCTTCTGCTT
*G

TruSeq Adaptor, Index 6

/5Phos/GATCGGAAGAGCACACGTCTGAACTCCAGTCACGCCAATATCTCGTATGCCGTCTTCTGCTT
*G

TruSeq Adaptor, Index 7

/5Phos/GATCGGAAGAGCACACGTCTGAACTCCAGTCACCAGATCATCTCGTATGCCGTCTTCTGCTT
*G

TruSeq Adaptor, Index 12

/5Phos/GATCGGAAGAGCACACGTCTGAACTCCAGTCACCTTGTAATCTCGTATGCCGTCTTCTGCTT
*G

TruSeq PCR 1

AATGATACGGCGACCAACCGA*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.

MeDIP-seq Blocking Oligo

AGATCGGAAGAGCGTC

IMPORTANT!!!!!!: The current batches of adapters from Illumina are methylated so you absolutely cannot use them. You must have your own oligos synthesized.

End Repair Enzyme Mix

30 µl 3 U/µl T4 DNA polymerase

6 µl 1 U/µl Klenow Fragment

30 µl 10 U/µl T4 DNA Polynucleotide Kinase

MeDIP Wash Buffer

PBS

0.05% Triton X-100

1 mM EDTA

Proteinase K digestion buffer

20 mM HEPES, pH 7.9

1 mM EDTA

0.5 % SDS

TE/10

10 mM Tris-HCl, pH 8.0

0.1 mM EDTA