dFOXO ChIP-on-chip protocol

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Day One

Crosslinking

1. Collect flies (15-day-old), about 200 mg per genotype, keep on ice for few minutes.
2. Grind flies in liquid nitrogen into powder with mortar and pestle.
3. Transfer the powder into a 15 ml Dounce homogenizer containing 5 ml 1x PBS and 135 ul 37 % formaldehyde (Fisher T-11730, final conc. is 1%). Turn on the time, set up as 20 min. Then homogenize the powder with ~20 strokes, about 5 min.
4. Transfer the homogenate into a 15 ml falcon tube. Rotate at RT for 15 min (total crosslinking time is 20 min). In the mean time, wash the mortar and Dounce homogenizer and homogenizer another sample.
5. Add 300 ul 2.5 M glycine (final conc. is 125 mM) to quench the formaldehyde and stop the crosslinking. Rotate at RT for 5 min.
6. Spin 1500 x g for 5 min at 4 ^{o}C. Dump the supernatant into a proper waster tank, not into the sink.
7. Wash the pellet three times with 5 ml 1x PBS with protease inhibitor (Sigma #P8340, 1000x dilution). After each wash, spin 1500 x g for 5 min at 4 ^{o}C.
8. Wash the pellet once with 5ml of basic lysis buffer with protease inhibitor, 0.1% of Na-deoxycholate and 1% of Triton-X100.
9. Spin 1500 x g for 5 min at 4 ^{o}C. Add 1 ml basic lysis buffer with protease inhibitor, 0.1% of Na-deoxycholate and 0.2% of sarkosyl (Fluka 10%).
10. Keep on ice for 10 min (rotate or not).

Sonication

1. Sonicate sample (in a 15 ml falcon tube) using Branson 450 sonicator (Power Set 4, duty cycle 100%, 4-6 times, 30 sec each with 1 min interval). During the sonication, keep falcon tube in a 50 ml beaker filled with ice.
2. Transfer the sonicated lysate to a 1.5 ml tube. Add 100 ul Triton-X100 (final conc. is 1%). Spin 16,000 x g for 15 min at 4 ^{o}C.
3. Transfer the supernatant (chromatin extracts) to a new 1.7ml prelubricated tube (Corning Costar #3207). Take 50 ul out to check the size and conc. of chromatin DNA. Keep the rest of the extracts at 4 ^{o}C if want to continue the immunoprecipitation on the same day, or at -80 ^{o}C for long-term storage.

Check chromatin size and conc.

1. Dilute 50 ul chromatin extracts with 50 ul TE buffer. Add 4 ul of 5 M NaCl and 1 ul of 10 mg/ml Proteinase K (Invitrogen #25530-015) (use a PCR tube).
2. Incubate at 65 ^{o}C for at lease 4 hr (using PCR machine) to reverse crosslink.

3. Add 2 ul of 10 mg/ml RNase A (Sigma #R6513), incubate at 37 ^{o}C for 30min- 1hr.
4. Purify DNA using Qiagen PCR purification column (or Minelute column). Use 20 ul EB to elute DNA. Measure conc. using Nanodrop or Invitrogen Qubit™ Fluorometer. Load the rest of DNA into the 1 % agarose gel. The chromatin should be a smear around 500 bp (using the loading dye containing only xylene blue).

Immunoprecipitation (keep everything cold from now)

1. Transfer 300-500 ul chromatin extracts to a 1.7ml prelubricated tube with 500-700 ul basic lysis buffer with 1%Triton-X100 and protease inhibitor. (Total 1 ml, sarkosyl final conc. is 0.1% or less).
2. Add 25 ul Dynal protean A beads (Invitrogen #100.02D) to a 1.7ml prelubricated tube. Collect beads using a magnetic rack and remove the bead buffer. Add 500 ul block solution (1 xPBS, 0.5% BSA, IgG-free BSA from Jackson ImmunoResearch #001-000-161). to block the beads. Rotate at 4 ^{o}C for 10 min.
3. Remove the block solution and transfer 1 ml chromatin extracts to the beads.
4. Rotate for 1 hr at 4 ^{o}C to get rid of non-specific binding (pre-clear).
5. Collect the chromatin extracts using a magnetic rack. Transfer pre-clear chromatin extracts to a new 1.7ml prelubricated tube.
6. Save 50 ul as input DNA.
7. Add 3 ul Heather's anti-dFOXO antibody or 5 ul Rondi's purified anti-dFOXO antibody (#9172) to pre-clear chromatin extracts. Rotate at 4 ^{o}C O/N.

Day Two

Bead blocking

1. After remove bead buffer, transfer 100 ul Dynal protean A beads to a new 1.7ml prelubricated tube with 1 ml block solution. Rotate for 1 hr at 4 ^{o}C.
2. Collect beads using a magnetic rack. Remove the old block solution and add 1 ml fresh block solution. Rotate for 1 min at 4 ^{o}C. Repeat two more times. Each time gently resuspend beads by inverting the tube.

Binding and washing

1. Transfer chromatin antibody mix to above blocked Dynal beads. Rotate for 2-4 hr at 4 ^{o}C.
2. Collect beads using a magnetic rack.
3. Add 1 ml ice-cold wash buffer (as Richard A Young's protocol) to the tube and gently resuspend beads. Rotate for 1 min at 4 ^{o}C. Repeat four more times.
4. Wash once with 1 ml ice-cold TE buffer with 10 ul 5 M NaCl (final conc. 50 mM).
5. Spin at 960 x g for 3 min at 4 ^{o}C and remove any residual TE buffer.

Elution

1. Add 220 ul of elution buffer (as Richard A Young's protocol) to the beads and incubate at 65 ^{o}C water bath for 15-30 min. Vortex briefly every 2 min.

2. Spin at 16,000 x g for 1 min at RT
3. Transfer 220 ul supernatant to a new tube. Save 20 ul to check the protein pull-down using western blotting (Only for the first time…)

Reverse crosslink

1. Reverse crosslink 200 ul immunoprecipitation DNA and 20 ul input DNA by incubating at 65 ^{o}C for 6-15 hr (in an oven, Hybridiser or PCR machine). Longer than 18 hr will result in increased noise in the microarray analysis.

Day Three

Purification of DNA

1. Add 200 ul TE buffer to dilute detergent.
2. Add 8 ul of 10 mg/ml RNase A. Incubate at 37^{o}C for 1hr.
3. Add 8 ul of 10 mg/ml Proteinase K. Incubate at 55 ^{o}C for 2hr.
4. Add 400 ul phenol:chloroform:isoamyl alcohol (Acros #327115000). Vortex. Transfer 400 ul aqueous layer to a new tube (or use heavy phase lock gel tube, 5 PRIME #2302810) containing 16 ul 5 M NaCl and 3 ul 10 mg/ml glycogen.
5. Add add 800 ul EtOH. Incubate for 20 min at -80 ^{o}C.
6. Spin at 16,000 x g for 15 min at 4 ^{o}C.
7. Wash the pellet with 75 % EtOH once and air dry. Resuspend the pellet in 50-100 ul Qiagen EB buffer.
8. It may be possible to measure DNA conc. using Invitrogen Qubit™ Fluorometer. Dilute half of input DNA to 0.5 ng/ul.

qPCR validation

1-2 ul DNA
1 ul 10uM Primer mix (amplifying 80-100 bp DNA fragment)
10 ul SYBR master mix
7-8 ul dH2O
20 ul in total

Program in ABI 7300

	Cycle	Temp	Time
Step 1	1	50 ^{o}C	2 min
Step 2	1	95 ^{o}C	3 min
Step 3	40	95 ^{o}C 60 ^{o}C	15 sec 30 sec
Step 4	1	Dissociation step	

For each DNA and primer set, set up replicate wells

For each DNA, test primers for the target gene promoter (e.g. 4E-BP) and Actin5C coding sequence (negative control).

Calculate the binding ratio of ChIP DNA vs. Input DNA for each specific genome region.
Formular: 2^-[Δt(ChIP DNA)- Δt(Input DNA)]

Buffer receipt:

Basic lysis buffer:

NaCl	140 mM
HEPES pH7.6	15 mM
EDTA	1 mM
EGTA	0.5 mM

Wash buffer (keep cold, fresh prepared):

HEPES buffer	50 mM (Invitrogen #15630-106)
LiCl	500mM
EDTA	1mM
NP-40	1%
Na-Deoxycholate	0.7%

Elution buffer:

Tris-HCl pH 8.0	50mM
EDTA	1mM
SDS	1%

TE buffer:

Tris-HCl pH 8.0	10mM
EDTA	1mM