Iyer Laboratory: Solid Tissue Chromatin Immunoprecipitation Protocol

Developed by BK Lee, Yunyun Ni and Amelia Weber Hall in January 2012 using mouse brain and liver samples (kindly donated by the Vokes lab). Used for profiling histone modifications across the genomes of glioblastoma brain tumors, and many other labs in the Central Texas area. Documented by Amelia Weber Hall, June 2017.

Equipment

Swinging bucket centrifuge with refrigeration
Microcentrifuge with refrigeration
Bioruptor sonicator with probe tips (Diagenode)
Glass dounce homogenizers (Wheaton)
Qubit, or other fluorescence-based method for assessing DNA concentration

Materials and suppliers

Aliquoting the tissues:

Phosphate Buffer Saline (PBS), 1x
Biopulverizer from BioSpec Products, Bartlesville, OK
15mL conical tubes, in polypropylene (for storage) and polystyrene (for sonication)
Sporicidin: hospital-grade disinfectant for cleaning all materials used to manipulate tissue
Liquid nitrogen
Dry ice

Cross-linking the tissues:

Phenyl methyl sulfonyl fluoride (PMSF) powder
Formaldehyde – 37%
2.5M glycine

Isolating and lysing the nuclei (lysis and sonication)

1M PIPES, pH 7.5
1M KCl
Phosphate Buffer Saline (PBS), 1x
10% NP-40
10% Na Deoxycholate
10% Sodium Dodecyl Sulfate (SDS)
Roche COmplete Protease Inhibitor tablets

Pre-clearing and Immunoprecipitation (IP):

Bovine surface albumin (BSA) powdered
Protein A or protein G agarose beads (Roche)
10% Triton X-100
0.5M EDTA pH 8.0

1M HEPES, pH 7.5
5M NaCl,
1M Lithium Chloride
1M Tris HCl pH 8.1
sodium bicarbonate powder

DNA Extraction:

Phenol-chroloform isoamyl alcohol (25:24:1) Ultrapure, from Life Technologies
5-Prime 2.0 mL phase lock tubes, heavy weight

Overall process notes:

Sterilize all liquids by vacuum filtration before making stock solutions.

Aliquoting the tissues

1. Harvest the tissues, rinse 1x in PBS and place in 15mL conical tubes or cryotubes and freeze by dropping into Dewar flask of liquid nitrogen (LN_2).
    a. If tissues have already been harvested and flash frozen, you can and should forgo the PBS rinse and just skip to preparing the biopulverizer.
2. Pulverizing tissues:
    a. Place the biopulverizer into a heat-insulating container and fill the container with LN_2.
    b. Fill the cup of the steel mortar with LN_2 and allow to boil off (or you won't be able to crush the tissues properly).
    c. Place frozen tissues in mortar.
    d. Smash by hitting the top of the mortal with a mallet (the rubberized hammer provided).
3. Divide the pulverized tissue:
    a. Put a cold freezer block (or a cooled heating block) on top of the scale and tare. Place weigh paper on the frozen block.
    b. Keep your tubes for the sample aliquots on dry ice. Make sure to label them before making them cold!
    c. Use a sterilized cold spatula (dip into LN_2 to keep cold).
    d. Working very quickly, aliquot 50-100 mg per conical tube.
        i. Pour more LN_2 into the mortar of the biopulverizer if it begins to get warm - don't let the tissues melt!
    e. Place tissue aliquots in the -80°C freezer, or proceed directly to cross-linking below.

Cross-linking the tissues

1. Cross-link DNA and protein in the tissues:
    a. Spin 3 min 500*g* at 4°C to pellet the tissues.
    b. Add 10mL PBS * PMSF to the tissues and use 250µl formaldehyde to cross link.
    c. Total cross-linking time can go for 10-20 minutes – 15 minutes seems to be a sweet spot (and what we've used in our solid tissue experiments).
2. After cross-linking, add 500µl 2.5M glycine to quench the reaction and shake at RT for 5 minutes.

3. Spin for 3 min 500*g*, wash 3x with PBS + PMSF, drain supernatant and place pellet in -80°C freezer until ready to start lysis.

Isolating and lysing the nuclei (lysis and sonication)

1. Prepare the following buffers:
    a. 1mL Farnham lysis buffer per 10^7 cells (5 mM PIPES, 85 mM KCl, 0.5% NP-40)
    b. 500µl RIPA buffer per 10^7 cells (1x PBS, 1% NP-40, 0.5% Na Deoxycholate, 0.1% SDS).
    c. One protease inhibitor tablet for every 50 mL of buffer prepared.
        i. Cut the tablets into pieces with a clean razor for ease of use.
        ii. Keep everything on ice and in the presence of protease inhibitor until DNA extraction.
2. Using a dounce tissue homogenizer, gently resuspend the pellet in 1 mL of Farnham lysis buffer (per IP). Use the loose rod, followed by the tight rod to resuspend in the Dounce homogenizer. Remove the sample with a pipet and let sit on ice for 10 minutes.
3. Centrifuge at 1200*g* at 4°C for 10 minutes. Remove the supernatant and resuspend the pellet in 500µl/IP of RIPA buffer.
4. Separate into 500µl aliquots in 15 mL polystyrene tubes and sonicate for 40 minutes using the bioruptor (four 10 min cycles, 30s on/60s off, replace ice in water bath every 10 minutes).

Pre-clearing and Immunoprecipitation (IP)

1. While the sample is sonicating, prepare protein A/G beads (use protein A for rabbit antibodies, protein G for mouse antibodies) to pre-clear the lysate:
    a. Make a solution of 5mL 1x PBS and 30mg BSA; add a small piece of protease inhibitor tablet.
    b. Wash the beads 3x in the 1x PBS + BSA (spin at 100g, 30s).
2. After sonication is completed, transfer samples to 1.5mL microfuge tubes and spin at top speed at 4°C in refrigerated desktop centrifuge.
    a. Remove and keep supernatant, add RIPA buffer to 1 mL total volume
    b. Add 60µl washed beads and nutate for 30-60 minutes in the cold room.
    c. Spin for 30 seconds at 100*g* (4°C), remove and keep the supernatant
    d. Remove an input sample: 100µl of lysate not exposed to antibody, freeze at -20°C)
    e. Add 10µl (or 10ug, the amount can be variable depending on the antibody) of antibody and nutate at 4°C overnight
3. The following morning, prepare protein A/G beads as above, add 60µl beads per ChIP sample and nutate the antibody with the beads for 30-60 minutes in the cold room.
4. All spins are 30s and 100*g* at 4°C, all washes rotate at 4°C for five minutes.
    a. Wash 2x with low salt buffer (0.1% Na Deoxycholate, 1% Triton X-100, 1mM EDTA, 50mM HEPES (pH 7.5), 150mM NaCl).
    b. Wash 1x with high salt buffer (0.1% Na Deoxycholate, 1% Triton X-100, 1mM EDTA, 50mM HEPES (pH 7.5), 500mM NaCl).
    c. Wash 1x with LiCl (250mM LiCl, 0.5% NP-40, 0.5% Na Deoxycholate, 1mM EDTA, 10mM TrisCl (8.1)).
    d. Wash 2x with TE buffer (10mM TrisCl (pH 8.1), 1mM EDTA) (the last spin can be at RT at the bench).
5. Get out input samples and treat in the same manner as IP samples.

6. Add 250µl 1% SDS and 0.1M NaHCO3 (sodium bicarbonate) to the beads and nutate for 15 minutes. Spin, remove and save the supernatant. Repeat once.
7. Combine the two 250µl aliquots in a single tube, add 20µl 5M NaCl and incubate at 65°C for at least 4h but up to overnight.
8. Place samples at -20°C until ready to extract DNA, or proceed directly to DNA extraction below.

DNA Extraction (phenol-chloroform)

1. Thaw samples and add:
    a. 5µl 0.5 mg/mL RNase A
    b. Incubate 30 minutes at 37°C
2. Spin the samples down briefly to collect condensate and add:
    a. 20µl 1M Tris, pH 6.8
    b. 10µl 0.5M EDTA, pH 8.0
    c. 3µl Protease K (20 mg/mL)
3. Incubate at 55°C for one hour.
4. Add an equal volume phenol-chloroform to the sample:
    a. The below description uses phase lock tubes, however these are not necessary.
    b. Centrifuge the phase lock tubes for 1 minute at 12-16K*g* to pellet the gel.
    c. Add sample and an equal volume of phenol-chloroform.
    d. Shake vigorously for ~30 seconds, until an emulsion between the organic, aqueous and gel solutions forms.
    e. Spin down at RT for 5 minutes at 12-16K*g*.
5. Re-extract with another volume of phenol-chloroform and re-spin.
6. Remove and save the aqueous supernatant.
7. Add 1 mL 100% ethanol and 2µl glycogen (10µg, 5µg/µl)
8. Incubate in the -80°C freezer for 20 minutes (no longer or the samples will likely freeze)
9. Spin in the cold room or in the refrigerated microfuge for 15 minutes at maximum speed, 4°C.
10. Wash the pellet with 70% EtOH and vortex gently.
11. Spin at maximum speed for five minutes at RT.
12. Remove 70% ethanol and let pellet dry for 5 minutes in a fume hood.
13. Resuspend pellet in water, taking into account what your downstream applications will be (qPCR, library prep and sequencing).
    a. 15µl/IP is a good starting volume.
14. Check the DNA concentration by Qubit.