Young Lab ChIP Protocol (Modified for MRC5 and IMR90 Human Cells)
Parisha Shah – Berger Lab, 10/2010

Note: *Protocol optimized for ChIPs with 500ug lysate protein.*

Crosslinking

1. Crosslink protein/DNA by addition of formaldehyde to 1% (271uL, 37% formaldehyde per 1, 10cm plate containing 10mL culture; adjust volumes accordingly).
2. Incubate with gentle rotation for 5 minutes at room temperature.
   Note: *Many protocols use 10-15 minutes of crosslinking; this protocol is optimized for use with short crosslinking to improve antibody accessibility in senescent cells. This step may require further optimization with different antibodies, cells, or experiments.*
3. Quench crosslinking reaction with addition of glycine to 125mM (642uL, 2.5M glycine per 1, 10cm plate containing 10mL culture + 271uL formaldehyde; adjust volumes accordingly).
4. Incubate with gentle rotation for 5 minutes at room temperature.
5. Scrape cells off of plate using disposable cell lifter (do not remove media from plate; skip this step if not using attached cells).
6. Collect cells in media and transfer to 15mL conical tube.
   Note: *Keep samples on ice after this step.*
7. Spin cells at 1200rpm for 5 minutes at 4°C.
8. Remove supernatant and resuspend cell pellet in cold 10mL 1xPBS.
9. Spin as in step 7.
10. Remove supernatant and freeze cell pellet at -80°C (pellets can be stored indefinitely at -80°C).
    Note: *4 cell pellets should yield enough lysate for 5-6 ChIPs, using 500ug lysate/IP, but the yield may vary depending on the cell type, crosslinking conditions, number of cells, etc.*

ChIP Day 1: Bead Preparation, Cell Lysis/Sonication, Setting up IP

Bead Preparation

1. Early in the day (at least six hours before setting up IPs), aliquot 30uL Protein G Magnetic Beads (Dynal) into 1.5mL microcentrifuge tube; set up 1 tube/IP.
2. Collect beads on magnet; put tubes on magnet, allow beads to separate for 30 seconds – 1 minute, and remove supernatant by aspiration.
3. Resuspend beads in 1mL Block Solution and mix by inverting tube.
4. Repeat Step 2 and 3.
5. Collect beads one final time as in Step 2 and resuspend beads in 250uL Block Solution.
6. Add 2ug antibody (or IgG/no antibody for control) per IP tube.
7. Rotate at 4°C for a minimum of six hours.

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Cell Lysis

Note about timing: *IPs will need to incubate overnight (12-18 hours). It takes approximately 1.5-2 hours to prepare lysates for the IPs (depending on the number of samples), so it is recommended to being cell lysis about 2 hours before setting up IPs.*

1. Remove frozen cell pellets from -80°C and put on ice.
   Note: *If processing multiple samples from the same cells, keep samples separate throughout the lysis procedure; lysates from the same samples can be combined after Step 8.*
2. Resuspend cell pellet in cold 10mL Lysis Buffer 1 and rock on platform rocker for 10 minutes at 4°C.
3. Spin cells at 3000rpm at 4°C for 5 minutes and aspirate supernatant.
4. Resuspend cell pellet in cold 10mL Lysis Buffer 2 and rock on platform rocker for 10 minutes at room temperature.
5. Spin and process pellet as in Step 3.
6. Resuspend pellet in 3.5mL cold Lysis Buffer 3 and sonicate using Bioruptor.
   a. Attach probes/lids to conical tubes for use with Bioruptor.
   b. Remove ½ of the water in the Bioruptor water bath and replace with ice.
   c. Insert tubes into the plastic ring and place ring in the Bioruptor.
   d. Close lid and sonicate for 7.5 minutes at high energy output, alternating between 30 seconds on and 30 seconds off.
   e. Repeat Steps b-d, resulting in a total of 15 minutes of sonication (7.5 minutes for 2 rounds of sonication).
      Note*: The sonication conditions have been optimized for use with the new Berger lab Bioruptor to yield average fragment size of 200-350bp fragments. Conditions may need to be optimized for other cell types and desired fragment size.*
7. Add 1/10 volume (350uL) 10% Triton X-100 to sonicated lysate.
8. Split sample evenly into 3, 1.5mL microcentrifuge tubes (~1.3mL/tube) and spin at full speed in microcentrifuge for 10 minutes at 4°C.
9. Combine supernatants from three tubes into a new 15mL conical tube; keep all samples on ice!
   Note: Lysates can be combined for same samples.
10. Measure approximate protein concentration in each sample by Bradford Assay.

Set Up IPs – Prepare Beads

1. Quick spin tubes containing beads/antibody mixture from to remove any liquid and beads from the lid.
2. Collect beads on magnet; put tubes on magnet, allow beads to separate for 30 seconds – 1 minute, and remove supernatant by aspiration.
3. Resuspend beads in 1mL Block Solution and mix by inverting tube.
4. Repeat steps 2 and 3 twice more (i.e. total of 3 washes).
5. Following final wash, resuspend each aliquot of beads in 50uL Block Solution; beads are ready to use.

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Set Up IPs – Combine Lysate and Beads

1. Remove (at least) 50uL aliquot from each lysate for input control and store at -20°C.
2. In microcentrifuge or 15mL conical tube, aliquot lysate corresponding to 500ug protein.
   Note: *If lysate volume is greater than 800uL, set up IPs in conical tubes, to allow maximum rotation of beads within the tube.*
3. Add 50uL bead mixture to tube containing lysate.
   Note: *Final IP mix contains 500ug lysate, 30uL beads (initial volume), and 2ug antibody (pre-bound to beads). If yield is not enough for 500ug lysate protein/IP, adjust all lysate amounts so that the same amount of protein is being used in every IP. All ChIPs must contain the same amount of input protein/IP to be comparable.*
4. Rotate IP tubes overnight at 4°C.

<u>Day 2: Washes, Elution, Reverse Cross-Linking</u>

Washes

1. Pre-chill microcentrifuge tubes on ice for ~15 minutes before beginning washes; set up 1 tube per IP.
2. Transfer 1.2mL IP/bead mixture to pre-chilled tube.
3. Collect beads using magnet; put tubes on magnet, allow beads to separate for 30 seconds – 1 minute, and remove supernatant by aspiration.
4. Repeat steps 2 and 3 until all of the IP/bead mixture is transferred to the tube.
5. Add 1mL cold RIPA Wash Buffer, mix tube by inversion, and incubate briefly on ice (1-2 minutes).
   Note: *If processing many samples at once, keep tubes on ice during washes; it is fine for the samples to sit longer than 2 minutes in wash buffer on ice.*
6. Collect beads using magnet; put tubes on magnet, allow beads to separate for 30 seconds – 1 minute, and remove supernatant by aspiration.
7. Repeat Steps 5 and 6 four times, resulting in a total of 5 RIPA washes.
8. Resuspend beads in 1mL Final ChIP Wash Buffer.
9. Collect beads as in Step 6.
10. Spin tubes at 1500rpm for 3 minutes in microcentrifuge at 4°C.
11. Collect beads on magnet as in Step 6 to remove any residual buffer from beads.

Elution

1. Resuspend beads in 210uL Elution Buffer.
2. Incubate tubes at 65°C for 30 minutes.
   Note: *Use a Thermomixer with gentle agitation (approx. 400rpm) otherwise, flick/invert tubes every 5 minutes to keep beads in suspension.*
3. Spin tubes at top speed for 1 minute in microcentrifuge at room temperature.
4. Collect beads using magnet; put tubes on magnet, allow beads to separate for 30 seconds – 1 minute.
   Note: DO NOT ASPIRATE SUPERNATANT! THIS CONTAINS ELUTED, IP'ED DNA!
5. Remove 200uL supernatant to fresh tube (remaining 10uL left behind to prevent transfer of beads).

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Reverse Crosslinking

1. Thaw tubes containing input DNA.
2. Add 150uL Elution Buffer to input DNA tubes (3 volumes more than input volume; adjust accordingly if using greater than 50uL input lysate).
3. Incubate all tubes (Input and IP) at 65°C overnight to reverse crosslinking.
   Note: *Incubate for at least 12 hours and not more than 18 hours to maximize DNA content, but minimize background signal resulting from excess reverse crosslinking.*

Day 3 – DNA Purification

Note: *These steps are optimized for 200uL samples; if using greater than 200uL volume for input sample, adjust reagent volumes accordingly.*

1. Add 200uL 1xTE to each tube.
2. Add 8uL 10mg/mL RNaseA (0.2mg/mL final concentration) to each tube and incubate at 37°C for 2 hours.
3. Add 4uL 20mg/mL Proteinase K (0.2mg/mL final concentration) to each tube and incubate at 55°C for 2 hours.
4. Add 400uL phenol:chloroform:isoamyl alcohol (P:C:IA) and vortex.
5. Pre-spin 2mL Phase Lock Heavy Gel tubes for 1 minute at top speed in microcentrifuge.
6. Pipet eluate/P:C:IA mixture onto the gel in the Phase Lock tubes and spin for 15 minutes at top speed in microcentrifuge.
7. Transfer aqueous layer into fresh 1.5mL microcentrifuge tube containing 16uL 5M NaCl and 1.5uL 20ug/uL glycogen (final 30ug).
8. Add 800uL cold 100% EtOH and mix well by inversion.
9. Incubate tubes at -20°C for 30 minutes to precipitate DNA.
10. Spin samples at top speed for 10 minutes in microcentrifuge at 4°C; note orientation of tube in microcentrifuge, as pellets may be difficult to see.
11. Carefully decant/aspirate supernatant (pellets may be loose).
12. Add 500uL 80% EtOH to wash DNA (pellets may become dislodged).
13. Spin as in step 10.
14. Remove all EtOH by pipet/aspiration.
15. Allow pellets to air dry for 10-15 minutes at room temperature.
16. Resuspend pellet in 60uL 10mM Tris-HCl, pH 8.0.
    Note: This volume is adjustable; 60uL is used here, to allow for part of the sample to be analyzed by qPCR and the remainder of the sample to be used for ChIP-seq. Depending on the amount of DNA being IP'ed and the use for the IP'ed DNA, volumes can be adjusted accordingly.

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Block Solution (Make fresh each time, store at 4°C)
1xPBS
0.5% BSA (powdered stock at 4°C)

Lysis Buffer 1 (Filter and store at 4°C, add PI before using)
50mM HEPES-KOH, pH 7.5
140mM NaCl
1mM EDTA
10% Glycerol
0.5% NP-40
0.25% Triton X-100
1x Protease Inhibitors – Add Fresh Before Using

Lysis Buffer 2 (Filter and store at 4°C, add PI before using)
10mM Tris-HCl, pH 8.0
200mM NaCl
1mM EDTA
0.5mM EGTA
1x Protease Inhibitors – Add Fresh Before Using

Lysis Buffer 3 (Filter and store at 4°C, add PI before using)
10mM Tris-HCl, pH 8.0
100mM NaCl
1mM EDTA
0.5mM EGTA
0.1% Na-Deoxycholate
0.5% N-lauroylsarcosine
1x Protease Inhibitors – Add Fresh Before Using

RIPA Wash Buffer (Filter and store at 4°C)
50mM HEPES-KOH, pH 7.5
500mM LiCl
1mM EDTA
1% NP-40
0.7% Na-Deoxycholate

ChIP Final Wash Buffer (Filter and store at 4°C)
1xTE
50mM NaCl

ChIP Elution Buffer (Filter and store at room temperature)
50mM Tris-HCl, pH 8.0
10mM EDTA
1% SDS