

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 ($\text{Na}_2\text{P}_2\text{O}_4$) | 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°C . We purchased the premade one instead. (Sigma 93482-250 ml-F), which we adequate and store at 4°C .

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

Phosphatase 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-phosphatases.

- 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 -20⁰C.

This procedure depolymerizes the vanadate, converting it into more potent inhibitor of protein tyrosine phosphatase. 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 4⁰C. (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(μ L) | DTT(μ L) | 25X Phosphatase(μ L) |
|-----------|----------------|---------------|---------------------------|
| 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, Na₃VO₄, and Na₂P₂O₄

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:

*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 one 15 mL Falcon tubes, each containing 2×10^7 of frozen cross-linked cells. Add 1 mL of cold PBS into each tube. Thaw cells on ice.

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

* Use the 7 mL dounce homogenizer

4. Transfer lysate to 2 mL microfuge tubes. Centrifuge at 600 g (rcf) at 4 °C for 5 min, Discard supernatant.

5. Resuspend nuclear pellets in 1 mL 1X RIPA buffer. Centrifuge at 600 g (rcf) at 4 °C for 5 min, Discard supernatant.

6. Resuspend nuclear pellets in 2 mL 1X RIPA buffer, Transfer nuclear lysate into 15ml Bioruptor tube with 300ul sonication beads (Diagenode C01020031), (Beads washed with 1ml 1X PBS once and 1X RIPA buffer once) Incubate for 30 min on ice.

7. **Sonication cycles 30sec on / 30sec off , 16 rounds.** Vortexed every 4 cycles.

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

8. Centrifuge tubes at 3000 rpm for 5 minutes at 4 °C.

9. Transfer lysate to 2 ml microfuge tubes and clarify by centrifugation at 14,000 rpm at 4 °C for 15 minutes. Pool supernatants back together in one 15 ml Falcon tubes. Save 100 uL of lysate, label "input". Store overnight at 4 °C, then carry through with rest of samples from Day 2 to Day 3, i.e. reverse crosslink at 65 °C, RNase, proteinase K, and spin column steps. Split pooled supernatants into 2 mL aliquots in 15 mL BD tubes for parallel immunoprecipitations.

*Snap freeze unused sheared chromatin in liquid nitrogen (2ml lysate containing 2 X 10⁷ cells per tube). Store @-80 °C for later use.

10. Add 5ug GFP antibody . Incubate at 4 °C on a neutator for 12-16 hours (overnight).

Day 2:

1. Remove 80 µL of Protein A/G-agarose (Glat antibody use 80 ul Protein G agarose beads) for each sample (2X10⁷ 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 °C.

2. Resuspend 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 °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 °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 °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 °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 °C.

Day 3:

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

2. Add 5.0 µL of 20 mg/mL Proteinase K to each sample. Incubate at 45 °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 ₂ O | | 23.0 µL |
| Total Volume | | 50.0 µL |

6. PCR amplify DNA using the following conditions:

- Step 1: 94 °C ---4 minutes
- Step 2: 94 °C ---30 seconds
- Step 3: 52 °C ---30 seconds
- Step 4: 72 °C ---30 seconds
- Repeat step 2-4 for 28 times.**
- Step 5: 72 °C ---10 minutes
- Step 6: 4 °C ---forever

7. Load 10 µL PCR product on gel.

All antibodies followed the “PREPARTION OF ChIP FOR ILLUMINA SEQUENCING”.

Bravo automatic library preparation

Step 1: End Repair (for 24 reactions) TruSeq-End-Repair protocol

10X buffer: 140 ul

ATP: 140 ul

dNTP: 140 ul

enzyme: 28 ul

Mix the above reagents in one tube and transfer 53 ul of the mixture to the A3-H3 wells of a 1ml Nunc plate.

Transfer 34 ul of ChIP DNA to Twin-Tec Eppendorf 96-well plate A1-H1, A2-H2, A3H3.

Put plates on Bravo.

Step 2: Purification--SPRISelect reagent (Beckman Coulter B23318) –TruSeq 04

AmPure Purification

90 ul beads was used for each well reaction

Step 3: Addition of ‘A’ base to 3` Ends—TruSeq 07 A-Tailing

NEB buffer 2: 140 ul

1mM dATP: 280 ul

Klenow: 28 ul

Mix the above reagents in one tube and transfer 53 ul of the mixture to the A4-H4 wells of a 1ml Nunc plate.

Step 4: Purification--SPRISelect reagent (Beckman Coulter B23318) –TruSeq 06

AmPure Purification

90 ul beads was used for each well reaction

Step 5: Adapter Ligation—TruSeq 08 Adapter Ligation

2 x buffer: 420 ul

Enzyme: 56 ul

Mix the above reagents in one tube and transfer 56 ul of the mixture to the A5-H5 wells of a 1ml Nunc plate.

Load 2 ul H₂O and 1 ul of adapter 1, 2, 3, 4, 7, 9 to the A1-H1, A2-H2, A3-H3 well of Twin-Tec Eppendorf 96-well plate.

Step 6: Size selection--SPRISelect reagent (Beckman Coulter B23318) Nugen Chip-Seq 03 purification

50 ul beads was loaded to A1-H1, A2-H2, A3-H3 wells of 1ml Nunc plate.

14 ul beads was loaded to A1-H1, A2-H2, A3-H3 wells of Twin-Tec Eppendorf 96-well plate.

Step 7: PCR Amplification

2 x phusion: 1 ml

TruSeq primer 1.0: 20 ul

TruSeq primer 2.0: 20 ul

H₂O: 35 ul

Mix the above reagents in one tube and transfer 43 ul of the mixture to 8-strip tubes and add 37 ul DNA to each well (total 80 ul).

Amplify using the following PCR protocol:

Step 1: 98 °C---30 seconds

Step 2: [98 °C---10 seconds] --> [65 °C---30 seconds] -->[72 °C---30 seconds]

(Go to Step 2): 17 more cycles

Step 3: 72 °C---5 minutes

Step 4: Hold at 4 °C

Step 8: Purification--SPRISelect reagent (Beckman Coulter B23318) – Nugen Chip-Seq 05 purification

40 ul beads was loaded to A1-H1, A2-H2, A3-H3 wells of 1ml Nunc plate.

11.2 ul beads was loaded to A1-H1, A2-H2, A3-H3 wells of Twin-Tec Eppendorf 96-well plate.

Step 9: Measure the DNA concentration (ng/μL) using Qubit.