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ATAC-Me Library Construction Protocol

Materials

- Non-adapter complexed Tn5 Transposase Enzyme (Homemade or EZ-Tn5™ Transposase [Lucigen, TNP92110])
- DNA Clean & Concentrate-5 (Zymo, D4004)
- EZ DNA Methylation-Lightning Kit (Zymo, D5030)
- TrypLE™ Express Enzyme (1X), no phenol red (Gibco, 12604013)
- T4 DNA Polymerase (NEB, M0203S)
- Ampligase® Enzyme and Buffer (Lucigen, A3202K)
- 2x KAPA HiFi HotStart Uracil+ ReadyMix (Roche, 07959052001)
- IGEPAL® CA-630 (Sigma, I8896)
- Trizma® hydrochloride solution (Sigma, T2319)
- Dimethylformamide (Thermo Fisher Scientific, D119-1)
- 100% Glycerol
- 100% Ethanol, molecular biology grade
- Nuclease free water
- See table below for required oligos (5' to 3', sequences formatted for ordering input at IDT):

Tn5mC-Apt1	T/iMe-dC/GT/iMe-dC/GG/iMe-dC/AG/iMe-dC/GT/iMe-dC/AGATGTGTATAAGAGA/iMe-dC/AG
Tn5mC1.1-A1block	/5Phos/CTGTCTCTTATACA/3ddC/
Tn5mC-RepIO1	/5Phos//iMe-dC/TGT/iMe-dC/T/iMe-dC/TTATA/iMe-dC/A/iMe-dC/AT/iMe-dC/T/iMe-dC//iMe-dC/GAG/iMe-dC//iMe-dC//iMe-dC/A/iMe-dC/GAGA/iMe-dC//3InvdT/
Index N701	CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTCTCGTGGGCTCGG
Index N702	CAAGCAGAAGACGGCATACGAGATCTAGTACGGTCTCGTGGGCTCGG
Index N703	CAAGCAGAAGACGGCATACGAGATTTCTGCCTGTCTCGTGGGCTCGG
Index N704	CAAGCAGAAGACGGCATACGAGATGCTCAGGAGTCTCGTGGGCTCGG
Index N705	CAAGCAGAAGACGGCATACGAGATAGGAGTCCGTCTCGTGGGCTCGG
Index N706	CAAGCAGAAGACGGCATACGAGATCATGCCTAGTCTCGTGGGCTCGG
Index N501	AATGATACGGCGACCACCGAGATCTACACTAGATCGCTCGTCGGCAGCGTC
Index N502	AATGATACGGCGACCACCGAGATCTACACCTCTCTATTCGTCGGCAGCGTC
Index N503	AATGATACGGCGACCACCGAGATCTACACTATCCTCTTCGTCGGCAGCGTC
Index N504	AATGATACGGCGACCACCGAGATCTACACAGAGTAGATCGTCGGCAGCGTC
Index N505	AATGATACGGCGACCACCGAGATCTACACGTAAGGAGTCGTCGGCAGCGTC

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Index N506	AATGATACGGCGACCACCGAGATCTACACACTGCATATCGTCGGCAGCGTC

Buffers

5x Tris-DMF (50 mM Tris-HCl pH 7.5, 25 mM $MgCl_2$, 50% Dimethylformamide)

STE Buffer (10 mM Tris-HCl pH 8.0, 50 mM NaCl, 1mM EDTA, pH 8.0)

ATAC Lysis Buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 0.1% IGEPAL CA-630)

Transposome Preparation:

** **Critical:** Many recommend adding Magnesium to help stabilize two annealing oligos. This is not recommended for the adapter oligo annealing. Magnesium ions are the catalytic ion required for Tn5 transposase activity. Any Magnesium ion content during transposome assembly can result in concatemers of fully methylated adapter sequences.

1. Dilute oligos to 100 µM in STE buffer.

2. In a 200µl PCR tube prepare oligo mixture:

10µl 100µM Tn5mC-Apt1 oligo
10µl 100µM Tn5mC1.1-A1block oligo
\+ 80µl Nuclease Free Water
100µL total volume

3. Incubate oligo mixture in a PCR thermocycler to anneal adapters as follows:

95°C, 3 min
65°C, 3 min
Ramp down to 24°C, -1°C/cycle, 30 sec/cycle
Hold at 24°C

During incubation heat approximately 500µL of 100% glycerol to 98°C for 2 min. While glycerol is still hot pipette 100µL into a clean eppendorf tube and cool on ice for 5 min.

4. After incubation add 100µL oligo mixture into the cooled 100µL volume of 100% glycerol. Mix well by vortexing and stirring with a micropipette tip. This is the final adapter mixture at 50% glycerol, 5µM adapters.

5. Mix as follows:

10 µl 5µM Adapters
\+ 10 µl Tn5 (4-5mg/mL in Tn5 storage buffer)
20µL transposome assembly

6. Let mixture stand at room temp for 30-60min, then immediately place on ice. Any remaining assembled Tn5 can be stored at -20°C.

Cell preparation

**** Critical:** This section assumes preparation of a single cell suspension. Large numbers of cell clumps can cause inaccurate cell counts and inefficient Tn5 transposase activity. If harvesting adherent cells the TrypLExpress reagent is recommended followed by a PBS wash. Cells should be resuspended in cold PBS or culture media without trypsin contamination.

7. Count PBS cell suspension with a manual or automated cell counter. Trypan blue is recommended to assess cell viability.

8. According to the cell count, pipette a volume of PBS cell suspension corresponding to $2x10^5$ cells into a pre-chilled 1.5mL eppendorf tube.

9. Centrifuge cells in eppendorf tube at 500g for 5 min at 4°C.

10. Remove the supernatant and add 150µL ice-cold ATAC lysis buffer

**** Critical:** Cells should not be allowed to incubate with lysis buffer for extended durations. It is recommended to maintain a sample number less than 15 to reduce sample processing times.

11. Resuspend the cell pellet by gently pipetting 10 times with a 200µL micropipette tip.

12. Immediately centrifuge cells in eppendorf tube at 500g for 10 min at 4°C.

13. Remove supernatant being careful not to disturb pelleted nuclei and continue immediately to transposition reaction.

**** Critical:** When removing supernatant from the nuclei pellet it is best to avoid removing the entire volume to avoid accidentally aspirating away nuclei. Generally leaving <= 5µL of supernatant is compatible with this protocol.

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Transposition Reaction:

14. Prepare transposition master mix of:

150µl	Nuclease free water
+ 40µl	5x Tris-DMF
190µL	total volume

15. Resuspend the nuclei pellet by gently pipetting up/down with a 200µL micropipette tip 3 times in 190µL of transposition reaction master mix.

16. Lastly, add 10µL assembled transposome and flick gently to mix.

17. Incubate the transposition reaction at 37°C for 30 min at 700RPM in an Eppendorf Thermomixer. The shaking is not crucial but enhances the tagmentation reaction. Alternatively one can flick the tubes every 5-10 min in a water bath.

18. Stop the reaction by adding 1mL Zymo DNA Binding Buffer from the Zymo DNA clean & concentrator-5 kit and vortexing briefly.

**** Pause point:** Samples may be stored at -20°C in Zymo DNA binding buffer for several months. Samples must be allowed to warm to room temperature again when processing further as DNA binding buffer components usually precipitate at -20°C.

19. Pipette 500µL transposition reaction and DNA binding buffer mixture into a Zymo DNA Clean and Concentrator-5 spin column.

20. Centrifuge at 12,500g for 30 sec at room temp. Discard flow through.

21. Repeat step 20 with the same spin column to load the remaining volume of transposition reaction and DNA binding buffer transposition mixture. Discard flow through.

**** Critical:** Old or improperly stored DNA wash buffer containing ethanol can result in inefficient DNA recovery during purification. It is recommended to always properly seal the wash buffer storage container or prepare aliquots of fresh wash buffer as needed.

22. Wash spin column by adding 200µL Zymo DNA wash buffer that has been previously supplemented with molecular grade 100% ethanol according to manufacturer instructions and centrifuging at 12,500g for 30 sec at room temp. Discard flow through.

23. Repeat wash step 22

24. Transfer spin column to a clean 1.5mL eppendorf and add 15µL nuclease free water directly to the column matrix.

25. Incubate spin column at room temp for 2 min.

26. Centrifuge column at 12,500g for 1 min to elute DNA. Store the eluted DNA on ice or at -20°C.

Gap Repair:

27. Prepare the following gap repair master mix on ice:

	2µl	10µM Tn5mC-Repl01 oligo
	2µl	10x Ampligase buffer
+	2µl	10mM dNTPs (2.5mM of each 4 dNTPs)
	6µl	total volume/gap repair reaction

28. Mix the following on ice in a PCR tube:

	11µL eluted DNA from transposition reaction
+	6µL of gap repair master mix
	17µL total volume

29. Incubate the mixture as follows:

50°C for 1 min
45°C for 10 min
Ramp down to 37°C at -0.1°C/second
Hold at 37°C

30. Add 1uL T4 DNA Polymerase and 2.5uL Ampligase to the incubated sample without removing the tube from the thermocycler. Do not make a master mix of these two enzymes, as their manufacturer storage buffers are likely incompatible. Add them in two separate additions. Mix well by pipetting up/down 10 times with a 20µL micropipette or multi-channel micropipette.

31. Incubate as follows:

37°C for 30 min
Hold at 4°C

32. Reserve 2µL for a test amplification to determine library distribution pre-bisulfite conversion. It is recommended to reserve as 2µL reaction mix + 8µL nuclease free water. Immediately use this material to prepare a pre-bisulfite PCR.

33. Add 2µL 250mM EDTA to stop the gap repair reaction.

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Bisulfite conversion:

34. Mix the following in a 1.5mL eppendorf and vortex briefly:

	20µl	gap repaired DNA fragments
+	130µl	CT conversion reagent (Zymo EZ DNA Methylation-Lightning Kit)
	150µl	total reaction volume

35. Spin down eppendorf tube to ensure no droplets are on the sides or lid of eppendorf tube

36. Distribute 150µL of DNA and conversion reagent mixture across three PCR tubes, 50µL/tube

37. Spin down PCR tubes to ensure no droplets are on the sides or lid of eppendorf tube.

38. Incubate the conversion reagent mixture as follows:

98°C for 8 min
54°C for 60 min
Hold at 4°C

**** Critical:** Do not spin down after denaturation, only before. Droplets on the sides of the tube are likely to be inefficiently denatured/bisulfite converted.

39. Add 600µl of M-Binding Buffer from the Zymo EZ DNA Methylation-Lightning Kit to the supplied spin column.

40. Recombine the 3 separate 50µL bisulfite conversions into a single 150µL volume and add to spin column with the M-Binding buffer.

41. Invert the capped spin column 5-7 times to mix M-Binding Buffer with bisulfite converted DNA

42. Centrifuge at 12,500g for 30 sec. Discard flow through.

43. Add 100 µl M-Wash Buffer to the spin column

44. Centrifuge at 12,500g for 30 sec.

45. Add 200µl L-Desulphonation Buffer from the Zymo EZ DNA Methylation-Lightning Kit, let column stand at room temp for 20 min.

46. Centrifuge at 12,500g for 30 sec. Discard flow through.

47. Wash column with 200 µl M-Wash buffer from the Zymo EZ DNA Methylation-Lightning Kit that has been previously supplemented with molecular grade 100% ethanol according to manufacturer instructions and centrifuge at 12,500g for 30 sec at room temp.

48. Repeat wash step 47.

49. Transfer spin column to a clean 1.5mL eppendorf tube.

50. Add 25µL M-Elution Buffer directly to the column matrix.

51. Incubate spin column at room temp for 2 min.

52. Centrifuge column at 12,500g for 1 min to elute DNA.

PCR Amplification and barcoding:

53. Assemble the following PCR reaction:

25µl 2x Kapa HiFi HotStart Uracil + Ready Mix
1.5µl 10µM Forward Primer (N7 index)
1.5µl 10µM Reverse Primer (N5 index)
\+ 22µl gap repaired, bisulfite converted DNA eluate
50µL total reaction volume

54. Incubate PCR reaction as follows:

1 cycle of:
98°C for 45 sec

10 cycles of:
98°C for 15 sec
62°C for 30 sec
72°C for 30 sec

Final extension 72°C for 2 min

55. Mix 250µL of Zymo DNA Binding Buffer with the 50µL PCR reaction in a 1.5mL eppendorf tube

56. Pipette DNA binding buffer mixture into a Zymo DNA Clean and Concentrator-5 spin column.

57. Centrifuge at 12,500g for 30 sec at room temp. Discard flow through.

58. Wash spin column by adding 200µL Zymo DNA wash buffer that has been previously supplemented with molecular grade 100% ethanol according to manufacturer instructions and centrifuging at 12,500g for 30 sec at room temp. Discard flow through.

59. Repeat wash step

60. Transfer spin column to a clean 1.5mL eppendorf and add 22µL nuclease free water directly to the column matrix.

61. Incubate spin column at room temp for 2 min.

62. Centrifuge column at 12,500g for 1 min to elute DNA. Store the eluted DNA on ice or at -20°C.

Library Analysis:

63. Analyze DNA libraries on an Agilent Tape Station using a D5000 screentape. Alternatively a 5% bis-acrylamide DNA gel can be used.

Example D5000 tapestation electrophoresis image of a successful ATAC-Me libraries: