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Revised for P19, 05/11/2010 Ivy Chen

# ChIP Protocol for EZ-Magna ChIP™ A—anti-GFP, P19 cells

*70% confluent 6cm dish contains ~6.5 million P19 cells.
*Centrifugal force for spinning in Section A is 800g, or 1600rpm when using the small centrifuge in the -80°C room.

## A. *In vivo* Crosslinking and Lysis

-One 6cm dish of P19 cells (requires ~$10^6$ cells per ChIP sample)
-DMEM/F12
-Ice-cold 1X PBS
-10X Glysine
-Trypsin (warmed up)
-MEF medium (or any other one that contains serum)
-Cell scraper (from BD Falcon, REF 353085)
-Cell lysis buffer
-Nuclear lysis buffer at RT
-Protease inhibitor cocktail II at RT

1. Wash cells twice w/ DMEM/F12.
2. Add 110ul of 37% formaldehyde to 4ml of cells (Final Conc.= 1%). Gently swirl to mix. Sit at RT for 10min.
3. Meanwhile, prepare 500ul of ice-cold 1X PBS with 2.5ul Protease inhibitor cocktail II to a separate tube for one dish. Put on ice.
4. Add 400ul of 10X Glysine to the dish. Swirl to mix and sit at RT for 5 min.
5. Place the dish on ice.
6. Aspirate, remove as much medium as possible, careful not to disturb the cells.
7. Wash twice with 2ml ice-cold 1X PBS.
8. Add 1ml of 0.05% trypsin to the dish, and sit at 37°C for 5min.
9. Add 1ml MEF medium to stop trypsin reaction.
10. Scrape cells off into two Eppendorf tubes (1ml each) using a cell scraper.
11. Rinse the dish with 1ml MEF medium, and add 500ul to each tube.
12. Spin cells down at 1600rpm, 4°C, for 5min. Discard supernatant.
13. Wash one of the tube with 500ul ice-cold 1X PBS, move the cells into the other tube, rinse the empty tube with another 500ul ice-cold 1X PBS, and then join the leftover cells into the other tube. Spin the cells down at 1600rpm, 4°C, for 5min.
14. Resuspend the cell pellet with 500ul ice-cold PBS w/ 1X Protease inhibitor cocktail II (from step 3). Spin down at 1600rpm, 4°C, for 5min.
15. Meanwhile, if not freezing the cells at -80°C, prepare 500ul of cell lysis buffer 2.5ul of protease inhibitor cocktail II for one dish.
16. Remove supernatant.

[Store @-80°C]

17. Resuspend the pellet in 500ul of cell lysis buffer w/ 1X protease inhibitor cocktail II (from step 15). Incubate on ice for 15min, vortex every 2min.
18. Spin the cells at 1600rpm, 4°C, for 5min.

19. Meanwhile, prepare 500ul of nuclear lysis buffer w/ 2.5ul of protease inhibitor cocktail II for one tube.
20. Remove supernatant, resuspend the pellet in 500ul of nuclear lysis buffer w/ 1X protease inhibitor cocktail II (from step 19).

## B. Sonication to Shear DNA

1. Sonicate cell lysate on wet ice using Misonix cup horn in June's lab, setting 4, 15sec, for 20 cycles. Place sample on ice for 1min in between sonication cycles (may need to reduce the number of cycles if samples were frozen before).
2. Spin at 10,000g~15,000g, at 4$^{o}$C, for 10min.
3. Move supernatant to fresh microfuge tubes in 100ul aliquots (~$10^6$ cell equivalents of lysate per tube).

[Store @-80$^{o}$C]

## C. Immunoprecipitation of Crosslinked Protein/DNA

-Protease inhibitor cocktail II at RT
-Dilution buffer
-Protein A magnetic beads, fully suspended.
-Antibodies (positive/negative control, target antibody)

1. Prepare 900ul of Dilution buffer w/ 5ul of Protease inhibitor cocktail II for each sample (at least 3—positive, negative, experimental).
2. Take crosslinked chromatin from Section B, and keep on ice.
3. Add 900ul of Dilution buffer w/ Protease inhibitor cocktail II to each 100ul sample (from Section B).
4. Save 10ul (1%) of the supernatant from each sample as "Input" for Section D, step 1. Store at 4$^{o}$C.
5. Add the antibody and 20ul well-mixed protein A magnetic beads to each sample.
   (i) Positive control: anti-acetyl H3, add 5.0ug/tube.
   (ii) Negative control: Normal rabbit IgG, add 5.0ug/tube.
   (iii) Target antibody: 1-10ug/tube.
   (ex. rabbit anti-GFP, 2ug/ul)
6. Incubate for 1hr to overnight at 4$^{o}$C w/ rotation.
7. Pellet Protein A magnetic beads w/ a magnetic separator, remove supernatant.
8. Wash beads w/ 0.5ml of each of the **cold buffers**, incubate for 3-5min on a rotating platform prior to magnetic clearance and carefully remove supernatant:
   (i) Low salt immune complex wash buffer, once.
   (ii) High salt immune complex wash buffer, once.
   (iii) LiCl immune complex wash buffer, once.
   (iv) TE buffer, once.

## D. Elution of Protein/DNA Complexes and Reverse Crosslinks

-Proteinase K at RT
-ChIP elution buffer at RT

1. Prepare 100ul ChIP Elution buffer w/ 1ul Proteinase K for each tube (including all IP tubes and Input tubes).
2. Incubate at 62$^{o}$C for 2hr w/ shaking.
3. Incubate at 95$^{o}$C for 10min.
4. Cool to RT.
5. Separate beads w/ a magnet stand and move supernatant to a new tube.

## E. DNA Purification

-Prepare one spin filter in collection tube and one separate collection tube for each sample.
-Bind reagent "A"
-Wash reagent "B"
-Elution buffer "C"

1. Add 500ul of Bind reagent "A" to each 100ul DNA sample (from Section D) and mix well.
2. Transfer the mixture to the spin filter in collection tube. Spin at 10,000g~15,000g for 30sec.
3. Discard supernatant from collection tube. Put the spin filter back and add 500ul of Wash Reagent "B" to the filter.
4. Spin at 10,000g~15,000g for 30sec.
5. Discard supernatant from collection tube. Put the spin filter back and spin again at 10,000g~15,000g for 30sec.
6. Discard supernatant **AND** collection tube. Put the spin filter into a clean collection tube.
7. Add 50ul of Elution buffer "C" directly to the filter membrane. Sit or 1min.
8. Spin at 10,000g~15,000g for 30sec. Discard spin filter.
9. Eluate in the collection tube is now purified DNA. Store at -20$^{o}$C.

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**PCR**

-Filter-tip pipette tips recommended
-At least 5 200ul PCR tubes (PCR control, +/-/input controls, and samples)

| **Reagent** | **ul/reaction** |
|---|---|
| DNA | 2.0 |
| $H_2O$ | 13.2 |
| 10X PCR Buffer (w/o $MgCl_2$) | 2.0 |
| 25mM $MgCl_2$ | 1.2 |
| 10mM dNTP | 0.4 |
| 5uM Primer Set (F+R) | 0.8 |
| 5U/ul Hot-Start™ Taq Polymerase | 0.4 |
| Total | 20 |

Program:

94°C 3min

94°C 20sec
59°C 30sec
72°C 30sec
} 32 cycles

72°C 2min

Store at -20°C

Gel: 2% agarose, use 10ul of each PCR reaction sample.