Chromatin Immunoprecipitation (ChIP) Protocol

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-Agaorse, Fast Flow (10 mL, Upstate, Cat#16-156)
Protein G-Agaorse, 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	Sigma 450243-50 g
10 mM Sodium fluoride (NaF)	Ser/Thr and Acidic phosphatase	S6776-100 g

Reagent Preparation:

100 mM PMSF: Dissolve 0.87 g in 50 mL of iso-propanol, aliquot (~550 μL), and store at -20 ^{0}C. We purchased the premade one instead. (Sigma 93482-250 ml-F), which we adequate and store at 4 ^{0}C.

1M DTT: Dissolve 1.54 g DTT in 10 mL PBS, aliquot (~110 μL), and store at -20 ^{0}C

***Phophatase Inhibitor*:**

Sodium pyrophosphate: Make 200 mM stock solution in water

Sodium orthovanadate: See full protocol for preparation of 200 mM stock
-Should be activated for maximal inhibition of protein phosphotyrosyl-phophatases.
1) Prepare a 200 mM solution of sodium orthovanadate.
2) Adjust the pH to 10.0 using either 1N Na OH, or 1N HCl. The starting pH of the solution may vary with lots of the chemical. At pH10.0, the solution will be yellow.
3) Boil the solution until it turns colorless (approximately 10 minutes).
4) Cool to room temperature.
5) Readjust the pH to 10.0 and repeat steps 3 and 4 until the solution remains colorless and the pH stabilize at 10.0.
6) Aliquote and store the activated sodium orthovanadate at -20^{0}C.
This procedure depolymerizes the vanadate, converting it into more potent inhibitor of protein tyrosine phophatase. Please note that adding DTT rapidly inactivates sodium orthovanadate.
Reference: Gordon, J.: Methods Enzymol. (1991)201:477-482
Sodium fluoride: Make 1M stock solution in water

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

Volum(mL)	PMSF(μL)	DTT(μL)	25X Phosphatase(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

Note:
25X Phosphatase Inhibitor: NaF, Na_3VO_4, and $Na_2P_2O_4$

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.

Day 1:

Note:
* This protocol is for 4 TF and 1 IgG control ChIP experiment.
*Needed to add protease inhibitor, PMSF, and DTT to the Hypotonic buffer. Besides protease inhibitor, PMSF, DTT, also add the phophatase inhibitors to 1X RIPA through out the protocol if there is no specific instruction.
*All cross-linked cells are in ORANGE tubes!!
*8 samples (including the control) is the maximum number we will handle per experiment.

1. Take out one 15 mL Falcon tubes, each containing 1 X 10 ^8 of frozen cross-linked cells. Add 6 mL of cold PBS into each tube. Thaw cells in a cold room for 1 hour on neutator rocking.

*Change the volume accordingly when number of cells is differ from 1 X 10^8 cells.

2. Spin the cells at 1,500 rpm for 3 minutes. Remove PBS and add 6 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, 30 strokes, tot break open the cells. Keep the total time to 15 minutes for hypotonic lysis/homogenization steps.

*2 sizes of homogenizer; 7 & 15 mL. Use the 7 mL dounce homogenizer when
the volume is less than 7 mL.

4. Aliquot lysate into 2 mL microfuge tubes. Centrifuge at 600 g (rcf) at 4 ^{0}C for 5 minutes (K562, HeLa S3, and HepG2), or 8 minutes (GM12878) to pellet nuclei. Discard supernatant and wash nuclear once with hypotonic buffer.

5. Resuspend nuclear pellets in 1 mL 1X RIPA buffer. Combine the nuclear lysate to 15 mL Falcon tube, bring up to 6 mL 1X RIPA/10^8 cells. Incubate 30 minutes on ice. If the cell lysis is incomplete, debris will clog the sonicator tips and cause foaming.

*Remove the bubbles before sonication.

6. Transfer **3 ml** nuclear lysate (1X10^8 cells) into a 15 ml Falcon tube (BD 352196). Sonicate each sample with a Branson 250 Sonifier to shear the chromatin. **Constant duty cycle, output control 3-4, 20 seconds pulses**, incubating on ice for 2 min between pulses to cool down. **12 rounds**. Keep the tip few millimeters above the bottom of the tube to avoid foaming.

*It will be 5 X 10^7 cells per tube when starts with 1 X 10^8 cells.

*Make sure to remove the bubbles before sonication.

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

7. Transfer lysates to 2 mL microtubes and clarify by centrifugation at 14,000 rpm for 15 minutes at 4 ^{0}C. Pool supernatants back together in 50 mL falcon tube, and add more 1X RIPA buffer (with protease inhibitor, DTT, PMSF, phosphatase inhibitors) to bring volume up to **10 mL** (***save 100 uL of lysate, label "input". Store overnight at 4 ^{0}C, then carry through with rest of samples from Day 2 to Day 3 with rest of samples,i.e. reverse crosslink at 65 ^{0}C, RNase, porteinase K, and spin column steps***). Split each sample into 2 mL aliquots in 15 mL tubes for parallel immunoprecipitations.

*Snap freeze the unused sheared chromatin in liquid nitrogen. Stored @ -80^{0}C.

8. Add 5ug TF antibody or 5ug normal IgG (control sample) 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 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. Respend the beads in 80 μL in 1X RIPA. Add Protein A/G-agarose to tubes containing Ag-Ab complex (use another 100 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 protein A/G-agarose beads 3 times with 10 mL of **fresh, ice cold** 1X RIPA buffer (with protease inhibitor, add PMSF and DTT 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 Protein A/G-agarose 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 bead 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 (1% SDS, 1X TE)** to each tube, resuspend Protein A/G-agarose. Incubate at 65 ^{0}C for 10 minutes 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 (0.67% SDS, 1X TE)** to Protein A/G-agarose 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 Protein A/G-agarose, 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 sample.

9. Reverse cross-linked ChIP DNA and input DNA overnight at 65 ^{0}C.

*IgG ChIP:	250 µL
TF ChIP:	250 µL
Input DNA:	250 µL

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 the samples to 15 mL tube. Purify ChIP and Input DNA using Qiagen PCR purification column (adjust the volume of elution buffer accordingly). Elute DNA in 50 or 35 µL EB (when working with Pol II antibody; elute DNA in 50 µL EB).

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

*Use 2 µL to read!

5. Follow the next procedure in blue when working with Pol II. The rest of antibodies will skip onto ***the "PREPARTION OF ChIP FOR ILLUMINA SEQUENCING".***

*The following PCR ONLY for Pol II ChIP validation! Proceed to "Preparation of ChIP for Illumina Sequencing".

To determine enrichment of target sequences in the ChIP DNA preparations set up a PCR assay using primers designed from known target genes. Dilute input DNA to 10 ng/µL. Set up PCR reactions with CHIP DNA prepared using specific antibody and normal IgG and input DNA.

PCR reaction mixture:

Tag Mastermix	Qiagen	25.0 µL
F/R primers	10 µM (at a final concentration of 1µM)	1.0 µL
Template	ChIP DNA and Input DNA 10 ng/µL	1.0 µL
H_2O		23.0 µL
Total Volume		50.0 µL

6. PCR amplify DNA using the following conditions:

Step 1: 94 ^{0}C ---4 minutes

Step 2: 94 ^{0}C ---30 seconds

Step 3: 52 ^{0}C ---30 seconds

Step 4: 72 ^{0}C ---30 seconds

Repeat step 2-4 for 28 times.

Step 5: 72 ^{0}C ---10 minutes

Step 6: 4 ^{0}C ---forever

7. Load 10 μL PCR product on gel.

All antibodies followed the "PREPARTION OF ChIP FOR ILLUMINA SEQUENCING".

Step 1: End Repair:

Use ChIP DNA prepared from 2 x 10^7 cells or gel purified ChIP DNA of 0.1-0.3 kb in size.

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

ChIP DNA to be end-repaired	1-34 μL (however much DNA you isolated from the band)
10X End-Repair Buffer	5 μL
2.5 mM dNTP Mix	5 μL
10 mM ATP	5 μL
Sterile water	*X* μL to bring reaction volume to 49 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.

*Follow QIAquick PCR purification protocol.
*Use the column stored at room temperature.

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	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.

*Follow QIAquick PCR purification protocol.
*Use the column stored at 4 ^{0}C.

Step 3: Adapter Ligation

a) Dilute the Illumina adapters 1:10 with water. **DO NOT** reuse diluted adapters.

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 (1:10)	1 µL
DNA ligase	2 µL
Total reaction volume	30 µL

* Truseq Index (1,2,3,4,7,9)

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.

*Follow QIAquick PCR purification protocol.

*Use the column stored at 4 ^{0}C.

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

Day 4:

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

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

f) 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.

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

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