APEX-Seq

Introduction

Get started by giving your protocol a name and editing this introduction.

Materials

- Labeling Media
  - 500 µM Biotin-phenol
  - in cell culture media (normal DMEM + FBS)
- 2X Quenching Buffer
  - 10 mM Trolox
  - 20 mM sodium ascorate
  - in DPBS (for cell culture)
- H_2O_2 (to start reaction)
  - 100 mM H_2O_2
  - in H_2O
- RNA Digest Buffer
  - 500 mM Tris, pH 7.5
  - 10 mM EDTA
  - H_2O
- Buffer 1
  - PBS
  - 1 mM $MgCl_2$
  - 0.5 % Sodium Deoxycholate
- Blocking Buffer
  - 5X Deinhardt's Reagent (Thermo Fisher Scientific: cat #: 750018)
  - 150 µg/ml Poly IC (InvivoGen)
  - Make up in Buffer 1
- Buffer 2
  - 6M Urea, pH 8
  - 0.1% SDS
  - In PBS
- Buffer 3
  - 2% SDS

- In PBS

- Buffer 4
  - 750 mM NaCl
  - 0.5% sodium deoxycholate
  - 0.1% SDS
  - In PBS
- Buffer 5
  - 150mM NaCl
  - 0.5% sodium deoxycholate
  - 0.1% SDS
  - In PBS
- RNase T1/A
  - thermo fisher scientific
- AMPure XP Beads
- RNA cleanXP beads
- NEB Ultra RNA II Library Prep

Procedure

in vivo labeling (protein or RNA)

1. Plate cells in 15 cm dish (for protein labeling) or 10 cm dish (for RNA labeling) & TET induce fusion protein for appropriate amount of time.
2. Incubate cells for 30 minutes with Biotin-phenol containing media at 37°C
3. Add H_2O_2 to 1 mM final concentration and incubate for 1 minute with gently but consistent shaking of plate
4. Immediately add 2X quenching reagent.
5. Lyse cells with ~ 800 µL TRIzol (Qiagen) and use a cell scraper to collect cells.
6. Vortex tubes well to make sure there are no tissue clumps.

RNA precipitation

Note: I prefer performing a TRIzol cleanup rather than a Direct-zol clean up, since you end up with more material.

7. Add 200 µL of Chloroform and vortex for 15 seconds well.

8. Incubate at room temp for 5 minutes.
9. Centrifuge at 20,000g for 20 min at 4°C
10. Collect aqueous phase, and mix with 0.1X sodium acetate, and ~500 µL isopropanol and chill at -80°C for 1 hour.

    Note: in principle, biotinylated proteins can be collected from the organic phase in order to perform both protein and RNA labeling from the same experiment. I have not had time to test this, however.

11. Precipitate RNAs by centrifuging at 20,000g for 20 min at 4°C
12. Perform two cold ethanol (70%) washes and resuspend pellet in H_2O.

DNase I treatment

13. Perform a scaled up DNase I digest following NEB DNase I protocol.
14. While performing DNase I clean up, bring RNA CleanXP beads to room temp
15. Add 1.8X bead volume to reaction volume, and incubate for 5 min at room temp
16. Load samples onto magnetic stand and incubate for 5 min.
17. Perform two 70% ethanol washes, being careful not to disturb the beads.
18. Let beads dry for ~5 minutes, but make sure not to overdry the beads as this will affect elution.
19. Elute RNA from beads with H_2O (elution volume will depend on how initial volume of beads used. I tend to elute in ~ 30 µL)

RNA fragmentation

20. Set up reaction in PCR strip tubes and perform reaction on ice.
21. CRITICAL Dilute RNase T1/A to 10^{-5} U. Use diluted (to 10^{-5} U) RNAse T1/A for reaction
22. Normalize RNA samples and set up a reaction where the final concentration is 1 µg/µL of RNA. A 100 µg reaction should look something like this:

Table5

	A	B	C	D	E	F
1	**Name**	**to 100 µg (µL)**	**10X Buffer (µl)**	**RNase T1/A (µl)**	**H2O (µl)**	**Total**
2	Sample 1	21.6	10	10	58.4	100 µL

23. CRITICAL Add RNase T1/A to reaction last.

    Note: I prefer to add RNase T1/A to the caps of the tubes and I then spin down the PCR strip to ensure all tubes received RNase at the same time.

24. Briefly flick PCR strip tube and spin down again (briefly)

25. Incubate in thermocycler for 10 min at 37°C

26. Immediately place the samples back on ice and quench reaction with 400 µL of TRIzol.

27. Add 500 µL more of TRIzol for a total of 1 ml.

28. Perform TRIzol clean up like that mentioned above.

29. CRITICAL After clean up, check the RIN value for the RNA in a bioanalyzer.

30. Aliquot ~ 20 ng RNA prior to pulldown for later sequencing.

Streptavidin Pulldown

31. C1 Streptavidin beads (10 µl per sample; Thermo Fisher Scientific) were washed three times with Buffer 1

32. Washed once and blocked for 30 minutes with Blocking Buffer

33. Remove Blocking Buffer and replace with extracted RNA samples in fresh Blocking Buffer

34. Incubate samples at room temperature for 1 hour and then wash twice with Buffer 2

35. Wash samples once with Buffer 3

36. Wash samples once with Buffer 4

37. Wash samples once with Buffer 5

38. RNAs were eluted from Streptavidin beads by denaturation in 300 µl TRIzol.

39. RNAs can be cleaned up using directo-zol kit (adding 300 µL of 100% EtOH, and following manufacturer recommendation).

40. Elute RNA in 6 µl H_2O

41. Follow NEB ULTRA II RNA library prep according to manufacturers intrusctions.

42. If you are performing rRNA depletion, do so at this step.

    Note: I tend to get on average 5X more reads if I perform rRNA depletion. NEB has a rRNA depletion kit that works for low input material which I recommend, but it is not cheap. If you do perform rRNA depletion, I would quantify samples because it will be useful downstream at PCR step. If you choose to skip rRNA depletion, I would still quantify samples after Streptavidin pulldown because it will be useful at the PCR step.

RNA Fragmentation and Priming Starting from Intact or Partially Degraded RNA

43. Assemble the following fragmentation and priming reaction on ice

Table1

	A	B
1		µl
2	RNA	5
3	NEBNext First Strand Synthesis Reaction Buffer (5X)	4
4	Random Primers	1

44. Mix thoroughly by pipetting up and down several times

45. Place the sample on a thermal cycler and incubate the sample at 94°C following the recommendations in Table 2.5.1 below for fragment sizes ~200 nt.

46. CRITICAL Digest for **7min at 94°C** (RIN values should be larger than 2 and less than 7). Otherwise, see NEB protocol for referring to different RIN values. For highly degraded samples (RIN = 1 to 2) (e.g. FFPE), which do not require fragmentation, follow the library preparation protocol in Chapter 3.

47. Immediately transfer the tube to ice and proceed to First Strand cDNA Synthesis

First strand cDNA synthesis

48. Assemble the first strand synthesis reaction on ice by adding the following components to the fragmented and primed RNA.

Table2

	A	B
1		µl
2	Fragmented and Primed RNA	10
3	NEBNext Strand Specificity Reagent	8
4	NEBNext First Strand Synthesis Enzyme Mix	2

49. Mix thoroughly by pipetting up and down several times

50. Incubate the sample in a preheated thermal cycler with the heated lid set at ≥ 80°C as follows:

    CRITICAL Note: If you are following recommendations in Appendix A (NEB RNA ULTRA II), for longer RNA fragments, increase the incubation at 42°C to 50 minutes in Step 2. I always use 15 min at 42C, however.
    10 min at 25C
    15 min at 42C
    15 min at 70C
    Hold at 4C

51. Proceed directly to Second Strand cDNA Synthesis

Perform Second Strand cDNA Synthesis

52. Assemble the second strand cDNA synthesis reaction on ice by adding the following components into the first strand synthesis product from

Table10

	A	B
1		µl
2	First Strand Synthesis Product	20
3	NEBNext Second Strand Synthesis Reaction Buffer with dUTP (10X)	8
4	NEBNext Second Strand Synthesis Enzyme Mix	4
5	Nuclease-free Water	48

53. Keeping the tube on ice, mix thoroughly by pipetting up and down several times.

54. CRITICAL Set thermalcycler lid to ≤ 40°C

55. Incubate in a thermal cycler for 1 hour at 16°C with a ≤ 40°C lid.

Purify the Double-stranded cDNA

56. Vortex AMPure XP Beads to resuspend.

57. Add 144 µl (1.8X) of resuspended AMPure XP Beads to the second strand synthesis reaction (~80 µl). Mix well on a vortex mixer or by pipetting up and down at least 10 times

58. Incubate for 5 minutes at room temperature

59. Quickly spin the tube in a microcentrifuge to collect any sample on the sides of the tube. Place the tube on an appropriate magnetic rack to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets

60. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

61. Repeat wash for a total of 2 washing steps

62. Air dry the beads for 5 minutes while the tube is on the magnetic rack with lid open.

63. CRITICAL Caution: Do not over dry the beads. This may result in lower recovery of DNA target.

64. Remove the tube from the magnetic rack. Elute the DNA from the beads by adding 53 µl 0.1X TE Buffer (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down several times. Quickly spin the tube and incubate for 2 minutes at room temperature. Place the tube on the magnetic rack until the solution is clear.

65. Remove 50 µl of the supernatant and transfer to a clean nuclease-free PCR tube

66. PAUSE Note: If you need to stop at this point in the protocol samples can be stored at –20°C.

End Prep of cDNA Library

67. Assemble the end prep reaction on ice by adding the following components to the second strand synthesis product

Table4

	A	B
1		µl
2	Second Strand Synthesis Product	50
3	NEBNext Ultra II End Prep Reaction Buffer	7
4	NEBNext Ultra II End Prep Enzyme Mix	3

68. Set a 100 µl or 200 µl pipette to 50 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

    CRITICAL Note: It is important to mix well. The presence of a small amount of bubbles will not interfere with performance.

69. Incubate the sample in a thermal cycler with the heated lid set at ≥ 75°C as follows

    30 minutes at 20°C
    30 minutes at 65°C
    Hold at 4°C

70. Proceed immediately to Adaptor Ligation

Adaptor Ligation

71. Dilute the •(red) NEBNext Adaptor* prior to setting up the ligation reaction in ice-cold Adaptor Dilution Buffer and keep the adaptor on ice

Table3

	A	B
1	**TOTAL RNA INPUT**	**DILUTION REQUIRED**
2	1,000 ng - 101 ng	5-fold dilution in Adaptor Dilution Buffer
3	100 ng - 10 ng	25-fold dilution in Adaptor Dilution Buffer
4	5 ng	200-fold dilution in Adaptor Dilution Buffer

72. The adaptor is provided in NEBNext Singleplex (NEB #E7350) or NEBNext Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E6609 or #E7600) Oligos for Illumina

73. Assemble the ligation reaction on ice by adding the following components, in the order given, to the end prep reaction product

Table11

	A	B
1	**LIGATION REACTION**	**µl**
2	End Prepped DNA	60
3	Diluted Adaptor	2.5
4	NEBNext Ligation Enhancer	1
5	NEBNext Ultra II Ligation Master Mix	30

74. CRITICAL Note: The Ligation Master Mix and Ligation Enhancer can be mixed ahead of time and is stable for at least 8 hours @ 4°C. **We do not recommend premixing the Ligation Master Mix, Ligation Enhancer and adaptor prior to use in the Adaptor Ligation Step**.

75. Set a 100 µl or 200 µl pipette to 80 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

76. CRITICAL Caution: The NEBNext Ultra II Ligation Master Mix is very viscous. Care should be taken to ensure adequate mixing of the ligation reaction, as incomplete mixing will result in reduced ligation efficiency. The presence of a small amount of bubbles will not interfere with performance.

77. Incubate 15 minutes at 20°C in a thermal cycler.

78. Add 3 µl • (blue) USER Enzyme to the ligation mixture from Step 2.10.4, resulting in total volume of 96.5 µl.

79. Mix well and incubate at 37°C for 15 minutes with the heated lid set to ≥ 45°C.

80. Proceed immediately to Purification of the Ligation Reaction

Purification of the Ligation Reaction

81. Use AMPure XP Beads (or NEBNext Sample Purification Beads)

    Note: If you are selecting for larger size fragments (> 200 nt) follow the size selection recommendations in Appendix A of NEB ULTRA RNA II manual

82. Add 87 µl (0.9X) resuspended AMPure XP Beads and mix well on a vortex mixer or by pipetting up and down at least 10 times.

83. Incubate for 10 minutes at room temperature.

84. Quickly spin the tube in a microcentrifuge and place the tube on an appropriate magnetic rack to separate beads from the supernatant. After the solution is clear (~ 5 minutes), discard the supernatant that contains unwanted fragments. Caution: do not discard the beads.

85. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

86. Repeat Step once for a total of 2 washing steps.

87. Briefly spin the tube, and put the tube back in the magnetic rack.

88. Completely remove the residual ethanol, and air dry beads until the beads are dry for 5 minutes while the tube is on the magnetic rack with the lid open.

    CRITICAL Caution: Do not overdry the beads. This may result in lower recovery of DNA.

89. Remove the tube from the magnetic rack. Elute DNA target from the beads by adding 17 µl 0.1X TE (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down. Quickly spin the tube and incubate for 2 minutes at room temperature. Put the tube in the magnet until the solution is clear

90. Without disturbing the bead pellet, transfer 15 µl of the supernatant to a clean PCR tube and proceed to PCR enrichment.

91. PAUSE Note: If you need to stop at this point in the protocol samples can be stored at –20°C.

PCR Enrichment of Adaptor Ligated DNA

92. Check and verify that the concentration of your oligos is 10 µM on the label.

93. **Follow Section 2.12.1A** if you are using the following oligos (10 µM):

    NEBNext Singleplex Oligos for Illumina (NEB #E7350) NEBNext Multiplex Oligos for Illumina (Set 1, NEB #E7335) NEBNext Multiplex Oligos for Illumina (Set 2, NEB #E7500) NEBNext Multiplex Oligos for Illumina (Set 3, NEB #E7710) NEBNext Multiplex Oligos for Illumina (Set 4, NEB #E7730) NEBNext Multiplex Oligos for Illumina (Dual Index Primers, NEB #E7600)

94. Set up the PCR reaction as described below based on the type of oligos (PCR primers) used.

Table12

	A	B
1		**µl**
2	Adaptor Ligated DNA	7.5
3	NEBNext Ultra II Q5 Master Mix	25
4	Universal PCR Primer /i5 Primer*, **	5
5	Index (X) Primer/i7 Primer*,***	5
6	H2O	7.5
7	total	50

95. Mix well by gently pipetting up and down 10 times. Quickly spin the tube in a microcentrifuge

96. Place the tube on a thermocycler with the heated lid set to 105°C and perform PCR amplification using the following PCR cycling conditions (refer to Table 2.12.3A and Table 2.12.3B)

Table13

	A	B	C	D
1	**CYCLE STEP**	**TEMP**	**TIME**	**CYCLES**
2	Initial Denaturation	98	30s	1
3	Denaturation	98	10s	**7-16*,****
4	Annealing/Extension	65	75s	
5	Final extension	65	5 min	1
6	Hold	4		

97. Total RNA input recommended PCR cycles

Note: I guide myself based on the amount of RNA I added to the fragmentation and priming step (i.e. fragmentation starting at library prep stage after streptavidin pulldown). If you perform rRNA depletion, I would not use the amount that went into the rRNA depletion to decide how many cycles to perform. Instead I would use amount after rRNA depletion.

Table6

	A	B
1	**Total RNA Input**	**Recommended PCR Cycles**
2	1000 ng	7-8
3	100 ng	11-12
4	10 ng	14-15
5	5 ng	15-16

98. Purification of the PCR Reaction using SPRIselect Beads or NEBNext Sample Purification Beads

Purification of the PCR Reaction

99. Vortex AMPure XP Beads (or NEBNext Sample Purification Beads) to resuspend

100. Add 45 µl (0.9X) of resuspended beads to the PCR reaction (~ 50 µl). Mix well on a vortex mixer or by pipetting up and down at least 10 times

101. Incubate for 5 minutes at room temperature.

102. Quickly spin the tube in a microcentrifuge and place the tube on an appropriate magnetic rack to separate beads from the supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

103. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

104. Repeat once for a total of 2 washing steps.

105. Air dry the beads for 5 minutes while the tube is on the magnetic rack with the lid open.

106. **Caution: Do not overdry the beads. This may result in lower recovery of DNA**

107. Remove the tube from the magnetic rack. Elute the DNA target from the beads by adding 23 µl 0.1X TE (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down several times. Quickly spin the tube in a microcentrifuge and incubate for 2 minutes at room temperature. Place the tube in the magnetic rack until the solution is clear.

108. Transfer 20 µl of the supernatant to a clean PCR tube, and store at –20°C.

109. **Assess Library Quality on a Bioanalyzer**