Human location analysis protocol **version 1.8 10/08/04**

PROCEDURE

I. Cell cross-linking

Use $5x10^7$ – $1x10^8$ cells (70-80% confluency for adhesion cells of 8-12 15 cm^2 plates or 175 cm^2 flasks) for each location analysis reaction.

Adherent cells:

1. Add 1/10 volume of fresh 11% formaldehyde solution to plates.
2. Swirl plates briefly and let them sit at RT for 10 min.
3. Add 1/20 volume of 2.5 M glycine to plates to quench formaldehyde.
4. Rinse cells twice with 5 ml 1X PBS. Harvest cells using silicon scraper.
5. Spin cells at 4k for 10' at 4°C.
6. Transfer cells to 15ml conical tubes and spin 4k 10' at 4°C.
7. Flash freeze cells in liquid nitrogen and store pellets at –80 °C.

Suspension cells:

1. Add 1/10 volume of fresh 11% formaldehyde solution to flasks.
2. Swirl flasks briefly and let them sit at RT for 20 min.
3. Add 1/20 volume of 2.5 M glycine to flasks to quench formaldehyde.
4. Spin cells at 2500rpm for 10' at 4°C.
5. Rinse cells with 100ml 1x PBS and spin cells at 2500rpm for 10' at 4°C.
6. Rinse cells with 10ml 1x PBS and transfer to 15ml conical tubes and spin 4k 10' at 4°C.
7. Flash freeze cells in liquid nitrogen and store pellets at –80 °C.

II. Preblock and binding of antibody to magnetic beads (@4°C preferred)

1. wash 100 µL Dynal magnetic beads (per reaction) in 1 ml fresh BSA/PBS
2. collect the beads by spinning at 3000 RPM for 3 minutes.
3. wash beads in 1.5 ml BSA/PBS 2 times, collect the beads with the magnet.
4. Add 6-10 µg of Ab + 250µL of PBS/BSA.
5. incubate 4 hr to O.N. on a rotating platform at 4°C.
6. wash beads 3 times in 1.5 ml PBS/BSA.
7. Resuspend in 100µL PBS/BSA.

REAGENTS AND CHECKPOINTS

11% Formaldehyde Solution

50 mls:	Final concentration
14.9 ml 37% Formaldehyde	11%
1 ml 5M NaCl	0.1M
100 µl 0.5 M EDTA (pH 8)	1 mM
50 µl 0.5 M EGTA (pH 8)	0.5 mM
2.5 ml 1M Hepes (pH 8)	50 mM

2.5 M glycine

-Dissolve glycine in water with constant stirring.
-don't adjust pH

BSA/PBS Solution

100 mL:
10 mL 10x PBS
500 mg BSA
90 mL H20
(good for at least a week)

III. Cell Sonication

Note: Add protease inhibitors to all lysis buffers before use. If using a protease inhibitor cocktail tablet from Complete, dissolve one tablet in 2 ml H_2O for 25x solution. Store in aliquots at -20°C.

1. Resuspend each tube of cells in 5-10 ml of Lysis buffer I. Rock at 4°C for 10'. Then spin at 4000rpm in a table top centrifµge, 5 min at 4°C.
2. Resuspend each tube of cells in 5-10 ml of buffer 2. Rock gently at 4°C for 5 min. Pellet nuclei in tabletop centrifµge by spinning at 2.5K rpm, 5 min at 4°C.
3. Resuspend pellet in each tube in 3 ml buffer 3.
4. Sonicate the suspension in 15ml conical tube with a microtip attached to Branson 250 sonifier, output setting at 5, constant power. Sonicate 12-15 times with 30 sec, allowing the suspension to cool on ice for 1 min between pulses. Automatic sonicator power should be power 7.
5. Add 1/10 volume of 10% Triton X-100. Transfer to 1.5 ml centrifuge tubes. Spin out debris 14K 4°C 10 min.
6. Save 100 µL (or at least 1/50 vol) of cell lysate from each sample as input control. Store at -20°C.

Lysis Buffer 1 (LB1)

100 ml:	final concentration
5 ml 1M Hepes-KOH, pH 7.5	50 mM
2.8 ml 5M NaCl	140 mM
0.2 ml 0.5M EDTA	1 mM
10 ml glycerol	10%
5 ml 10% NP-40	0.5%
0.25 ml Triton X-100	0.25%

Lysis Buffer 2 (LB2)

100 ml:	final concentration
4 ml 5M NaCl	200 mM
0.2 ml 0.5M EDTA	1 mM
0.1 ml 0.5M EGTA	0.5 mM
0.5 ml 2M Tris pH 8	10 mM

Lysis Buffer 3 (LB3)

100 ml:	final concentration
0.2 ml 0.5M EDTA	1 mM
0.1 ml 0.5M EGTA	0.5 mM
0.5 ml 2M Tris-HCl, pH8	10 mM
2 mL 5M NaCl	100 mM
1 mL 10% Na-Deoxycholate	0.1%
500 mg N-lauroyl sarcosine	0.5%

IV. Chromatin Immunoprecipitation

1. Add 100 µL Ab prebound Dynal magnetic beads from step III.
2. Rock 4°C O/N.

*NOTE: DO **NOT** 'PRE-CLEAR' THE LYSATE BEFORE OVERNIGHT ChIP WITH PROTEIN G OR A BEADS.*

V. Washing, eluting, and reverse cross-linking

1. Transfer to centrifuge tubes, continue working in the cold room until step 6.
2. Use the magnetic stand to precipitate the beads.
3. Wash 4-8 times with 1 mL wash buffer
4. Wash once with 1 ml TE-plus-50 mM NaCl or 1 mL TBS.
5. Spin 3k for 2-3 min and aspirate any residual TE/TBS.
6. Add 100 µl of elution buffer.
7. Elute DNA-protein complexes from beads at 65°C for 10-15 min with brief vortexing every 2 min.
8. Spin down beads 14k for 1 min.
9. Remove all 100 µl of supernatant.
10. Reverse x-link O/N 65°C.
11. Thaw input from step III(6), add 3 vol of elution buffer and reverse x-link O/N 65°C.

Wash buffer (RIPA buffer)

100ml:	final concentration
5ml of 1M Hepes (pH 7.6)	50 mM
200µL of 0.5M EDTA	1 mM
7 ml of 10% DOC (Na deoxycholate)	0.7%
10 ml of 10% NP-40 (IPGEL)	1%
10 ml of 5M LiCl or 2.12g powder	0.5 M

Elution buffer:
50mM Tris pH8
10mM EDTA
1% SDS

Western analysis

-Elute 2nd time from beads with elution buffer and use a portion of elution to reverse x-link
-Check for precipitated protein by Western analysis after crosslink reversal

VI. RNase, Proteinase K

1. Add 1 vol of TE to IP and input fraction.
2. Add RNase A so final is 0.2µg/µL (~5 µL/250 µL rxn). Incubate 37°C 1-2hr.
3. Add proteinase K so final is 0.2µg/µL (~2.5 µL/250 µL rxn). Incubate 55°C 2hr.
4. Extract once w/ 1 vol of phenol.
5. Extract once w/ 1 vol of phenol:chl:IA (made by mixing 1 vol of phenol w/ 1 vol of Chloroform:isoamyl alcohol).
6. extract once w/ 1 vol of chl:IA.

4-6. Or instead extract 1x w/ 1 vol phenol:chl:IA using phaselock tubes

7. add 20 µg (1 µL) of glycogen.
8. Add 5M NaCl so final is 0.2M (10 µL/250 µL rxn).
9. Precipitate DNA with a 10 min 14K spin, and wash pellet with 500 µL 75% EtOH.
10. Dry and resuspend pellets in 60 µL 10mM Tris HCl pH 8. Save 5 µL of IP sample for checkpoints to the right. Normalize the input/wce fraction to 100 ng/µL using the Nanodrop.

Fragment Size Verification

After sonication, the spread of size fragments can be checked by running 1 µL of input/wce control DNA on a 1% agarose gel. Spread should extend no higher than 2 kb.

Gene specific PCR

Check for IP enrichment specific for transcription factor by performing gene specific PCR with 2-3 µL of IP sample and a dilution series of input sample. Enrichment should be at least 2 fold over a non specific gene.

VII. T4 Fill-in and blunt-end ligation

A. T4 DNA polymerase filled in

1. To 55 µL of IP sample, and 200 ng (=2 µL) of the normalized input/wce diluted to 55 µL:
   - 11 µL 10X T4 DNA pol buffer
   - 0.5 µL BSA (NEB 10mg/ml)
   - 1 µL 10mM dNTP mix
   - 0.2 µL T4 DNA pol (NEB 3U/µL)
   - up to 110 µL H_2O
2. Transfer sample to PCR tube and incubate 12°C 20 min (program 12/20).
3. Add 11.5 µl 3 M NaAc and 0.5 µL (=10 µg) glycogen.
4. Extract 1x with 120 µL phenol:chl:IA.
5. Precipitate with 250 µL EtOH.
6. Spin and wash with 500 µL 75% EtOH.
7. Dry pellet and resuspend in 25 µL H_2O.

B. Blunt-end ligation

1. Make at 4°C, 25 µL ligase mix per reaction:
   - 7.8 µL H_2O
   - 10 µL 5X ligase buffer (Gibco)
   - 6.7 µL annealed linkers (*thaw at 4°C*)
   - 0.5 µL T4 DNA ligase
2. add mix to 25 µL of sample.
3. incubate 4°C or 12°C O/N, cover with foil.
4. Next day add 6µL of 3M NaOAc and 130 µL EtOH.
5. Spin and wash with 500 µL 75% EtOH.
6. Dry and resuspend pellet in 40 µL PCR reaction mix below.

Oligos for blunt end ligation:

oJW102: 5'-GCGGTGACCCGGGAGATCTGAATTC
oJW103: 5'-GAATTCAGATC
Final buffer/concentrations for annealing:
50 mM Tris pH 7.5
50 mM NaCl
17.5 µM each oligo
After mixing, boil 5 minutes, and anneal slowly from a 100°C heat block to 25°C, then cool to 4°C overnight, aliquot, and freeze.

VIII. Ligation mediated PCR

1. for PCR: use pellets from blunt ligation.
2. Make PCR mix per rxn:
   - 35 µL H_2O
   - 4 µL 10X Thermopol buffer (NE)
   - 0.5 µL 25 mM dNTP mix
   - 0.5 µL 100 µM oligo oJW102
3. Dissolve pellet in mix above and start PCR program CHIPCHIP: 55 °C _ 4'; 72°C _ 3'; 95°C _ 2'; 95°C _ 30''; 60°C _ 30''; 72°C _ 1'; goto step 4 24 times; 72°C _ 5'; 4°C _ hold.
4. During step 1 (55 °C _ 4') add TAQ mix:
   - 8 µL H_2O
   - 1 µL 10X ThermoPol buffer
   - 0.5 µL TAQ (5U/µL)(Gibco)
5. Cleanup PCR product with Qiagen PCR column. Wash several times with PE buffer. Elute DNA with 60 µL EB buffer.
   5 (alternate) Or, add 25µL of 7.5M NH_4OAc and 225µL EtOH. Spin and wash pellet with 500 µL 75% EtOH. Redissolve in 50 µL H2O.
6. Normalize [DNA] to 100 ng/µL.

Product Size and Amount Verification

Remove 2 µL of PCR product and run it out on 2% agarose gel. Product sizes should be around 250-350 bp. Amount of IP PCR product should be roughly equal to input PCR product. Check the concentration using the Nanodrop, expecting ~50-100 ng/µL.

IX. Cy3-Cy5 Random Primer labeling

1. From the normalized IP and wce PCR DNA above, 1 µg of DNA will be fluorescently labeled (=10µL of LM-PCR product).
2. Add 36 µL water to above.
3. Add 40 µL of 2.5 X random primer solution (Invitrogen Bioprime labeling kit).
4. Boil 5 min in heatblock (Use cap lock). Place tubes in icewater. Incubate 5 min.
5. Add 10 µL 10X low T dNTP mix (1.2 mM dATP, dCTP, dGTP each and 0.6mM dTTP).
6. Add 2 µL of cy5-dUTP to IP tube and 2 µL cy3-dUTP to input tube.
7. Add 2 µL of high concentration Klenow (40U/µL, Bioprime kit).
8. Incubate 20 °C 6-12 hrs (keep samples in dark as much as possible from here on).
9. Cleanup labeling rxn with Qiagen PCR purification column. Wash several times with PE buffer. Elute with 50 µL H_2O.
   8&9 (alternate). Incubate at 20°C 4-12 hours and purify by adding 30µL of 7.5M NH4OAc, and re-ppt once by resuspending pellet into 100 µL of 2.5 M NH4OAc, and adding 3X EtOH.
10. Dry samples at 37°C (>10 min)
11. Dissolve colored pellet in 16.5 µL H_2O

Dye incorporation

Use Nanodrop microarray program to measure cy3 (550 nm) and cy5 (650 nm) incorporation. Ideally 100 pmol or more per slide, 50 pmol is the minimum.

X. Chip hybridization

A. Prehybridization.

1. Make 50 ml of prehybridization buffer per 4 arrays, take out 1mL for hybe buffer.
2. Pour remaining prehybe buffer into Coplin jar. Pre-heat at 50°C 30 minutes.
3. Remove slides from vacuum and place them in the Coplin jar w/ prehyb buffer.
4. Incubate slides at 50°C for a *maximum* of 45 minutes.
5. Wash slides twice w/ RO water and dry slides by spinning 1000 rpm 2 min.
6. Fill two holes of bottom hybe chamber with water.
7. Place dried slides onto bottom chamber.

B. Probe preparation.

1. Combine cy5 labeled IP probes w/ cy3 labeled Input probes.
2. Add 20 µg of human Cot-1 DNA (1mg/ml) and 5 µL yeast tRNA (8 mg/mL).
3. Add 30 µL of NH4OAc 7.5 M and 130 µL EtOH
4. ppt DNA and wash with 500 µL 75% EtOH.
5. Dry and resuspend pellet in 50 µL hybe buffer saved from pre-hybe step.
6. Denature probes 95 °C for 5 min, spin briefly.
7. Incubate at 50°C until slides are ready (up to 5 min), spin briefly.
8. *Spot 47 µL of probes onto slides.
9. *Place cover slip (not too steep an angle) and gently press down (from top end of slide) to evenly spread the probe solution so that it covers all the spots on the array.
10. Gently place the top hyb chamber and secure chamber tightly w/ metal brackets.
11. Gently place chamber in 50°C water bath for at least 16 hr.

Prehybridization solution (make fresh)

50ml:	final concentration
12.5 ml formamide	25%
12.5 ml 20x SSC	5x
0.5 ml 10% SDS	0.1%
0.1 g BSA or acetylated BSA	0.2%

(remove 1 mL of pre-hybe to be used as hybe buffer)

*This step is an art and will take considerable practice. Some find it easier to spot the hybe solution down the center of a cover slip then gently lower the array toward the coverslip. Place the coverslip on a tip box or other raised surface, spot the hybe solution in several drops down the center of the slip, invert a slide (array facing down) and carefully lower it toward the coverslip. When the array touches the liquid it will pull the slip against the array (don't drop the array onto the slip). Simply turn the array over so the coverslip is on top and place into the chamber as usual. Once the coverslip is seated DO NOT TOUCH IT. Bubbles often remove themselves, your finger will just destroy the liquid film.

XI. Slide washing and scanning

A. Washing

1) Pre-heat Wash I to 37°C-50°C if desired.
2) Remove coverslip from slide by submerging slides on a rack in a 300 mL glass dish containing washing solution I and gently agitating. Do NOT force the cover slip off—you will damage the array spots.
3) Shake 65 rpm for 5 min RT.
4) Transfer rack with slides to glass dish containing washing solution II. Shake for 5 min.
5) Transfer slides to new glass dish containing wash III. Shake 1 min.
6) Repeat step 5 two more times. (3 x 1 minute washes, use new dish each time)
7) Dry slides by spinning at 1000 rpm for 2 min or by spraying with N_2 gas.
8) Store slides in the dark.

B. Scanning

1. Turn on Axon scanner and open Genepix
2. Place slide upside down in scanner.
3. Prescan.
4. During pre scan, adjust the cy3 channel(532 nm PMT voltage under hardware settings) to a background ~ 100 or less by pointing the cursor to area where there is no spot and viewing the p (pixel) value on the side bar.
5. Set up the proper scanning window
6. Scan.
7. Adjust the cy5 channel (635 nm) so the intensity of the spot signals of both channels approximately match, using combination of techniques below
    a. use histogram to overlap green and red graphs
    b. intensity ratio around 1
    c. toggle between 2 wavelength images to adjust by eye
8. when set, stop scan and rescan image
9. Save image file as multi-channels in Tif format.
10. load proper gps template (For 12k array, template is called hu13K.master2, location: settings folder of E:drive).
11. align template properly over all spots.
12. inspect all spots closely and adjust diameters of spots appropriately.
13. For spots that are bad, flag them as "flag not found."
14. click to analyze and save results as .gpr files.

Washing solution I

100ml:	final concentration
10 ml 20 X SSC	2X
2 ml 10% SDS	0.1%

Washing solution II

300ml:	final concentration
1.5 ml 20 X SSC	0.1X
3 ml 10% SDS	0.1%

Washing solution III

1L:	final concentration
5 ml 20X SSC	0.1X