Chromatin IP (Isw2)

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Reagents

Fix soln: 11% formaldehyde, 0.1 M NaCl, 1 mM EDTA, 50 mM Hepes-KOH pH 7.6. Freshly prepared. Do not store in glass bottles.

2.5 M glycine.

TBS: 20 mM Tris pH 7.6, 150 mM NaCl.

Breaking buffer: 100 mM Tris pH 8.0, 20% glycerol, 1 mM PMSF (added fresh).

Acid washed glass beads 425-600 μ (Sigma G-8772).

FA buffer: 50 mM Hepes-KOH pH 7.6, 150mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate. NaCl conc must be determined for each combination of tagged factor and epitope tag. Add protease inhibitors (100x) just before use. [100X cocktail: 0.1 M PMSF, 0.2 mM pepstatin A, 60 μM leupeptine, 0.2 mg / ml chymostatin, 0.2 M benzamidine in 100% EtOH. Store at -20 °C].

FA-HS buffer: same as FA buffer but contains 500 mM NaCl.

RIPA buffer: 10 mM Tris pH 8.0, 0.25 M LiCl, 0.5% NP40, 0.5% sodium deoxycholate, 1 mM EDTA.

3XFLAG peptide (Met-Asp-Tyr-Lys-Asp-His-Asp-Gly-Asp-Tyr-Lys-Asp-His-Asp-Ile-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys. 5 mg / ml stock in Buffer H 0.1.) Crude peptide is sufficient.

Dynabeads Protein G (Dynal, cat# 100.04).

FLAG M2 antibody (Sigma).

0.1 M sodium Phosphate buffer pH7.0

0.1 M sodium Phosphate buffer pH7.0 + 0.01% Twwn 20

2x Stop buffer: 20 mM Tris pH8.0, 100 mM NaCl, 20 mM EDTA, 1% SDS.

Chromatin Prep

1. On the day before chromatin prep, inoculate yeast so that the culture will be OD_{600}=0.5 at the time you want to start chromatin prep. I routinely prep chromatin from 50-300 ml culture.

2. At OD_{600}=0.5, add 1/10 volume of Fix soln to culture, and shake slowly @ room temp for 20 min. Optimum time for fixation must be determined for each epitope tag and tagged protein. We have done 5 or 20 min fix for Isw2 and saw the same results.

3. Add 18 ml of 2.5 M glycine to every 100 ml of culture to terminate fixation. Shake culture for 5 min at room temp. If you are taking time course, you can keep the cells on ice for a while (up to a couple of hors) at this stage.

4. Pellet cells (5,000 rpm 5 min in JA-10), and wash twice with ice cold TBS (10 ml ea) in 14 ml tubes. You can freez cell pellet in liquid N2 and store in -80 °C at this stage.

5. Re-suspend cells in breaking buffer (500 μl buffer for 300 ml culture) and transfer to flat bottom 2 ml microfuge tubes. Jairo uses 300 μl breaking buffer for cells from 50 ml culture.

6. Add glass beads (500 μl for 300 ml culture, ~100 μl for small scale) and bead beat 2-5 minutes. It's very important not to over bead beat. Check cells under microscope, and stop when 70-80 % of cells are broken. Optimum time widely varies among different strains.

7. Puncture one hole each in the bottom then top of the tube by a needle, and put tubes on Falcon 2059 (14 ml snap cap) or equivalent tubes. Centrifuge @ 1,000 for 2 min to recover cell lysates.

8. Add 1 ml FA buffer to lysates, mix, and transfer to 2 ml microfuge tubes, and spin at 14,000 rpm for 1 min at 4 °C and discard sup.

9. Suspend ppt in 1 ml FA buffer by gentle pipetting. Repeat step 8 twice to partially purify chromatin. suspend chromatin in 1 ml FA buffer.

10 Sonicate up in Biorupter. I normally do 15 min sonication twice at 50% cycle. For some prep, 30 minutes sonication is too much. If so, reduce it to 25 minutes total.

11. Centrifuge in microfuge tubes at 14,000 rpm for 30 min twice (turn tubes in between) at 4 °C, and transfer the sup into a new eppi tube. The sup is the chromatin sample. Go to IP reaction, and/or freeze chromatin in liquid N_2. Store at -80 °C.

Beads Prep

1. Take 20 μl of Pro G beads/sample (IP reactions) into a siliconized eppi tube.

2. Concentrate the beads on a concentrator (MPC) and remove sup. To avoid clamping, do not leave beads on MCP for more than a couple of minutes.

3. Add 0.5 ml of 0.1 M sodium phosphate pH7.0 (or PBST).

4. Rotate at room temp for 2~3 min. Concentrate on MPC and remove sup.

5. Repeat step 4 twice (total 3 washes).

6. Take up beads in 20~100 μl 0.1 M sodium phosphate pH7.0 (or PBST) and suspend beads. Add anti-FLAG Ab (normally ~2 μl/IP: need to be optimized for each protein). If multiple IP is to be done, steps 6-8 should be done in one tube.

7. Constantly mix at room temp for 30 minures.

8. Concentrate on MPC and remove sup. Add 0.5 ml 0.1 M sodium phosphate pH7.0 + 0.01% Tween 20 (or PBST), suspend beads and rotate 2~3 minutes. Concentrate on MPC. Repeat beads wash x1 in 0.5 ml FA biffer, and suspend beads at 20 μl/IP. Keep beads on ice until use.

IP reaction

1. Thaw chromatin in room temp water, centrifuge at the top speed for 5 minutes and take the amount needed. I usually take 240 μl (200 μl for IP and 20 μl each for DNA and western analyses).

2. Save 20 μl each of chromatin for DNA and western blotting ("input" sample). Add 20 μl of 2 x Stop buffer or 2 x SDS/PAGE sample buffer to DNA and western samples, respectively.

3. Add 200 μl of chromatin from step 2 to 20 μl of Protein G Dynabeads as prepared above in siliconized microfuge tubes. Rotate at room temp for 90 min for binding.

5. Concentrate beads on MPC. Save 20 μl of sup for western ("unbound" fraction) and add 20 μl 2 x SDS/PAGE sample buffer.

6. Add 1 ml FA buffer, suspend beads by flicking and rotate 5 min at room temp. Concentrate on MPC and remove the buffer.

7. Do washing (step 6) for total 3 times with FA buffer, 2 times with FA-HS buffer and 1 times with RIPA buffer.

8. Elute by adding 45 μl of x2 Stop buffer and incubating at 65 °C for 15 minutes with occasional vortexing.

12. Save 5 μl of eluate for western from each eluate ("bound" fraction). Add 20 μl 2 x SDS/PAGE sample buffer and 15 μl H2O.

13. For western blotting, incubate samples for 30 min on 95 °C heat block. Determine how much of Isw1/2 was precipitated by comparing signals for “input”, “unbound” and “bound”.

14. For all DNA samples, add 1 μl of 20 mg / ml glycogen per tube. Add 20 μl of 2x Stop buffer and 20 μl H2O (total 80 μl). Incubate @ 75 °C for at least 6 hrs, then add 2 μl of 10 mg / ml proteinase K. Incubate at 50-55 °C for several hrs to overnight. Phenol-chloroform extract twice and EtOH ppt DNA (20 μl 10 M NH4OAC and 250 μl EtOH).

14*: Alternatively, you can prepare DNA by Qiagen PCR Purification Kit. Elute in 50 μl.

***Ultra-rapid DNA prep method** [ref: Nelson et al., Nature Protocols **1**:179-185, 2006).

Required reagent: 10 % (wt/vol) Chelex 100 (Bio-Rad, cat # 142-1253) in distilled water.

1. Input DNA (20 μl at the step 3 above): add 60 μl EtOH. Cfg at max for 15 minutes and wash ppt twice with 80% EtOH. Suspend DNA in 100 μl 10 % (wt/vol) Chelex 100 and dissolve DNA.

2. Bound DNA: after the last wash (RIPA at the step 7 above), add 100 μl 10 % Chelex 100 directly to the beads. Vortex.

3. Boil both input and bound DNA 30 minutes on 95 °C heat block. Take 10 μl for western blotting ("bound" sample).

4. Add 2 μl 10 mg/ml PK to each tube and incubate 55 °C 30 min to overnight. I have done 30 minutes, a few hours and overnight, and all worked fine.

5. Boil 10 minutes on 95 °C heat block. Vortex, cfg and recover sup.

6. Add 100 μl water to beads, vortex, cfg and take the sup. Combine samples from steps 5 and 6, add 1 μl glycogen and EtOH ppt. Rinse ppt twice with 80 % EtOH and dissolve them in 50 μl TE.

PCR

1. Take up DNA in 50 μl of TE. Take 3 μl of " input " samples, treat with RNase A and run on 1.0-1.3 % agarose gel to check size of DNA. You should see smears centered around 300-500 bp.

2. For control, I usually use 5-fold serial dilution of " input " sample. Try 10, 50, and 250-fold dilution. For "bound", use undiluted sample.

3. Typical PCR reaction (20 μl).

   2 μl DNA sample
   1X buffer
   1.5 mM $MgCl_2$
   20 pmole each primers
   200 μM each 4dNTP

   95 °C 2 min--[95 °C 20 sec - 59 °C 40 sec - 72 °C 30 sec] x 26-28 cycles--72 °C 5 min **(RoboCycler).**

4. Run on 2 % agarose in 0.5 x TBE.

*:You may need to try different number of cycles for different primers/samples. For Isw1/2, I adjust amount of DNA so that 26 cycles would be sufficient to see signals. Higher numbers of cycles are not recommended because reactions tend to go out of linear range.

**: For radioactive PCR for quantitation (strongly recommended), I add 0.5 μl [α-^{32} P] dCTP (3,000 Ci/mmol, 10 μCi/μl) per 100 μl PCR reaction. Run DNA on 2% agarose in 0.5 x TBE.

***: For CHIP on DNA microaray, I follow Rich Young lab's protocol (http://staffa.wi.mit.edu/cgi-bin/young_public/navframe.cgi?s=12&f=ChIPArray) after step 1.