Chromatin Immunoprecipitation (ChIP) Protocol
Bioruptor TruSeq
ENCODE4 - Version 1

Materials:

1X PBS
KCl (SIGMA)
HEPES (Invitrogen 15630) (Adjust pH to 7.9)
0.5 M EDTA, pH 8.0 (American Bioanalytica)
Glycerol (99%; America Bioanalytica)
DTT (BioRad)
PMSF (SIGMA 93482-250 mL-F))
Protease Inhibitor (Roche Complete tablets, Cat#1697498)
10X RIPA Buffer (Upstate, Cat#20-188)
Nuclease Free Water
Proteinase K (Ambion, Cat#2546)
RNase A (Qiagen)
Protein A-Agarose, Fast Flow (10 mL, Upstate, Cat#16-156)
Protein G-Agarose, Fast Flow (10 mL, Upstate, Cat#16-266)
50X TE (100 mL, USB, Cat#45834)
20% SDS (American Bioanalytica)
Qiagen PCR Purification Kit (Cat#28106)
Tag Mastermix (Qiagen)
Phosphatase inhibitors (as of 8/1/10)

Inhibitors	Function	Cat Number (Sigma)
1 mM Sodium pyrophosphate ($Na_2P_2O_4$)	Ser/Thr phosphatase	221368-100 g
2 mM Sodium orthovanadate (Na_3VO_4)	Tyr and Alkaline phosphatase	Boston BioProducts BP-440
10 mM Sodium fluoride (NaF)	Ser/Thr and Acidic phosphatase	S6776-100 g

Reagent Preparation:

100 mM PMSF: aliquot and store at 4 ^{0}C.

1M DTT: Dissolve 1.54 g DTT in 10 mL PBS, aliquot and store at -20 ^{0}C

***25X Phophatase Inhibitor*:**

Sodium pyrophosphate: Make 100 mM stock solution in water
Sodium orthovanadate (100 mM): Boston BioProducts BP-440
Sodium fluoride: Make 1M stock solution in water

Mix 50ml of sodium pyrophosphate (100 mM) and 50ml of sodium fluoride (1 M) first and then add 100ml of sodium orthovanadate (100 mM). Aliquot and freeze at -20 ^{0}C.

Hypotonic Solution:

Materials	Volume (mL)	Final Concentration (FC)
1M Hepes, pH 7.9	1.0	20 mM
1M KCl	0.5	10 mM
0.5MEDTA, pH8.0	0.1	1 mM
50% Glycerol	10.0	10%
Nuclease-free water	38.0	
Total Volume	50.0	

Dissolve 2 Protease Inhibitor tablet in 50 mL hypotonic buffer (need time to dissolve, do it half hour before the cells are ready for next step.) Just before use, add 250 µL of 100 mM PMSF and 50 µL of 1M DTT.

1X RIPA Buffer
Dilute 100 mL of 10X RIPA Buffer in 900 mL Nuclease-free water. Store at 4 ^{0}C. (Alternative: Prepare 1X RIPA buffer accordingly with the cold nuclease-free water before use.)

Add protease inhibitor tablets, PMSF, and DTT as for the hypotonic buffer, also add phosphatase inhibitors.

Hypotonic Buffer

Volume (mL)	PMSF (µL)	DTT (µL)
5	25	5
10	50	10
15	75	15
20	100	20
25	125	25
30	150	30
35	175	35
40	200	40
45	225	45
50	250	50

1X RIPA Buffer

Volume (mL)	PMSF (µL)	DTT (µL)	25X Phosphatase inhibitor (µL)
5	25	5	200
10	50	10	400
15	75	15	600
20	100	20	800
25	125	25	1000
30	150	30	1200
35	175	35	1400
40	200	40	1600
45	225	45	1800
50	250	50	2000

Elution Buffers **(PREPARE FRESH BEFORE USE EVERY TIME!!)**

Elute 1: 1% SDS, 1X TE

Total Volume (mL)	50 X TE (mL)	20% SDS (mL)	Nuclease Free Water (mL)
1	0.02	0.05	0.93
2	0.04	0.10	1.86
3	0.06	0.15	2.79
4	0.08	0.20	3.72
5	0.10	0.25	4.65
6	0.12	0.30	5.58
7	0.14	0.35	6.51
8	0.16	0.40	7.44
9	0.18	0.45	8.37
10	0.20	0.50	9.30

Elute 2: 0.67% SDS, 1X TE

Total Volume (mL)	Elute 1 (1% SDS, 1X TE) (mL)	1X TE (mL)
1	0.67	0.33
2	1.34	0.66
3	2.01	0.99
4	2.68	1.32
5	3.35	1.65
6	4.02	1.98
7	4.69	2.31
8	5.36	2.64
9	6.03	2.97
10	6.70	3.30

http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Protein-Expression-and-Analysis/Protein-Sample-Preparation-and-Protein-Purification/ProteinSPProteinIso-Misc/Protein-Isolation/Immunoprecipitation-using-Dynabeads-Protein-A-or-Protein-G

Antibody Compatibility Table:
+++: strong binding, ++: medium binding, +: weak binding, -: no binding.

Species	Ig Subclass	Protein A	Protein G
Human	IgG1, IgG2, IgG4	+++	+++
	IgG3	+	+++
	IgD	-	-
	IgD	+	-
	Fab	+	+
	ScF_v	+	-
Mouse	IgG1	+	++
	IgG2a, IgG2b, IgG3	+++	+++
	IgM	-	-
Rat	IgG1	+	++
	IgG2a	-	+++
	IgG2b	-	+
	IgG2c	+++	+++
Goat	IgG1	+	+++
	IgG2	+++	+++
Sheep	IgG1	+	+++
	IgG2	+++	+++
Cow / Bovine	IgG1	+	+++
	IgG2	+++	+++
Horse	IgG(ab)	+	-
	IgG(c)	+	-
	IgG(T)	-	+++
Rabbit	Total Ig	+++	+++
Dog	Total Ig	+++	+
Cat	Total Ig	+++	+
Pig	Total Ig	+++	+
Guinea pig	Total Ig	+++	+
Chicken	Total Ig	-	-

Protein G and protein A exhibit variation in binding strength to different Immunoglobulins (Ig). This variation exists both between different species and between different antibody subclasses from the same species. (As an example, human IgG3 will bind strongly to protein G, but only weakly to protein A.) The table above gives an overview of binding strengths of protein G and protein A to different Ig species and subclasses.

ChIP Preparation:

Day 1:

Note:
*Needed to add protease inhibitor, PMSF, and DTT to the Hypotonic buffer. Besides protease inhibitor, PMSF, DTT, also add the phosphatase inhibitors to 1X RIPA throughout the protocol if there is no specific instruction.

1. Take out 2 X 10 ^7 of frozen cross-linked cells for each ChIP. Add 5 mL of cold PBS. Thaw cells in a cold room on neutator rocking.

2. Spin the cells at 1,500 rpm for 3 minutes. Remove PBS and add 3 mL of hypotonic buffer. Resuspend cells and leave the tubes on ice for 10 minutes to swell the cells.

*Tap to bottom of the tube to dislodge the cells before adding the buffer which makes the task easier.

3. Dounce homogenize on ice using 7 ml glass homogenizer, 30 strokes, to break open the cells. Keep the total time to 15 minutes for hypotonic lysis/homogenization steps.

4. Aliquot lysate into 2 mL microfuge tubes. Centrifuge at 600 g (rcf) at 4 ^{0}C for 7 minutes to pellet nuclei. Discard supernatant and wash nuclear once with hypotonic buffer.

5. Resuspend nuclear pellets in 2 mL 1X RIPA buffer for each sample and transfer to 15ml Bioruptor tube with sonication beads (Diagenode C01020031) (use 300 µl beads) Incubate 30 minutes on ice. Centrifuge tubes at 3000 rpm for 5 minutes at 4 ^{0}C.

6. Sonicate each sample with Bioruptor Pico. Sonication cycles 30sec on / 30sec off, 16 rounds. Vortex every 4 cycles.

*Optimal sonication condition needed to be determined for any new cell line.

7. Centrifuge tubes at 3000 rpm for 5 minutes at 4 ^{0}C.

8. Transfer 2 ml lysate to two 2 ml or 1.5 ml tubes and clarify by centrifugation at 14,000 rpm at 4 ^{0}C for 15 minutes. Pool supernatants back together in one 15 ml Falcon tubes. Save 100 µl of lysate, label “input”. Store overnight at 4 ^{0}C, then carry through with rest of samples from Day 2 to Day 3, i.e. reverse crosslink at 65 ^{0}C, RNase, proteinase K, and spin column steps.

*sheared chromatin could be snap freeze in liquid nitrogen and stored at -80^{0}C.

9. Add 5ug GFP antibody to each 2 ml lysates (2 X10^7 cells). Incubate at 4 ^{0}C on a neutator for 12-16 hours (overnight).

Day 2:

1. Remove 80 μL of Protein A/G-agarose beads (if the species of GFP antibody is goat, only use protein G agarose beads) for each sample (2X10^7 cells) to 1.5 ml microfuge tubes and wash twice with 1 ml of ice cold 1X RIPA buffer (containing protease inhibitor, DTT, PMSF, and phosphatase inhibitors). Spin at 5,000 rpm for 1 minute at 4 ^{0}C.

2. Resuspend the beads in 80 μL in 1X RIPA. Add beads to tubes containing Ag-Ab complex (use another 80 μL 1X RIPA to wash out the beads) and incubate for 1 hour at 4 ^{0}C with neutator rocking.

3. Centrifuge the tubes at 1,500 rpm for 3 minutes, wash the agarose beads 3 times with 10 mL of fresh, ice cold 1X RIPA buffer (with protease inhibitor, add PMSF, DTT and phosphatase inhibitors just before use), and once with ice-cold PBS. 15 minutes each wash.

*For washing, only use ONE tablet of protease inhibitor per 50 mL 1X RIPA buffer.

4. Resuspend agarose beads in 800 μL ice-cold 1X PBS and transfer the beads from 15 mL tube to a 1.5 mL Eppendorf tube. Wash out the beads in 15 mL tube again with 400 μL ice-cold 1X PBS (to be sure all the beads are collected) and transfer them to Eppendorf tube.

5. Centrifuge Eppendorf tube at 5,000 rpm for 1 minute. Remove PBS completely, add 100 μL of Elute 1 solution (1% SDS, 1X TE) to each tube, resuspend agarose beads. Incubate at 65 ^{0}C for 10 minutes with gentle mixing every two minutes.

6. Spin at 5,000 rpm for 1 minute (room temperature), and remove supernatant to an Eppendorf tube (Elute1). Add 150 μL of Elute 2 solution (0.67% SDS, 1X TE) to beads pellet in each tube. Incubate again at 65 ^{0}C for 10 minutes with occasional gentle vortexing. Spin at 5,000 rpm for 1 minutes (Elute 2). Combine the second elute with the first one.

7. To remove any residual beads, spin tubes at 14,000 rpm for 2 minutes at room temperature. Transfer the supernatants to new 1.5 mL tube (avoid transferring any beads.)

8. Remove input DNA tube (from Day1) stored at 4 ^{0}C and add 150 μL of 1% SDS, 1X TE.

*Hint: Add 1.5X of 1% SDS, 1X TE to input sample.

9. Put ChIP DNA and input DNA overnight at 65 ^{0}C to reverse cross-link.

Day 3:

1. Add 250 µL 1X TE containing 100 µg RNase to each of the sample. Incubate for 30 minutes at 37 ^{0}C.

2. Add 5.0 µL of 20 mg/mL Proteinase K to each sample. Incubate at 45 ^{0}C for 30 minutes.

3. Transfer samples to 15 mL tube. Purify ChIP and Input DNA using Qiagen PCR purification column. Elute DNA in 50 or 35 µL EB (adjust the volume of elution buffer accordingly).

4. Determine the concentration of "Input DNA" (Nanodrop).

*Use 2 µL to read!

Library Preparation:

Step 1: End Repair

Use ChIP DNA prepared from 2 x 10^7 cells

a) Combine and mix the following components in a microfuge tube.

ChIP DNA to be end-repaired	34 µL
10X End-Repair Buffer	5 µL
2.5 mM dNTP Mix	5 µL
10 mM ATP	5 µL
End-Repair Enzyme Mix	1 µL
Total reaction volume	50 µL

b) Incubate at room temperature for 45 minutes.

c) Purify on one QIAquick column using the QIAquick Purification Kit and protocol. Elute in 34 µL EB.

Step 2: Addition of 'A' base to 3` Ends

Prepare stocks of 1 mM dATP using NEB 100 mM dATP, e.g. add 5 µL of 100 mM dATP to 495 µL sterile RNase free Gibco water; then make 50 µL aliquots of this and freeze at -20 ^{0}C.

a) Combine and mix the following components in a microfuge tube.

DNA from step 1	34 μL
Klenow buffer (NEB2)	5 μL
1 mM dATP	10 μL
Klenow (3' to 5' exo minus)	1 μL
Total reaction volume	50 μL

b) Incubate at 37 ^{0}C for 30 minutes.

c) Purify on one QIAquik MinElute column, using the MinElute PCR purification Kit and protocol. Elute in 12 μL EB.

Step 3: Adapter Ligation

a) Dilute the Illumina adapters 1:10 with TE buffer.

b) Combine and mix the following components in a microfuge tube.

DNA purified from Step 2	12 μL
2X DNA ligase buffer	15 μL
*TruSeq Adapter oligo mix with index (1:10)	1 μL
DNA ligase	2 μL
Total reaction volume	30 μL

c) Incubate for 15 minutes at room temperature.

d) Purify on one QIAquik MinElute column, using the MinElute PCR purification Kit and protocol. Elute in 19 μL EB.

Step 4: Size selection

a) Run adapter ligated DNA on a 2% Agarose EX-Gel.

b) Excise gel in the range of 450-650 bp with a clean scalpel. Be sure to take photos of the gel before and after the gel slices are excised.

c) Purify the DNA from the agarose slices using Qiagen Gel Extraction Kit. Elute in 24 μL EB.

Step 5: PCR Amplification

a) Use 0.5 μL of each primer (TruSeq 1.0 and 2.0) (25 μM) in a 50 μL reaction.

b) Combine and mix the following components in a PCR tubes (or plate).

DNA from Step 4	24 μL
Phusion DNA polymerase	25 μL
TruSeq 25uM PCR primer 1.0	0.5 μL
TruSeq 25uM PCR primer 2.0	0.5 μL
Total reaction volume	50 μL

c) Amplify using the following PCR protocol:

Step 1: 98 ^{0}C---30 seconds

Step 2: [98 ^{0}C---10 seconds] --> [65 ^{0}C---30 seconds] --> [72 ^{0}C---30 seconds]

(Go to Step 2): 14 more cycles

Step 3: 72 ^{0}C---5 minutes

Step 4: Hold at 4 ^{0}C

Step 6: **Purification of PCR product by gel cutting**

a) Purify on one QIAquik MinElute column, using the MinElute PCR purification Kit and protocol. Elute in 19 μL EB.

b) Run PCR product on a 2% Agarose EX-Gel.

c) Excise gel in the range of 350-550 bp with a clean scalpel. Be sure to take photos of the gel before and after the gel slices are excised.

d) Purify the DNA from the agarose slices using Qiagen Gel Extraction Kit. Elute in 12 μL EB.

e) Measure the DNA concentration (ng/μL) using Qubit.

f) Run each library on Bioanalyzer

Step7: sample pooling for sequencing

Pool equal mole of different barcode's libraries into one tube and submit for sequencing.