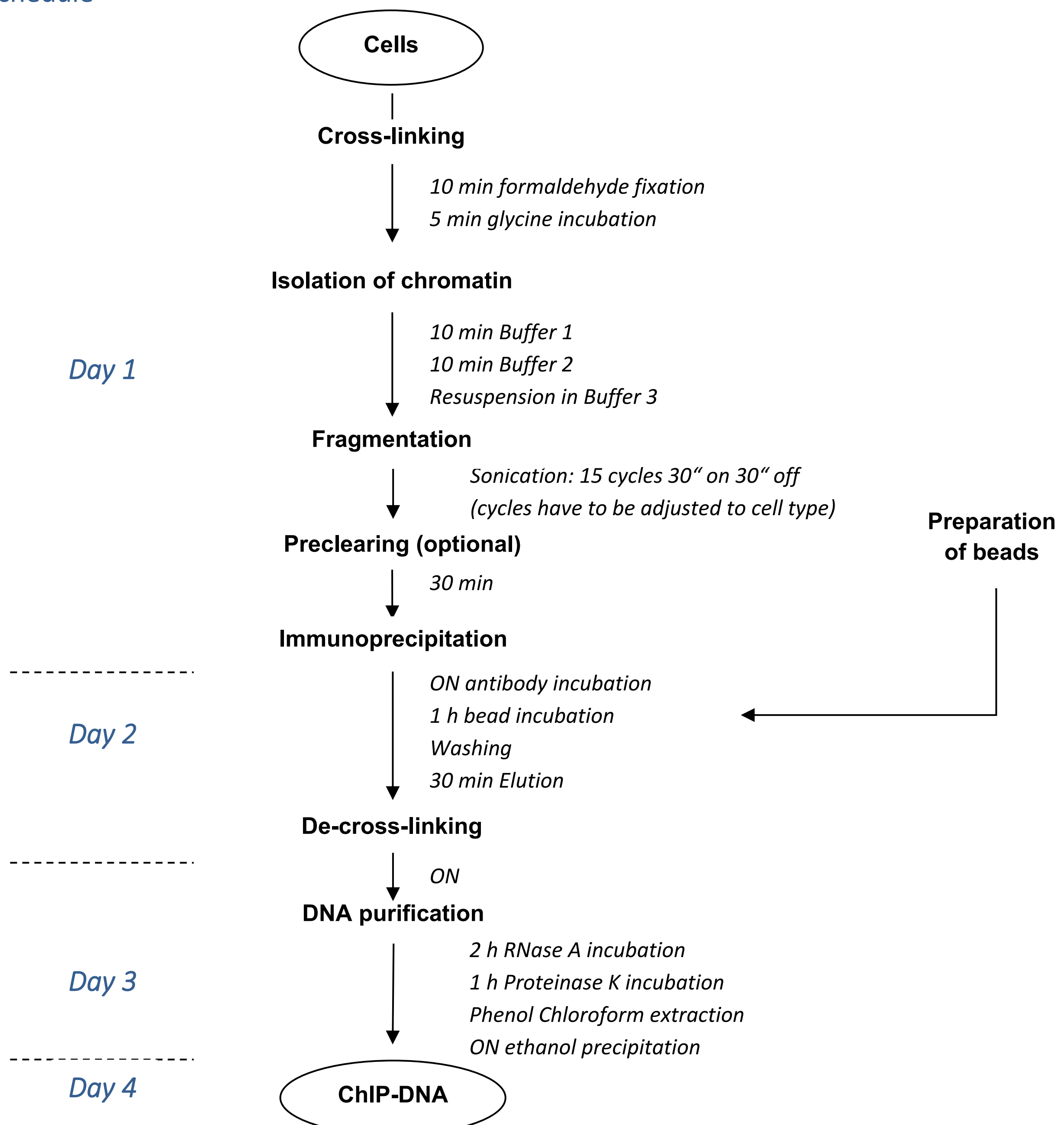
Chromatin Immunoprecipitation (ChIP)

For any questions regarding this protocol, feel free to send an email to info@deep-dv.org

Starting Material

The starting material can be infected or transfected cells expressing the target factor and harboring the target factor binding sites of interest. In general, 1x 10^6 cells per IP are required for experiments with antibodies against modified histones. Additionally, plan to use chromatin from another 1x 10^6 cells for an IgG control and 1/4th of the number of cells used per IP (i.e. 2,5x 10^5 cells) as an input control. For transcription factors or other non-histone DNA binding sites, which are of significantly lower abundance than histones, the cell number used per IP may be increased.

Schedule



Protocol

A: Cross-linking

For adherent cells

1. Trypsinize cells and incubate them at 37°C until cells detach from the plate, resuspend cells in 8 mL cell culture medium and determine the cell number
2. Transfer the cell suspension into a 15 mL tube and add 37 % formaldehyde to a final concentration of 1 %, invert the suspension for several times
   270 µL formaldehyde to 10 mL cell suspension

For suspension cells

1. Determine the cell number
2. Transfer the cell suspension into a 15 mL tube and add 37 % formaldehyde to a final concentration of 1 %, invert the suspension for several times
   270 µL formaldehyde to 10 mL cell suspension
3. Incubate for 10 min at RT
4. Quench the reaction by adding 1/10th the volume of a 1,25 M glycine stock solution and invert the suspension for several times
   1 mL glycine to 10 mL cell suspension
5. Incubate for 5 min at RT
6. Centrifuge for 3 min at 300 g and 4°C and discard the supernatant
7. Resuspend the cell pellet in 950 µL PBS and transfer the suspension into a 1,5 mL tube
8. Centrifuge for 3 min at 300 g and 4°C and discard the supernatant
9. Repeat the last two steps
10. Resuspend the cell pellet in 40 µL PBS
11. Continue with the chromatin isolation or store the pellet at -20°C

B: Isolation of chromatin

12. The following steps should be performed on ice
13. Add 1000x Pefabloc (to 1x final) and 25x Protease Inhibitor Cocktail (to 1x final) to Buffer 1, Buffer 2 and Buffer 3
14. Resuspend the cell pellet in 950 µL Buffer 1 and mix carefully by pipetting up and down
15. Rotate for 10 min at 4°C
16. Centrifuge for 5 min at 2.000 g and 4°C
17. Discard supernatant by pipetting
18. Resuspend the cell pellet in 950 µL Buffer 2 and mix carefully by pipetting up and down
19. Rotate for 10 min at 4°C
20. Centrifuge for 5 min at 2.000 g and 4°C
21. Discard supernatant by pipetting
22. Resuspend the cell pellet in Buffer 3, disrupt the nuclear membrane by pipetting up and down at least 30 times
    Volume of Buffer 3 depends on the cell number; dilute the chromatin ideally to 1x 10^6 cells/100 µL (add 950 µL to 1x 10^7 cells)
23. Continue with the fragmentation or store the suspension at -20°C

C: Fragmentation

For less than 600 µL suspension

24. Switch on the Bioruptor and the attached cooling device (Bioruptor NGS, diagenode)
25. Split the suspension into 50-100 µL aliquots in 0,65 mL Microtubes for DNA Shearing

26. Place the tubes on the corresponding tube holder and carefully place the tube holder on the holding plate
27. Shear the chromatin for 15 cycles (30 sec "on" and 30 sec "off", using highest settings)
28. Continue with step 29

<u>For more than 600 µL suspension</u>

24. witch on the Bioruptor and the attached cooling device (Bioruptor I, diagenode)
25. Split the suspension into 600-1000 µL aliquots in 15 mL Falcon tubes
26. Rinse a metallic bar with EtOH and destH_2O and insert it into the sample
27. Place the tubes on the corresponding tube holder, insert an aluminium ring and carefully place the tube holder on the holding plate
28. Shear the chromatin for 15 cycles (30 sec "on" and 30 sec "off", using highest settings) (adjust cycles according to cell type)
29. Transfer the solution into 1,5 mL tubes
30. Add 1/10 V of Triton X-100 and vortex
31. Centrifuge for 10 min at 20.000 g and 4°C
32. Transfer supernatant (chromatin) into a new 1,5 mL tube
33. Continue with the preclearing or store the fragmented chromatin at -80°C

D: Preperation of beads

33. Add 1000x Pefabloc (to 1x final) and 25x Protease Inhibitor Cocktail (to 1x final) to Dilution Buffer
34. Vortex beads until they are completely resuspended and transfer a sufficient amount of beads into a 1,5 mL tube
    50 µL beads per ChIP experiment
35. Place the tube on a magnetic stand and wait until the supernatant is clear
36. Discard the supernatant with the storage solution and resuspend the beads in 950 µL Dilution Buffer by pipetting up and down
37. Repeat the last two steps
38. Place the tube on a magnetic stand and wait until the supernatant is clear
39. Discard the supernatant and resuspend the beads in 950 µL Dilution Buffer and 50 µL 20 mg/mL BSA by pipetting up and down
40. Rotate ON at 4°C
    Beads can also prepared freshly and rotated for 1 h at 4°C
41. Place the tube on a magnetic stand and wait until the supernatant is clear
42. Discard the supernatant with the storage solution and resuspend the beads in 950 µL Dilution Buffer by pipetting up and down
43. Place the tube on a magnetic stand and wait until the supernatant is clear
44. Resuspend the beads in Dilution Buffer by pipetting up and down
    Use the same volume as in step 34

E: Preclearing (optional when using magnetic beads)

45. Add 1000x Pefabloc (to 1x final) and 25x Protease Inhibitor Cocktail (to 1x final) to Dilution Buffer
46. Vortex beads until they are completely resuspended and transfer a sufficient amount of beads into a 1,5 mL tube
    50 µL beads per chromatin sample
47. Place the tube on a magnetic stand and wait until the supernatant is clear
48. Discard the supernatant with the storage solution and resuspend the beads in 950 µL Dilution Buffer by pipetting up and down
49. Repeat the last two steps

50. Place the tube on a magnetic stand and wait until the supernatant is clear
51. Resuspend the beads in Dilution Buffer by pipetting up and down
    Use the same volume as in step 42
52. Add 50 µL beads to the chromatin sample
53. Rotate for 30 min at 4°C
54. Place the tube on a magnetic stand and wait until the supernatant is clear
55. Transfer the supernatant into a 1,5 mL tube
    supernatant contains the precleared chromatin

F: Immunoprecipitation

56. Set aside a (precleared) chromatin aliquot containing 1/4th of the volume that is used for the IP in step 58 as an input control
    If the concentration of the chromatin is adjusted to 1x10^6 cells/100 µL, use 100 µL for the IP and set aside 25 µL as an input control
57. Continue with the immunoprecipitation or store the (precleared) chromatin at -80°C
58. Dilute the chromatin 1:10 in Dilution Buffer
    If the concentration of the chromatin is adjusted to 1x10^6 cells/100 µL, dilute 100 µL chromatin in 900 µL Dilution Buffer for one ChIP experiment
59. Add 4 µg of the specific antibody or the corresponding control IgG antibody
60. Rotate ON at 4°C
61. Add 50 µL blocked beads
62. Rotate for 1 h at 4°C
63. Add 1000x Pefabloc (to 1x final) and 25x Protease Inhibitor Cocktail (to 1x final) to Low-Salt Wash Buffer, High-Salt Wash Buffer and LiCl Wash Buffer
64. Place the tube on a magnetic stand and wait until the supernatant is clear
65. Discard the supernatant, resuspend the beads in 950 µL Low-Salt Buffer by pipetting up and down and transfer the suspension into a new 1,5 mL tube
66. Place the tube on a magnetic stand and wait until the supernatant is clear
67. Discard the supernatant and resuspend the beads in 950 µL High-Salt Buffer by pipetting up and down
68. Place the tube on a magnetic stand and wait until the supernatant is clear
69. Discard the supernatant and resuspend the beads in 950 µL LiCl Wash Buffer by pipetting up and down
70. Place the tube on a magnetic stand and wait until the supernatant is clear
71. Discard the supernatant and resuspend the beads in 950 µL TE Buffer by pipetting up and down
72. Place the tube on a magnetic stand and wait until the supernatant is clear
73. Discard the supernatant, resuspend the beads in 950 µL TE Buffer by pipetting up and down and transfer the suspension into a new 1,5 mL tube
74. Place the tube on a magnetic stand and wait until the supernatant is clear
75. Discard the supernatant and resuspend the beads in 210 µL Elution Buffer by pipetting up and down
76. Incubate for 30 min at 65°C and 1.000 rpm in a thermo shaker
77. Centrifuge briefly and place the tube on a magnetic stand
78. Transfer 200 µL supernatant into a new 1,5 mL tube

G: De-cross-linking

79. Thaw the input control from step 56 and adjust the volume to 200 µL with Elution Buffer
80. Add 8 µL 5 M NaCl to input and ChIP samples
81. Incubate ON at 65°C and 1.000 rpm in a thermo shaker

H: DNA purification

82. Centrifuge briefly, add 200 µL TE Buffer and vortex
83. Add 2,6 µL RNase A and vortex
84. Incubate for 2 h at 37°C and 1.000 rpm in a thermo shaker
85. Centrifuge briefly, add 7 µL 300 mM $CaCl_2$ and vortex
86. Add 4 µL Proteinase K and vortex
87. Incubate for 1 h at 55°C and 1.000 rpm in a thermo shaker
88. Prepare Phase Lock tubes by centrifuging for 1 min at 18.000 g and 4°C
89. Centrifuge samples briefly and add 400 µL to PhaseLock tubes
90. Add 400 µL PCI solution and vortex
    Work under fume hood; low surface tension
91. Centrifuge for 4 min at 18.000 g and 4°C
92. Add 400 µL PCI solution and vortex
93. Centrifuge for 4 min at 18.000 g and 4°C
94. Add 400 µL chloroform and vortex
95. Centrifuge for 4 min at 18.000 g and 4°C
96. Transfer aqueous phase (phase above the Phase Lock gel) into a new 1,5 mL tube without disturbing the gel
97. Add 24 µL 5 M NaCl and 3 µL glycogen and vortex
98. Add 1055 µL 100 % ethanol and vortex
99. Incubate ON at -80°C
100. Centrifuge for 15 min at 20.000 g and 4°C
101. Discard the supernatant by decanting
102. Add 500 µL 70 % ethanol and shake gently until the pellet is detached from the tube
103. Centrifuge for 15 min at 20.000 g and 4°C
104. Discard the supernatant by pipetting
105. Remove residual fluid in a heat block
106. Collect DNA in 55 µL EB Buffer and store the samples at -20°C

Materials

Cell culture medium
Trypsin
37 % formaldehyde, *Order No. F8775-25ML, Sigma-Aldrich*
Phosphate-buffered saline (PBS), *Order No. D8537, Sigma-Aldrich*
Protein A/G Magnetic Beads, *Order No. 26162, Life Technologies*
BSA, *Order No. B9000S, New England Biolabs*
Ribonuclease A from bovine pancreas, *Order No. R4642-50MG, Sigma-Aldrich*
Proteinase K, *Order No. 732-3276, VWR*
Phenol/Chloroform/Isoamyl alcohol (25:24:1), *Order No. A156.2, Roth*
Trichlormethan/Chloroform, *Order No. 3313.1, Roth*
Glycogen, *Order No. AM9510, Life Technologies*
Ethanol, *Order No. 5054.2, Roth*
EB Buffer, *Order No. 19086, Qiagen*

Pefabloc® SC-Protease-Inhibitor, *Order No. A154.1, Roth*
for a 1000x stock solution dissolve 100 mg in 1 mL DEPC, store for 3 months at -20°C

cOmplete™ Protease Inhibitor Cocktail, *Order No. 11697498001, Roche*
for a 25x stock solution dissolve 1 tablet in 2 mL DEPC, store at -20°C

0.65 ml Bioruptor® Microtubes, *Order No. C30010011, Diagenode sa*

2 mL Phase Lock heavy tubes, *Order No. 2302830, Quantabio*

Solutions and Buffers

Stock Solutions

1,25 M	glycine *(75,07 g/mol)*	*weigh in 4,7 g of glycine, adjust to 50 mL with distilled water, sterile-filter, store at 4°C*
1 M	Hepes-KOH *(238,31 g/mol)*	*weigh in 23,8 g of Hepes, adjust pH with KOH to 7,5 adjust to 100 mL with distilled water, sterile-filter, store at RT*
5 M	NaCl *(58,44 g/mol)*	*weigh in 146,1 g of NaCl, adjust to 500 mL with distilled water, store at RT*
0,5 M	EDTA *(372,24 g/mol)*	*weigh in 46,5 g EDTA, adjust pH with NaOH to 8,0, adjust to 250 mL with distilled water, store at RT*
0,5 M	EGTA *(380,35 g/mol)*	*weigh in 2,9 g EGTA, adjust pH with NaOH to 8,0, adjust to 15 mL with distilled water, store at RT*
1 M	Tris-HCl, pH 8 *(121,14 g/mol)*	*weigh in 60,6 g Tris-HCl, adjust pH with HCl to 8,0, adjust to 500 mL with distilled water, store at RT*
10 %	NP-40	*adjust 25 mL 100 % NP40 to 250 mL with distilled water, store at RT*
10 %	Triton X-100	*adjust 25 mL 100 % Triton X-100 to 250 mL with distilled water, store at RT*
10 %	SDS	*weigh in 25 g SDS, adjust to 250 mL with distilled water, store at RT*

Buffer 1		***250 mL***	
50 mM	Hepes-KOH, pH 7,5	*12,5 mL 1 M Stock*	
140 mM	NaCl	*7 mL 5 M Stock*	
1 mM	EDTA	*500 µL 0,5 M Stock*	
10 %	glycerol	*25 mL 100 % Stock*	store at 4°C
0,5 %	NP-40	*12,5 mL 10 % Stock*	
0,25 %	Triton X-100	*6,25 mL 10 % Stock*	
		in distilled water, sterile-filtered	

Buffer 2		***250 mL***	
10 mM	Tris-HCl, pH 8	*2,5 mL 1 M Stock*	
200 mM	NaCl	*10 mL 5 M Stock*	
1 mM	EDTA	*500 µL 0,5 M Stock*	store at 4°C
0,5 mM	EGTA	*250 µL 0,5 M Stock*	
		in distilled water, sterile-filtered	

Buffer 3		***250 mL***
1 %	SDS	*25 mL 10 % Stock*

10 mM	EDTA, pH 8	*5 mL 0,5 M Stock*	store at RT
50 mM	Tris-HCl,pH 8	*12,5 mL 1 M Stock*	
		in distilled water, sterile-filtered	

Dilution Buffer		***250 mL***	
0,01 %	SDS	*250 µL 10 % Stock*	
1,1 %	Triton X-100	*27,5 mL 10 % Stock*	
1,2 mM	EDTA	*600 µL 0,5 M Stock*	store at 4°C
16,7 mM	Tris-HCl	*4,175 mL 1 M Stock*	
167 mM	NaCl	*8,35 mL 5 M Stock*	
		in distilled water, sterile-filtered	

Low-Salt Wash Buffer		***250 mL***	
0,1 %	SDS	*2,5 mL 10 % Stock*	
1 %	Triton X-100	*25 mL 10 % Stock*	
2 mM	EDTA, pH 8	*1 mL 0,5 M Stock*	store at 4°C
20 mM	Tris-HCl, pH 8	*5 mL 1 M Stock*	
150 mM	NaCl	*7,5 mL 5 M Stock*	
		in distilled water, sterile-filtered	

High-Salt Wash Buffer		***250 mL***	
0,1 %	SDS	*2,5 mL 10 % Stock*	
1 %	Triton X-100	*25 mL 10 % Stock*	
2 mM	EDTA, pH 8	*1 mL 0,5 M EDTA*	store at 4°C
20 mM	Tris-HCl, pH 8	*5 mL 1 M Stock*	
500 mM	NaCl	*25 mL 5 M Stock*	
		in distilled water, sterile-filtered	

LiCl Wash Buffer		***250 mL***	
0,25 M	LiCl	*2,65 g*	
1 %	NP-40	*25 mL 10 % Stock*	
1 %	Na deoxycholate	*2,5 g*	store at 4°C
1 mM	EDTA, pH 8	*500 µL 0,5 M Stock*	
10 mM	Tris-HCl, pH 8	*2,5 mL 1 M Stock*	
		in distilled water, sterile-filtered	

TE Buffer		***1 L***	
10 mM	Tris-HCl, pH 8	*10 mL 1 M Stock*	
1 mM	EDTA, pH 8	*2 mL 0,5 M Stock*	store at RT
		in distilled water, sterile-filtered	

Elution Buffer		***50 mL***	
50 mM	Tris-HCl, pH 8	*2,5 mL 1 M Stock*	
10 mM	EDTA, pH 8	*1 mL 0,5 M Stock*	store at RT
1 %	SDS	*5 mL 10 % Stock*	
		in distilled water, sterile-filtered	

$CaCl_2$ Buffer		***50 mL***	
300 mM	$CaCl_2$	*2,2 g*	
10 mM	Tris-HCl, pH 8	*500 µL 1 M Stock*	store at RT
		in distilled water, sterile-filtered	