Reinke Lab ChIP Protocol (last updated by MK 05/24/13)

Worm Collection

1. Collect worms in a 50ml tube. Spin and wait until worms are collected at the bottom. Transfer sample to a 15ml tube and wash with M9 ~3 times until free of bacteria.

- Prior to next step, determine volume of worm pellet.

2. Transfer sample to a 50ml conical. Add M9 & formaldehyde according to the following ratio:

- For 0-500 µL worm, add M9 up to 23.8 ml + 1.4 mL 37% formaldehyde.
- For 500-1000 µL worm, add M9 up to 47.5 ml + 2.8 mL 37% formaldehyde.
- Do NOT crosslink more than 1 mL worms per 50 mL conical.

3. Rotate at RT for 28'. Spin down cross-linked sample and remove supernatant.

4. Make FA Buffer during step 3. 25ml of RT FA Buffer + 1 Roche Complete tablet (Cat# 11 697 498 001, Protease Inhibitor Cocktail tablets).

- Note: 25 mL FA is enough for 2 samples.

5. After 28' incubation, spin and remove supernatant. Wash with 50 mL 1M Tris pH7.5. Spin and remove supernatant.

6. Wash 2 times with 50 mL M9, spin, and remove supernatant.

7. Transfer to a 15ml conical with M9. Mark the 100 & 200 µL lines on the side of 15 mL conical. Spin and remove supernatant.

- Worms may stick to 15 ml conical so add just a drop of FA buffer (~20-100 µL) to release them from the sides of the tube.

8. If packed worm volume is greater than 200 µL, split sample into 200 µL aliquots per 15 mL conical. Wash with 12-15mls of FA Buffer. Spin and remove as much supernatant as possible.

9. Flash freeze in liquid nitrogen and store at -80˚C.

Chromatin Immunoprecipitation

Day 1

1. Add 1 Roche Complete tablet, 25ul 1M DTT, 125ul of 100mM PMSF to 25mL of chilled FA Buffer (for 4 samples). Rotate at 4˚C to dissolve pellet. Once dissolved, keep chilled on ice.

2. During step 1 rotation, thaw and always keep samples on ice. After thawed, add FA buffer to 1.5ml mark.

3. Turn on sonicator (Sonic Dismembrator FB-705; Fisher Scientific; Microtip (FB4418, 0.125 in.)) and program as follows.

- Select "YES" to "Are you using a Microtip?".
- Select "To select or modify a program or sequence, press here".
- Set parameters as follows:
- Amplitude = 10.
- Process Time = 00:03:20. (NOTE: This processing time equals 20 cycles of sonication, which will take ~24 min. total and will achieve 200-600 bp fragmentation)
- Pulse-On Time = 00:00:10.
- Pulse-Off Time = 00:01:00.

4. Prepare ice bath by filling 500ml plastic beaker with ice and add water. Stir to mix.

- If your sonicator is not located at 4˚C, add ethanol to your ice/water bath.

5. Clean microtip by washing with 95% ethanol and then di-water. Clean microtip for 3 sonication cycles using a conical containing 1.5 mL di-water.

- At all stages, place microtip to the 100 µL level on the 15 mL conical tube.

6. Place sample in chamber and secure the conical with the clamp, keeping the microtip away from the sides & bottom of the conical. Keep sample submerged in ice bath as much as possible. Close and tape door. Press start.

- Make sure to not touch microtip against conical wall at any time (both during assembly as well as during sonication processing).
- Use a folded kim wipe to help secure the clamp to the conical tube.
- Make sure the conical stays within the layer of ice.
- After first round of sonication, make sure the sample is not frozen.

7. Rinse microtip with water, ethanol, water. Clean microtip for 3 sonication cycles using a 15 mL conical containing 1.5 mL di-water between strains with different genotypes.

8. Repeat 6 & 7 until all samples are sonicated.

9. Clean microtip for 3 sonication cycles. Turn off sonicator by EXITing until you return to home screen that says "Are you using a Microtip?".

10. Put the 1.5ml sample into a 2ml tube. Spin at 13,000 x g for 15' at 4˚C.

11. Transfer supernatant to a new 2ml tube, avoiding carrying over any pellet.

12. Determine protein concentration by Bradford assay.

a) BSA Standard (2mg/ml): 8 standards from 2mg/ml – 0 mg/ml (2, 1, 0.5, 0.25, 0.125, 0.0625, 0.0313, 0). 20ul of appropriate BSA standard + 980ul of Bradford reagent

- 2mg/ml standard has 20ul of BSA standard. Do serial 1:2 dilutions for 1mg/ml-0.0313 standards. 0mg/ml has 20ul of water.

b) Sample: 2 sample tubes of 1:20 and 1:40 dilutions. 20ul of appropriate dilution +980 ul of Bradford reagent.

- 1.5ul of sample, in total volume of 30ul for 1:20 dilution and serial dilute to make 1:40.

c) Transfer to cuvette and spec.

Determine the protein concentration.

- Multiply protein concentration determined by the spec and dilution factor. Average the concentration.

13. Add extract corresponding to ~4.4mg protein to a 1.5ml tube and bring the volume to 400ul with chilled FA Buffer+ Protease Inhibitors. Then add 1:20 (v:v) 20% sarkosyl (20ul) and spin at 13,000x g for 5 minutes at 4°C.

- If preparing an IgG control, use 8.8mg protein in a total volume of 800ul and 40ul sarkosyl.
- Flash freeze remainder and store at -80˚C.

14. Transfer the supernatant to a new tube. Remove 40uL of the material (10% input) and store it at
-20°C until the following day, when it will be used to prepare input DNA.

15. Add appropriate amount of antibody (7.5ug of GFP antibody per IP) and rotate overnight at 4°C (16-20h).

- If preparing the IgG control, split the remaining extract between two tubes (~2.2mg each), one for the ChIP antibody (GFP antibody) and one for the control goat IgG antibody* (R&D Systems, Cat. #AB-108-C). 7.5ug of each antibody.
- For RPC-1 IP, Use 4ug RPC-1 antibody (from Jason Lieb's lab) per 4mg protein (in 800ul volume), incubate with 40ul protein A sepharose beads (GE Healthcare Cat. #17-5280-01), and still elute twice with 150ul elution buffer.

Day 2

1. Thaw the input samples from the previous day and add 2uL 10mg/mL RNase A. Digest at RT for 2 hours.

2. While the input samples are thawing, begin washing beads. Take 35uL (~20ul of actual beads) of protein G sepharose beads (GE Healthcare Cat. #17-0618-01) per ChIP/IgG sample and wash 4 times with 1mL FA buffer. Spin at 2500x g (rcf) for 2 minutes to collect the beads.

- Cut pipette tip using clean scissors or sterile razor-blade.
- Can use the FA buffer + protease inhibitors from the day before. Keep FA buffer on ice.
- Compare the volume of spun down beads with a 1.5 ml tube containing 20ul of water to ensure there is an equivalent amount of beads in each tube (~20 µL).
- Use vacuum and 1-10ul tips to remove supernatant.

3. Quickly spin down IP sample from previous day. Add entire IP sample to beads and continue to rotate at 4°C for 2 hours.

4. After the 2 hour RNase A treatment (Step 1 for the input samples), add 260uL elution buffer (or enough to bring volume up to 300uL), then add 4uL of 10mg/mL Proteinase K (Roche cat #03115836001) and put the input sample at 55°C for 2-4 hours.

- If there is precipitate in the elution buffer, put at 42˚C to return back to solution.

5. After the 2 hour bead incubation (Step 3 for the ChIP samples), wash beads at RT by adding 1 mL of each of the following buffers and incubating for the specified time on a rotator. Collect beads by spinning for 2 minutes at 2500x g:

- 2 times RT FA buffer (150mM) for 5 minutes
- 1 time FA-1M NaCl for 5 minutes. After this wash, transfer beads to new tubes with the next wash buffer.
- 1 time FA-500mM NaCl for 10 minutes
- 1 time TEL buffer for 10 minutes
- 2 times TE for 5 minutes

**<u>OPTIONAL:</u> For IP-Western, resuspend beads in 50uL of 2x sample buffer. Reverse the crosslinks at 65˚C for at least 1 hour prior to boiling sample off beads.

6. To elute the immunocomplexes, add 150uL elution buffer and place the tube in a 65°C heat block for 15 minutes. Vortex briefly every 5 minutes.

7. Spin down the beads at 2500x g for 2 minutes and transfer the supernatant to a new tube.
- Use western loading tips to remove about 150ul of supernatant. After the first elution, leave some supernatant behind so you don't disturb the beads.

8. Repeat elution and combine supernatants.
- Try to remove as much supernatant as possible.

9. Add 2uL of 10mg/mL Proteinase K to each ChIP sample. Incubate for 1-2 hours at 55°C.

10. Transfer all input and ChIP samples to 65°C for 12-20 hours to reverse crosslinks.

Day 3

1. Purify the DNA with Qiaquick MinElute PCR purification kit (Qiagen Cat. 28006). Elute with 12uL **H2O**.
2. Send to University of Chicago for library preparation and sequencing.
   - Freeze samples after purification at -20°C until it's time to ship.
   - If your downstream application is ChIP-qPCR, elute in 30- 50 ul of water using a QiaQuick PCR purification kit (Qiagen Cat. 28106).

Reagents

For Extract Preparation, Collection of the Immunocomplexes, and Washes. Filter sterilize FA and TEL Buffers and store at 4°C (Good for at least 6 months).

1) FA Buffer (150mM NaCl):
50mM HEPES/KOH pH7.5, 1mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 150mM NaCl

Recipe (500 ml):

1M HEPES-KOH, pH 7.5	25 ml
0.5M EDTA, pH 8.0	1 ml
Triton-x-100	5 ml
5% DOC (deoxycholic acid)	10 ml
5M NaCl	15 ml
Sterile water	444 ml

2)FA-1M NaCl Buffer:
50mM HEPES/KOH pH7.5, 1mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 1M NaCl

Recipe (500 ml):

1M HEPES-KOH, pH 7.5	25 ml
0.5M EDTA, pH 8.0	1 ml
Triton-x-100	5 ml
5% DOC (deoxycholic acid)	10 ml
5M NaCl	100 ml
Sterile water	359 ml

3)FA-500mM NaCl Buffer:
50mM HEPES/KOH pH7.5, 1mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 500mM NaCl

Recipe (500 ml):

1M HEPES-KOH, pH 7.5	25 ml
0.5M EDTA, pH 8.0	1 ml
Triton-x-100	5 ml
5% DOC (deoxycholic acid)	10 ml
5M NaCl	50 ml
Sterile water	409 ml

4)TEL Buffer:
0.25M LiCl, 1% NP40, 1% sodium deoxycholate, 1mM EDTA, 10mM Tris-HCl, pH8.0

Recipe (250ml):

5M LiCl	12.5 ml
NP-40	2.5 ml
5% DOC (deoxycholic acid)	50 ml
0.5M EDTA, pH 8.0	0.5 ml
1M Tris-HCl, pH 8.0	2.5 ml
Sterile water	182 ml

5) Elution Buffer:
1% SDS in TE with 250mM NaCl

Recipe (50 ml):

20% SDS	2.5 ml
5M NaCl	2.5 ml
TE	45 ml

6) 20% Sarkosyl Solution:

Recipe (100 ml):

N-lauroyl-sarcosine, sodium salt	20g
Sterile water	100 ml