Chromatin immunoprecipitation from embryo lysates

Embryo isolation and formaldehyde induced protein-DNA cross-linking:

1. Two to five grams of embryos were harvested by bleaching synchronized gravid hermaphrodites (Portman, D.S, Wombook, *Profiling C. elegans gene expression with DNA microarrays*).
2. Embryos were then collected in a 50 ml conical tube by centrifugation at 1000xg for 1 minute. Supernatant was aspirated and the pellet washed three times with 50 ml of M9.
3. Packed embryos were then washed once in 45 ml of 2% (v/v) formaldehyde (ACS grade, EMD Chemicals Inc, San Diego, CA) in M9 (made fresh prior to resuspension). After final centrifugation at 1000xg for 1 minute and aspiration of supernatant, the embryo pellet was resuspended to 50 ml with 2% (v/v) formaldehyde in M9 and rocked at room temperature for 30 minutes.
4. The cross-linked embryos were then washed once with 50 ml of 0.1 M Tris-HCl (pH 7.5) and washed twice with 50 ml of M9.
5. Embryos were then isolated by centrifugation at 1000xg for 1 minute and the supernatant aspirated. Embryos were washed in 50 ml of homogenization buffer. After centrifugation at 1000xg and aspiration of the supernatant, 1 ml of homogenization buffer per gram of packed embryo pellet was added for resuspension and the samples snap frozen in liquid nitrogen. Samples were then stored at -80^{o}C.

Preparation of extract for immunoprecipitation:

6. Five to ten milliliters of cross-linked embryos from step 5 above were thawed on ice and PMSF added to a final concentration of 1 mM.
7. The embryo suspension was sonicated on ice with ten 30 second bursts at 10% power output (Heat System XL2020 Sonicator with a standard 3 mm tapered microtip). A one minute pause between 30 second bursts was allotted to allow the lysate to cool.
8. Embryo debris was isolated by centrifugation at 5000xg for 20 minutes. Supernatant was then transferred to a clean 15 ml conical tube.
9. Sample was sonicated again as described in step 7 to shear the DNA. Debris was isolated by centrifugation at 25,000xg for 20 minutes. The supernatant was collected in 1 ml aliquots.
10. Lysates were incubated with 100 µl IgGsorb (The Enzyme Center, Malden, MA) per ml of lysate, for 30 minutes rocking at 4^{o}C. IgGsorb was removed by centrifugation for 2 minutes at ≤ 500xg. Supernatant was transferred to a new microfuge tube and snap frozen in liquid nitrogen. Samples were then stored at -80^{o}C.

Immunoprecipitation:

11. Embryo lysates from step 10 were thawed on ice and centrifuged at 14,000xg for 10 minutes at 4^{o}C. The supernatant was transferred to a new chilled tube and the DNA concentration was determined using a UV spectrophotometer.
12. Five to 10 µg of affinity purified antibodies were incubated with a volume of embryo lysate containing 2 mg of DNA for 2 hours rocking at 4^{o}C. For mock immunoprecipitation, an identical mass of pre-immune rabbit IgG was used in place of affinity purified antibodies.

    *While incubating, Dynabead Protein A was prepared. For each immunoprecipitation, 25 µl of Dynabead Protein A was aliquoted to a microfuge tube. Each sample was washed four times as per the manufacturer's protocol with

1 ml PBS supplemented with 5 mg/ml BSA (filter sterilized). After the final wash, the Dynabeads were resuspended with an equal volume of PBS+5 mg/ml BSA. The Dynabeads were then left on ice until step 15.

13. Non-specific precipitates were isolated by centrifugation at 14,000xg for 10 minutes at 4°C.
14. The supernatant was transferred to a microfuge tube containing 25 µl of Dynabead Protein A that were prepared in step 12. The tube was then incubated with rocking at 4°C for 30 minutes. Following incubation, samples were centrifuged at 500xg for 2 seconds to recover sample from the lid.
14. The antibody-antigen complex captured on the Dynabeads protein A beads were isolated by placing the tubes on a magnetic rack, as per the manufactures protocol, and the supernatant was then aspirated.
15. The Dynabeads were washed at 4°C eight times using the following buffer changes:
    4 times with 1 ml of ChIP buffer with 100 mM KCl
    2 times with 1 ml of ChIP buffer with 1 M KCl
    2 times with 1 ml of TE.
    The last wash was transferred to a new microfuge tube, the beads captured with the magnetic rack, and the supernatant removed.
16. Two hundred microliters of elution buffer [10 mM Tris-HCl (pH8), 1% (w/v) SDS] was then added and the tube placed on a magnetic rack. The eluate was transferred to a clean microfuge tube. The elution step was repeated. The two eluates (≈400 µl total vol) were then combined.
    *For input samples, 50 µl of embryo supernatant from step 11 was added to 400 µl of elution buffer.
17. Sixteen microliters of 5 M NaCl was added to each input and immunoprecipitated sample and incubated overnight at 65°C to reverse the formaldehyde cross-links.
18. The next morning, 8 µl of 0.5 M EDTA and 16 µl of 1 M Tris-HCl (pH 6.8) were added and mixed. To digest protein, 20 µg of proteinase K (Roche, Indianapolis, IN) was added for 1 hour at 45°C.

Immunoprecipitation cleanup:

19. Immunoprecipitation reactions and input samples from step 18 were extracted twice with 300 µl phenol (equilibrated with TE, pH 8.0), and the aqueous phase retained.
20. Immunoprecipitation reactions and input samples were then extracted once with 300 µl chloroform:isoamyl alcohol (24:1 v/v), and the aqueous phase retained (final volume should be approximately 320 µl).
21. To each tube, 13 µl of 5 M NaCl and 20 µg of glycogen (Ambion, Austin, TX) were added.
22. Seven hundred microliters of 100% ethanol was added and the tube vortexed to mix.
23. Samples were incubated at -80°C for 30 minutes.
24. Samples were then centrifuged at 14,000xg for 15 minutes at 4°C.
25. Supernatant was removed and the pellet washed with 500 µl of ice cold 70% ethanol. Samples were then centrifuged at 14,000xg for 5 minutes at 4°C.
26. Final supernatant was aspirated and centrifuged at 14,000xg for 2 seconds. All liquid was removed with a micropipette and the pellet air dried for 5 minutes.
27. A stock solution of RNase TE was prepared by combining 16.5 µl 10 mg/ml RNase A (Qiagen, Valencia, CA) with 483.5 µl TE. Each sample was resuspended in 30 µl RNase/TE and incubated for 2 hours at 37°C.

28. DNA was purified using a QIAquick PCR purification kit (Qiagen) as per the manufacturer's protocol and eluted in 25 µl of EB elution buffer. The 25 µl eluate was removed from the collection tube and replaced back on the column for a final, second elution.
29. Using a UV spectrophotometer the concentration of DNA of the input samples were determined and the input sample diluted to 20 ng/µl in EB elution buffer. Twenty nanograms of input sample was diluted to 25 µl EB elution buffer and was used in the blunting procedure below.
30. Samples were then stored at -20°C.

Blunting immunoprecipitated DNA ends:

31. Added to each the 25 µl immunoprecipitation sample:
    - 14 µl dH_2O,
    - 10 µl 5x DNA Terminator End Repair Buffer (DNA Terminator End Repair Kit; Lucigen, Middleton, WI)
    - 1 µl DNA Terminator End Repair Enzymes (DNA Terminator End Repair Kit; Lucigen)

    Each sample was mixed and incubated at room temperature for 30 minutes.
32. The DNA was purified using a QIAquick PCR purification kit (Qiagen) as per the manufacture's protocol, and eluted in 25 µl of EB elution buffer. The eluate was removed from the collection tube and replaced back on the column for a final second elution.

Oligonucleotide linker ligation:

33. Ligase mix was prepared on ice:
    - 5.0 µl 10x T4 ligase buffer (NEB, Ipswich, MA)
    - 6.7 µl 15uM annealed oligo linkers oJW102+oJW103 (see below)
    - 0.5 µl T4 DNA ligase (NEB)
    - 12.8 µl dH_2O
    - 25.0 µl total per reaction.
34. Twenty five microliters of ligase mix was added to each 25 µl blunted DNA sample on ice.
35. The sample was incubated overnight at 16°C.
36. Twenty micrograms of glycogen (Ambion, Austin, TX) and 6 µl 3M sodium acetate was added and mixed. One hundred and thirty microliters of ethanol was added to each tube and vortexed to mix.
37. Sample was incubated at -80°C for 30 minutes.
38. Sample was centrifuged at 14,000xg for 15 minutes at 4°C.
39. The supernatant was removed and the pellet washed with 500 µl ice cold 70% ethanol. Sample was then vortexed to mix and centrifuged at 14,000xg for 5 minutes at 4°C.
40. The supernatant was aspirated and centrifuged at 14,000xg for 2 seconds. All liquid was removed with a micropipette and the pellet air dried for 5 minutes.
41. The pellet was resuspend in 30 µl dH_2O. Five microliters from each sample was removed for pre-LMPCR qRTPCR quality check (see step 50 below), and the remaining sample stored at -20°C.

Linker mediated PCR:

43. PCR master mix was prepared on ice:

    For one reaction

10x ThermoPol Rxn Buffer (NEB)	4.00 µl
25 mM dNTP	0.50 µl
40 µM oJW102	1.25 µl
dH_2O	9.25 µl
Total volume	15.00 µl

Scale up PCR master mix for the total number of samples being run.

44. Fifteen microliters of PCR mix was added to each sample and mixed.
45. The polymerase master mix was prepared on ice:

For one reaction

10x ThermoPol Rxn Buffer (NEB)	1.00 µl
Taq 5U/µl (Qiagen)	1.00 µl
Pfu Turbo pol 5U/µl (Stratagene, La Jolla, CA)	0.01 µl
dH_2O	8.00 µl
Total volume	10.00 µl

Scale up PCR master mix for the total number of samples being run.

46. Ten microliters of polymerase master mix were added to each tube in step 44 and mixed.
47. PCR was performed with the following thermocycler program:
    step 1. 55°C for 2'
    step 2. 72°C for 5'
    step 3. 95°C for 2'
    step 4. 95°C for 1'
    step 5. 60°C for 1'
    step 6. 72°C for 2'
    step 7. Go to step 4 for 22 times
    step 8. 72°C for 5'
    step 9. 4°C forever
48. PCR product was purified with a QIAquick PCR purification kit (Qiagen) as per the manufacture's protocol. Sample was eluted with 30 µl of EB elution buffer.
49. Samples were examined on an agarose gel and quality examined with qRT-PCR (step 50 below).

Examining the success of immunoprecipitation by quantitative real-time PCR (qRT-PCR) of known DNA targets.

50. PCR master mixes for 13 reactions x 30 µl for each of your ChIP, mock, and input samples were prepared. In each master mix either 5 µl of the pre-LM-PCR sample or 130 ng of the LM-PCR template were added.

    *Each 27 µl PCR reaction (3 µl primers aliquot separately) should have the following components:

	1x	13x
25 mM dNTP	0.15 µl	2 µl
100x SyberGreen (Invitrogen, Carlsbad, CA)	0.30 µl	4 µl
10x Taq reaction buffer (Invitrogen)	3.00 µl	39 µl
pre-LM-PCR sample		5 µl
OR		

LM-PCR sample		130 ng
Taq polymerase (Invitrogen)	0.15 μl	2 μl
H_20 to a final volume of	27.00 μl	304 μl

After 27 μl aliquots were distributed in a 96 well plate, 3 μl of 3 μM primer sets were added to each reaction, to bring the total volume up to 30 μl. Each primer set was performed in triplicate. The four primer sets *him-1, fat-1, rex-4, her-1* (sequences below) were performed in triplicate yielding 12 PCR reactions per sample.

All quantitative PCR reactions were carried out and analyzed on a DNA Engine Opticon Real-Time PCR Detection System (MJ Research, Waltham, MA).

The following PCR thermocycler program was used for qRT-PCR:

1. 95°C for 5'
2. 94°C for 30''
3. 60°C for 30''
4. 72°C for 1'
5. 78°C for 10''
6. Plate Read
7. Go To Step 2 40 times

Oligonucleotide sequences:
oJW102 GCGGTGACCCGGGAGATCTGAATTC
oJW103 GAATTCAGATC
her-1 forward GAAGTTTCACCGCTAAGTTCG
her-1 reverse CCATTGTCTACGTCATCGTAC
him-1 forward CATCAGGAGCACCGGAAAG
him-1 reverse TTGTGCTCGTGAGCAACGG
rex-4 forward TTCTACGCGACTCAACCCC
rex-4 reverse TCGTTACCGCAGCTCTGAC
fat-1 forward CACTGAAGAGCCACGCATC
fat-1 reverse GTGCCGCAAAGTCTTGCAC

Annealing oligonucleotide linkers

1. 250 μl 1 M Tris, pH 7.9 and 375 μl each of 40 μM oJW102 and oJW103 oligonucleotides were combined in a 1.5 ml microfuge tube.
2. Sample was divided into 100 μl aliquots and place at 95°C for 5 minutes.
3. Tubes were transferred to a 70°C heat block.
4. The block was then removed from the heating unit and let stand to cool to room temperature, 25°C.
5. The samples were then placed at 4°C overnight and stored at -20°C the following day.

Buffer Recipes

M9 buffer: 6 g Na_2HPO_4, 3 g KH_2PO_4, 5 g NaCl, 0.25 g of $MgSO_4 \cdot 7H_2O$ per liter. Autoclave to sterilize.

Homogenization buffer: 50 mM HEPES-KOH, pH 7.6; 1 mM EDTA; 140 mM KCl; 0.5% NP-40; 10% glycerol. Protease inhibitor (Roche), 1 mM Phenylmethylsulfonyl fluoride (PMSF) (Sigma Al) and 5 mM dithiothreitol (DTT) are added fresh].

ChIP buffer: 50 mM HEPES-KOH, pH 7.6; 1 mM EDTA; 0.05% NP-40. Add KCl to the desired concentration. Protease inhibitor (Roche) 1 mM Phenylmethylsulfonyl fluoride (PMSF) (Sigma Al) and 5 mM dithiothreitol (DTT) are added fresh].