Crosslinking cells and nuclear extraction:

Day 0

1. Wash 500 million cells (5 15 cm plates of E14 cells/ 10 15 cm dishes for H9 and HEK) with PBS
2. Fix the cells with 7 mL 2mM DSG per 15 cm plate for 45 min at *Room Temp*
3. Remove DSG and wash cells once with PBS
4. Cells are crosslinked with 7 mL of 3% Formaldehde preheated to 37 C and incubated at 37 C for 10 minutes
5. Add 7 mL of 1 M Glycine to stop crosslinking. Incubate at 37 C 5 mins.
6. Wash the cells thrice with 15 mL **cold PBS** and collect in 5mL PBS
7. Spin at 2000g/ 5 minutes/ 4 C (Cells can be frozen at this step. If froze, gently thaw the cells)

Day 1

8. Resuspended in 10 mL of hypotonic **buffer** and incubate on ice for 10 minutes

Buffer component	Final concentration	Stock concentration	Amount of stock for preparing 10 mL
Tris, pH 7.5	10 mM	1 M	100 uL
NaCl	10 mM	5 M	20 uL
PIC	1 X	50 X	200 uL
Water			9680 uL

9. Cells were pelleted by spinning at 3300 g for 7 minutes
10. Cells were lysed by incubating with 10 mL of prechilled lysis buffer

Buffer component	Final concentration	Stock concentration	Amount of stock for preparing 10 mL
Tris, pH 7.5	10 mM	1 M	100 uL
NaCl	10 mM	5 M	20 uL
PIC	1 X	50 X	200 uL

NP40	0.1%	100%	10
Water			9670 uL

11. Provide 20 strokes of pestle A (10 times / 1min on ice / 10 times)
12. Spin cells at 3300 g/ 7 min/ RT
13. Nuclei were lysed by resuspending in buffer RLT (Qiagen) with 2% Sarksosyl.
14. Nuclei were split into ten 1 mL aliquots and sonicated in a covaris ultrasonicator for 5 mins.
15. After sonication lysate was combined and centrifuges at 16000g for 10 minutes to pellet the insoluble material. Only soluble material (supernatant) was used for downstream reactions.
16. 1X volume of isopropanol and 3 mL of silane beads were added to lysate to isolate complexes containing nucleic acids. Incubated with rotation for 2 hours at R.T
17. Washed 3X with buffer RWT and then 3X with 80% ethanol
18. SIlane beads were resuspended in 2 mL 1X PBS.
19. 50 mg NHS-PEG4-biotin was added and biotinylation carried out overnight.

Day 2

20. RNase I (New England Biolabs M0243S) was diluted 10,000x in PBS. 10 µl of diluted RNase I was added per ml of lysate and incubated at 37ºC for 3 minutes.
21. Nucleic acid contain complexes were rebound to the silane beads by adding 3.5 X volume of buffer RLT and 4.5X volume of isporanol. Incubated with rotation at R.T for 1 hour.
22. Beads were washed 3X with 80% ethanol.
23. Complexes were eluted once in 5mL of TE buffer (10 mM Tris pH 7.5, 1mM EDTA) plus 0.1% Tween-20 at 37ºC for 5 minutes, once in 5mL of TE buffer at 37ºC for 5 minutes, and once in 5mL of TE buffer plus 6M GuHCl (Thermo Fisher 24115) at 37ºC for 5 minutes.

Day 3:

Preparation of beads and Streptavidin pull down of digested chromatin

24. 2 mL of GE streptavidin beads were washed thrice with 1X PBST (0.01% Tween 20) and then resuspended in 25 mL of completely denaturing binding and washing buffer

Buffer component	Final concentration	Stock concentration	Amount of stock for preparing 50 mL
Tris.HCl pH 7.5	10 mM	1 M	500 uL
NaCl	200 mM	5 M	2 mL
GuHCl	6 M	8 M	37.5 mL
EDTA	10 mM	500 mM	1 mL
N-lauryl sarcosine	2 %	20 %	5 mL
PMSF	1 mM	200 mM	250 uL
Water			3750 uL

25. Beads suspension was added to chromatin sample from step 23 and rotated at RT overnight

Day 4:

26. Beads were washed thrice with denaturing binding and wash buffer and then thrice with following high salt wash buffer. All washes in 5ml tube

Buffer component	Final concentration	Stock concentration	Amount of stock for preparing 50 mL
Tris, pH 7.5	50 mM	1 M	2500 uL
NaCl	1 M	5 M	10 mL
EDTA	10 mM	500 mM	200 uL
NP40	0.1%	100%	1 mL
SDS	0.1 %	20 %	250 uL
deoxycholate	0.5 %	10 %	2500 uL

NP40	1%	100%	500 uL
PMSF	1 mM	200 mM	250 uL
Water			32800 uL

27. Beads were washed twice with TE wash buffer

Buffer component	Final concentration	Stock concentration	Amount of stock for preparing 50 mL
Tris, pH 7.5	50 mM	1 M	2500 uL
EDTA	1 mM	500 mM	50 uL
Water			47450 uL

28. Beads were washed twice with PNK wash buffer

Buffer component	Final concentration	Stock concentration	Amount of stock for preparing 50 mL
Tris, pH 7.5	20 mM	1 M	1000 uL
MgCl2	10 mM	1 M	500 uL
Triton X 100	0.2 %	10 %	1000 uL
Water			47500 uL

T4 PNK treatment

29. Beads pelleted and each tube was treated with T4 PNK as follows to dephosphorylate 3’ in a 1 mL reaction volume

Buffer	Final	Stock	Amount of stock

component	concentration	concentration	for preparing 1 mL
PNK phosphatase buffer	1X	5X	200 uL
T4 PNK		1 U/uL	50 uL
Triton X100	0.1%	10%	10 uL
Water			740 uL

5 X PNK dephosphorylation buffer is:

Component	Stock Concentration	Final Concentration	1mL
Tris-HCl pH 6.5	1 M	350 mM	350 µL
$MgCl_2$	1 M	50 mM	50 µL
DTT	1 M	10 mM	10 µL
H_2O	-	-	590 µl

30. Incubated at 37 C for 30 minutes 800rpm shaking in a thermocycler.

31. Beads were washed thrice with PNK wash buffer

End Repair module

32. DNA was blunt ended using NEB next end repair module. Beads were resuspended in 500 uL of following buffer

Buffer component	Volume to be added per tube
10 X End Repair reaction Mix	50 uL

10 X end repair enzyme mix	25 uL
Water	425 uL

33. Beads washed thrice with PNK wash buffer

dA Tailing

34. Following buffer components were added to the beads

Buffer component	Volume to be added per tube
NEBuffer 2	60 uL
Klenow Exo (-)	36 uL
10 mM dATP	6 uL
10 % Triton X 100	6 uL
Water	492 uL

35. Incubate at 37 C for 30 minutes with 800 rpm shaking on a thermocycler.

36. Wash 2 X with PNK wash buffer.

37. Resuspend the beads with the following buffer *(check pH by pH paper before adding to the beads, pH should be around 7):*

Component	**Stock Concentration**	**Final Concentration**	**2mL**
Tris-HCl pH 7.0	1 M	100 mM	200 µL

NaCl	5 M	150 mM	60 µL
EDTA	0.5 M	10 mM	40 µL
D-Biotin	50 mM	3 mM	120 µL
Tween-20	10%	0.01%	2 µL
H_2O	-	-	1580 µl

38. Incubate at RT with rotation for 20 minutes
39. Beads pelleted and each tube was washed 3 X with PNK wash buffer.

RNA Linker Ligation

Prepare this reaction in the morning

40. Linkers have been procured as two single stranded sequences from IDT. The linker sequence proposed is 5'App – NNAGATCGGAAGAGCGTCGTGTAGGGAGGATCCGTTCAGACGTGTGTGCTCTTCCGA/BIODT/CT The colored region will be double stranded. The sense strand was adenylated as follows:

Buffer component	Volume to be added per tube
10 X adenylation buffer	3 uL
1 mM ATP	3 uL
200 uM oligo	4.5 uL
MTH RNA ligase	18 uL
Water	1.5 uL

41. Incubate at 65 C for 1 hour.
42. Inactivate enzyme by incubating at 85 C for 5 minutes
43. Antisense strand is annealed by adding 9 uL of oligo to the mix and running the following program on thermocycler

95°C 2 min

71 cycles of 20 s, starting from 95°C and decreasing the temperature by 1°C each cycle down to 25°C

25°C hold

44. Linkers were purified using 200 uL silane beads. Silane beads were washed with Buffer RLT (Qiagen) and then resuspended in 3.5 sample volumes of buffer RLT. Nucleic acids were added to bead suspension. 4.5 sample volume of isopropanol was added and the tube contents were thoroughly mixed.
45. Sample was incubated with beads for 15 minutes at room temperature.
46. Beads were washed twice 80 % ethanol and let to air dry.
47. Sample was eluted in 30 uL water
48. Linker was ligated by resuspending experiment beads (from step 31) in 300 uL of the following buffer.

Buffer component	Final concentration	Stock Concentration	Amount for preparing 300 uL stock
RNA ligase buffer	10 X	1 X	30 uL
Linker			30 uL
T4 RNA ligase 2 trunc KQ		10 u/uL	15 uL
PEG 8000	20%	50%	120 uL
Triton X 100 (Optimize)	0.1%	10 %	3 uL
Water			112 uL

49. Incubated at 22 c for 6 hours and 16 C overnight 800rpm shaking in a thermocycler.

Day 5

50. Reaction stopped by adding 30 uL 500 mM EDTA and re-shake at 16C for 5min

51. Beads pelleted and washed 5 times with PNK wash buffer. Beads pelleted and each tube was treated with T4 PNK as follows to phosphorylate 5' ends

Buffer component	Final concentration	Stock concentration	Amount of stock for preparing 1000 uL
PNK phosphorylation buffer	1X	10X	100 uL
T4 PNK		1 U/uL	50 uL
ATP		10 mM	100 uL
Triton X100	0.1%	10%	10 uL
Water			740 uL

52. Incubated at 37 C for 30 minutes with shaking at 800 rpm on a thermocycler.

53. Beads were washed thrice with PNK wash buffer.

Ligation

54. Following components were added to each tube

Buffer component	Volume for master mix
Water	16.6 mL
10 X ligase buffer	2 mL
10 % Triton X 100	900 uL
100 X BSA	500 uL
T4 DNA ligase (2U/uL)	40 uL

55. Incubated with rotation at 16 C overnight

Day 6

56. Ligation stopped by adding 2000 uL of 0.5M EDTA and rotating at 16 C for 15 minutes
57. Beads pelleted and supernatant removed

Reverse Crosslinking and DNA/RNA extraction

58. Crosslinking was reversed by resuspending in 500 uL of following **extraction buffer**

Buffer component	Final concentration	Stock concentration	Amount of stock for preparing 500 uL
Tris.HCl pH 7	50 mM	1 M	25 uL
SDS	1%	20%	25 uL
EDTA	1 mM	0.5 M	1 uL
NaCl	100 mM	5 M	10 uL
Water			439 uL

59. 25uL of 20 mg/mL Proteinase K was then added to each tube and incubated at 55 C for 4 hours.

60. Nucleic acids extracted twice using equal volume of Phenol:Chloroform:Isoamyl alcohol (25:24:1) , pH 7.9 in a phase lock gel.
61. DNA was precipitated by adding $1/10^{th}$ volume of 3M Na Acetate and 3X volume of ethanol and incubating at -20 C overnight

Day 7

62. DNA was pelleted by spinning at 20000g for 20 minutes at 4 C
63. Pellet was washed with 500 uL of 70% Ethanol and spun down at 20000g for 10 minutes

64. Supernatant discarded and pellet was resuspended in 50uL water.

Removal of unligated Biotin

65. Measure concentration using broad range Qubit kit

66. DNA + RNA was separated into multiple tubes containing maximum 8 ug of DNA each (In PCR strip)

67. To remove unligated biotin following mix was added to each tube

Buffer component	Final concentration	Stock concentration	Amount of stock for preparing 150 uL X8.5
NEBuffer 2	1X	10X	15 uL 127.5
BSA	1 X	100 X	1 uL 8.5
Exo I			5 uL 42.5
RNase Inhibitor			2 uL 17
Water+DNA+RNA			To 144 uL 1224

68. Incubated at 37 C for 30 minutes.

69. Add the following components

Buffer component	Final concentration	Stock concentration	Amount of stock for preparing 150 uL X8.5
dATP	0.1 mM	10mM	1.5 12.75
dGTP	0.1 mM	10 mM	1.5 uL 12.75
T4 DNA polymerase			3 uL 25.5

70. Incubate at 12 C for 2 hours

71. combine the 8 trips and add 120 uL of 0.5 M EDTA to stop the reaction (in 1200ul)

Biotin Pull down of specific chimera

72. 200 uL Streptavidin C1 beads were prepared by washing with following 1X buffer thrice then resuspended beads in 2 X buffer. (recipe provided below)

Buffer component	Final concentration	Stock concentration	Amount of stock for preparing 50 mL
Tris.HCl pH 8	10 mM	1 M	500 uL
NaCl	2 M NaCl	5 M	20 ml
EDTA	1 mM	0.5 M	100 uL
Water			29.4 ml

73. Resuspend beads in 1200uL of 2X b& W buffer

74. The DNA + RNA was added to the beads and incubated at RT for 30 minutes

75. Pulled down with magnet

76. Washed 5 times, 5 min rotation each time, with following wash buffer

Buffer component	Final concentration	Stock concentration	Amount of stock for preparing 10 mL
Tris.HCl pH 7	10 mM	1 M	100 uL
NaCl	4 M NaCl	5 M	8ml
EDTA	1 mM	0.5 M	20 uL
Water			1880 uL

RNA Reverse Transcription

77. RNA reverse transcribed as follows:

Buffer component	Volume to be added per tube
Water	22 uL
5 X Superscript buffer	8 uL
10 mM dNTP	2 uL
100 mM DTT	2 uL
RNAseIn	2 uL
Superscript III	4 uL

78. Incubated at 50 C for 1 hour 800 rpm in a thermocycler

79. Chimera pelleted and washed with the following buffer twice

Buffer component	Final concentration	Stock concentration	Amount of stock for preparing 1 mL
Tris.HCl pH 8	5 mM	1 M	5 uL
NaCl	1 M	5 M	200 uL
Triton X 100	0.1%	10 %	10
EDTA	0.5 mM	0.5 M	1 uL
Water			784 uL

T4 PNK and DNA denaturation

80. Sample phosphorylated using T4 PNK

81. Wash twice with 1X bind & wash buffer

82. Double stranded DNA was then denatured in 100 uL of the following buffer

Buffer component	Final concentration	Stock concentration	Amount of stock for preparing 1 mL
NaOH	0.1M	1 M	100 uL
EDTA	0.1 mM	500 mM	0.2 uL

Water			899.8 uL

83. Incubated at RT for 15 minutes. (get the supernatant!)

84. Beads pelleted and the supernatant neutralized with 14 uL HCl and 10 uL 1 M Tris, pH 7 and then purified using 100 uL Silane beads. Before purification check pH with paper

Circularization

85. Resuspend the eluate in 20 µl ligation mix

Buffer component	Volume to be added per tube
ssDNA	15 uL
10 X CircLigase Buffer II	2 uL
1mM ATP	1uL
50 mM MnCl2	1 uL
CircLigase	1 uL

86. Incubate at 60 C for four hours.

87. Heat inactivate enzyme by heating to 80 C for 10 minutes, hold 4C

Day 8

Annealing and digestion

88. Add 27 µl oligo annealing mix. Cut_oligo is TCGTGTAGGGAGGATCCGTTCAGACGTGTGCTCT/3InvdT/

Buffer component	Volume to be added per tube
Water	23 uL
Cutsmart buffer	3 uL
10 uM Cut_oligo	1 uL

89. Anneal the oligos with the following program:

    95°C 2 min

    71 cycles of 20 s, starting from 95°C and decreasing the temperature by 1°C each cycle down to 25°C

    25°C hold

90. Add 3 µl BamHI (NEB) and incubate for 1 hr at 37°C.

Purification and PCR Amplification

91. Purify using 50 uL Silane beads. Elute in 25 uL water.

Testing and Production PCR:

92. Testing PCR: Use 1uL of cDNA to find the ideal number of PCR cycles for library preparation(test from 10 to 23 cycles)

    EX: 50ul reaction for 5 tests (10, 13, 15, 18, 22)

5ul DNA/25ul 2X NEBnext mastermix , 1ul of each primer, 18ul H2O

93. Production PCR: Use 5 uL of cDNA at the appropriate cycle number to get library for sequencing.