ChIP-chip Using Affymetrix GeneChip S. cerevisiae Tiling 1.0R Arrays
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Making the WCE:

1. Grow 100 ml culture in YPD overnight at 30°C to an OD600 ~1.0.
(If working with ts alleles, add equal volume of media (pre-warmed overnight at 42°C) and heat-shock at 35°C for 30 minutes.)

2. To crosslink, add 1% Formaldehyde (2.7 mls 37% per 100mls culture) for 15 minutes at 30°C (keep at 35°C for this step *and* next if heat-shocking).

3. Add Glycine to 125 mM for 5 minutes at 30°C or 35°C (5 mls of 2.5 M stock per 100 mls culture).

4. Harvest cells by centrifugation, wash with 50 mls cold TBS + 125 mM Glycine. Repeat wash with TBS (no Glycine). Resuspend cells in ~1-2 mls cold TBS, split volume between two fast-prep tubes. Spin down and aspirate TBS. Freeze pellet at -80.

5. Suspend each pellet in 600 ul ChIP Lysis Buffer (140mM NaCl) with fresh protease inhibitors added. Use ½ complete protease inhibitor tablet for 25 mls buffer (see materials). Add acid-washed glass beads to the top of the liquid (almost the top of the tube).

6. Break cells with glass beads in fast prep machine for 45 seconds at speed 4.0. Repeat for a total of 7 times, waiting a minute in between each run to allow samples to settle and cool down.

7. Puncture tube with needle and spin down in small glass tubes in clinical centrifuge at 2K for 2'.

8. Resuspend <u>everything</u> in glass tube and transfer to eppendorf (Note- you will still have two tubes for each sample at this point - sonication needs to be done in this smaller volume.)

9. Sonicate each tube at 30% output, 90% duty cycle for 5 pulses. Repeat for a total of 7 times, alternating tubes between dry and wet ice to make sure they remain cool.

10. Spin 5' at 14K in cold room, transfer supernatant to new tube and spin again for 20'. Transfer supernatant to fresh tube and combine duplicate samples. This is your chromatin solution and should be stored at -80°C if not using right away.

11. Quantitate protein levels by Bradford Assay.

Immunoprecipitation:

12. IP an equal amount of each sample (1mg) . Make sure to save enough chromatin for the Totals ($1/10^{th}$ of the amount of protein used for the IP- put in separate eppendorf- freeze at -80 until next day.)

Note: For ChIP-chip, duplicates of each IP need to be performed to ensure you have enough material for the arrays. Set up 2 mocks and 2 IPs for each strain. You will only need one total for each strain.

13. If IPing with antibody, add antibody to 1 mg protein and bring total volume up to 500 ul ChIP Lysis Buffer (140 mM NaCl) with protease inhibitors (can use same buffer from before if stored at -20^{o}C). For negative control, use an equal amount of pre-immune serum (best) or do mock IP (no Ab) in 500 ul total volume. IPs are rotated overnight at 4^{o}C.

(The amount of Ab to use depends on the particular antibody. If you are IPing a tagged protein (HA, FLAG), it's must easier to just use the antibody coupled beads (see below). For myc-tagged proteins, we use 5 ul of antibody overnight. For antibodies made to our proteins, we generally use 5-6 ul. You might have to experiment with this.)

14. If IPing with antibody-coupled beads, add 40 ul washed beads to 1 mg of protein in 500 ul total volume. (Beads are washed with 1 ml lysis buffer and spin at 5K for 30 seconds. Carefully pipet off buffer, repeat twice more. Prepare 50% slurry by adding equal volume of buffer to beads- add 40 ul of slurry to IP). IP at 4C for 3-4 hours.

15. If you had IPed with antibody, the next morning add 40 ul protein A sepharose beads (Amersham) to a new eppendorf for each IP. Wash each with 3 X 1 ml lysis buffer. For last wash, remove all buffer. Quickly spin IPs to get rid of any precipitate that may have formed. Remove supernatant to tubes with beads. IP at 4C for 2 hours.

16. Perform washes with quick vortexes and quick spins (~5 seconds). Leave a small amount of liquid on top of beads to prevent loss during each wash

2 X 1 ml ChIP Lysis Buffer (140mM NaCl).
2 X 1 ml ChIP Lysis Buffer (500mM NaCl)
2 X 1 ml LiCl Wash Buffer
2 X 1 ml 1 X TE, remove supernatant from final spin completely and carefully.

17. Add 75 ul ChIP elution buffer to pellet. Incubate at 65^{o}C for 10 minutes.

18. Spin, remove supernatant to fresh tube, elute again with 75 ul ChIP Elution buffer.

19. Combine sups, incubate 65^{o}C overnight to reverse the crosslinks. Do the same for the totals- see next step.

20. Add 150 ul ChIP elution buffer to totals from step 12. Incubate at 65°C overnight.

21. Spin tubes briefly, add 750 ul Qiagen PCR purification kit PB buffer.
(Warm about 1 ml (or 55 ul per sample) of water in 50°C water bath.)

22. Add 450 ul to a column over a collection tube, spin 2', 10K.

23. Dump liquid from collection tube, add remainder of sample, spin 2', 10K.

24. Dump liquid, add 750 ul ethanol wash buffer PE, spin 1', 10K.

25. Dump liquid, spin 2', 10K to dry.

26. Transfer column to new eppendorf, drop 50ul 50°C water in column center. Incubate at 50°C for 10 minutes.

27. Spin 2', 10K.

Materials:

TBS:
For 500 mls:
30 mls 5M NaCl
20 mls 1M Tris-Cl pH 7.5
450 mls water

ChIP Lysis Buffer (140mM NaCl):
50 mM Hepes pH 7.5
140 mM NaCl
1% Triton X-100
0.1% NaDeoxyholate (Cholic Acid)
protease inhibitors (add before use)

500 mls:
25 mls 1M Hepes pH 7.5
14 mls 5M NaCl
50 mls 10% Triton X-100
5 mls 10% NaDeoxycholate
406 mls water

ChIP Lysis Buffer (500mM NaCL):
50 mM Hepes pH 7.5
500 mM NaCl
1% Triton X-100
0.1% NaDeoxycholate (Cholic Acid)
protease inhibitors (add before use)

500 mls:
25 mls 1M Hepes pH 7.5
50 mls 5M NaCl
50 mls 10% Triton X-100
5 mls 10% NaDeoxycholate
370 mls water

** For protease inhibitors, I use Complete, EDTA-free Protease inhibitor cocktail tablets (Roche 11 873 580 001) Add 1 tablet for 50 mls, or ½ tablet for 25 mls buffer. Store unused buffer at -20 C, can reuse if within 4-6 weeks).

LiCl Wash Buffer:
10 mM Tris pH 8.0
250 mM LiCl
0.5% NP-40
0.5% NaDeoxycholate
1 mM EDTA

500 mls:
5 mls 1M Tris pH 8.0
5.3 g LiCl
25 mls 10% NP-40
25 mls 10% NaDeoxycholate
1 ml 0.5M EDTA
up to 500 mls with water

ChIP Elution Buffer:
50 mM Tris pH 8.0
1% SDS
10mM EDTA

10 mls:
0.5 ml 1M Tris pH 8.0
1 ml 10% SDS
0.2 ml 0.5M EDTA
8.3 mls water

Store at RT.

Library Preparation and Amplification of Samples for Tiling Arrays

The reagents for the following steps are found in the Sigma GenomePlex Complete Whole Genome Amplification Kit – ignore manufacturer instructions.

Library Preparation

28. Dry down duplicate mock and IP samples in speed vac on medium heat until all liquid is gone. Keep a close eye on samples, you don't want them to be in there for a long time after the liquid is gone. This usually takes somewhere between 1 – 2 hours. Do not dry down your "total" sample.

29. Resuspend dried pellet (you probably won't see anything) in 10 ul H_20. Add water to pellet and let sit for a few minutes to ensure the DNA is rehydrated.

30. Quantitate Total for each strain by making a 50-fold dilution (2 ul in 100 ul total volume). Record C (ug/ul) and ratio for each sample. You should get around 0.1 – 0.2 ug/ul. Transfer 1 ug of total DNA to new tube – bring total volume up to 10 ul with water. Do this in duplicate for each strain, so you will now have duplicates of mock, IPs, and totals, making 6 samples for each strain.

31. Add 2 ul 1X Library Preparation Buffer to each tube with 10 ul of material.

32. Transfer samples to strip tubes or individual thin walled 0.2 ml PCR tubes. DO NOT use the yellow tubes- the seal is not tight enough and you will lose volume. I find this easiest to do with the green individual tubes, but if you prefer strips, that will be ok as long as you use caution when opening and closing the strips- it is extremely important that NO volume is lost. Also- try your best not to introduce bubbles when adding reagents because the mini-centrifuge for PCR tubes doesn't get rid of them.

33. Add 1 ul Library Stabilization Solution, mix by pipetting. Quick spin if necessary and place at 95^o for 2 minutes in thermal cycler --> PROGRAM WGA1 under ROS.

34. Immediately remove samples and cool on ice. Run PROGRAM WGA2 under ROS with nothing in the thermal cycler to cool block to 16^oC while performing next step.

35. Add 1 ul Library Preparation Enzyme, mix by pipetting and quick spin if necessary.

36. Incubate in thermal cycler as follows: PROGRAM WGA3 under ROS
    - 16^o for 20' (cycler should be precooled to this temp)
    - 24^o for 20'
    - 37^o for 20'
    - 75^o for 5'
    - 4^o hold

37. Quick spin if necessary and either proceed to 1^{st} amplification or freeze at -20^o for no longer than overnight. Material is not stable at this step for long periods in the freezer.

1st Amplification

38. Prepare a master mix containing the following for each sample (For 6 samples, multiply by 6.2, for 12 samples, multiply by 12.4 to conserve as much of the reagents as possible but still have enough for pipetting).

10 X Amplification Master Mix	7.5 ul
H20	46.3 ul
25 mM dUTP	1.2 ul
WGA DNA Polymerase	5 ul
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	60 ul

39. Add 60 ul master mix to each sample, mix by pipetting and quick spin if necessary.

40. Incubate in thermal cycler as follows: PROGRAM WGA4 under ROS

95^{o} for 3' then 14 cycles of
94^{o} for 15"
65^{o} for 5', then
4^{o} hold

At this point, amplified material is stable and can be stored at -20^{o} indefinitely.

41. Purify samples using QIAquick PCR purification kit according to manufacturer instructions. Prewarm water for elution as done previously to increase elution efficiency. It is important to elute the samples in water- NOT the elution buffer in the kit. Also- DO NOT use the PBI buffer in the kit, use PB (in separate bottle). PBI buffer is not compatible with microarray analysis.

42. Quantitate amplified samples using 50-fold dilution (2 ul in 100 final volume). Determine amount of total DNA (ug) in remaining volume (approximately 48 ul). Total amount of DNA will probably range from 0.1 ug to 4 ug at this point depending on the sample. 7.5 ug is of amplified DNA is required for hybridization to the arrays. Even if you have 7.5 ug by combining samples for the IP and Totals, you need to do the a 2nd amplification for ALL reactions to ensure they are treated equally. You should not have obtained enough material from your Mock samples- if you did, the experiment did not work properly. There should be a significant enrichment of material in your IP samples compared to mock- something in the neighborhood of 20-fold. If the enrichment is much less than this- stop here and talk to Rebekka.

2nd Amplification

43. Remove 10 ng purified amplification product from each sample and bring volume to 10 ul with water. Set up reactions in PCR tubes. To avoid pipetting small volumes, make a 1:10 dilution of samples that amplified more so that you never have to pipet less than 1 ul to get 10 ng.

44. Prepare master mix exactly as before (6 samples X 6.2, 12 samples X 12.4)

10X Amplification Master Mix	7.5 ul
H20	46.3 ul
25 mM dUTP	1.2 ul
WGA DNA Polymerase	5 ul
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	60 ul

45. Add 60 ul mix to each sample, mix by pipetting and quick spin if necessary.

46. Incubate in thermal cycler exactly as with 1st amplification: PROGRAM WGA4 under ROS.

47. Purify DNA using QIAquick PCR purification kit as before.

48. Quantitate DNA using 50-fold dilution (2 ul in 100 ul total volume) and calculate total DNA. At this point, you should have amplified your material enough to have 7.5 ug for the Mock, IP, and Total of each strain. This may or may not involve combining your duplicate samples. Combine samples where necessary to have 7.5 ug of each sample.

Fragmentation
Next- samples are fragmented with UDG to obtain the proper fragment size for the array.

The reagents for the following steps can be found in the GeneChip WT Double Stranded DNA Terminal Labeling Kit.

49. The fragmentation step requires that the 7.5 ug of DNA be in a volume of no more than 39.45 ul. If the volume of your samples is larger than this, reduce the volume in the speed vac. You do not have to dry the samples all the way down, reduce the volume and then determine the final volume with a pipet.

50. Bring the total volume of DNA up to 39.45 ul with water – transfer sample to PCR tubes- here individual tubes are best, because they will given to Alyson in the core and there is less chance for sample confusion if they are individually labeled.

51. Prepare a mix with the following components:

Component	1X
10 X Fragmentation Buffer	4.8 ul

UDG, 10 U/ul	1.5 ul
APE 1, 100 U/ul	2.25 ul
Total	8.55 ul

For 6 samples, multiply by 6.5, for 12 samples, multiply by 13. This kit is extremely expensive, so you only want to waste as little as possible.

52. Incubate in thermal cycler (with heated lid) using the following protocol: PROGRAM TA5 under ROS

37^{o} for 1 hour
93^{o} for 2 min
4^{o} for 2-10 min (dont hold for longer- material isn't stable at 4^{o})

53. Mix by pipetting, transfer 45 ul of sample to new PCR tube. There should be a few microliters left in the tube, which will be analyzed to make sure the fragmentation worked properly. The samples can be taken to Alyson in the core – write on the rack "RNA 6000 LabChIP Kit – Agilent Array", your initials, and Auble Lab. Make sure she understands that you just want her to analyze the fragment size, as she has done for Rebekka's samples in the Auble Lab. Ask her when she will run the array and tell her you want to look at the results. She knows what the data should look like, but you should see it for yourself at least the first time, and I can go with you and make sure it looks ok.

The peak of the distribution should be between 25-100 bases. There should not be any detectable fragments above 200 bases, and you should be concerned if you have lot of fragments in the 100-200 base range. This won't efficiently hybe to the 25-mer probes on the array.

Labeling of Fragmented DNA

Fragments are now biotin end-labeled for hybridization.

54. Prepare a mix with the following components

Component	1X
5X TdT Buffer	12 ul
Tdt, 30 U/ul	2 ul
DNA Labeling Reagent, 5 mM	1 ul
Total	15 ul

55. Add 15 ul mix to 45 ul of the fragmented DNA samples, mix by pipetting.

56. Incubate reactions in thermal cycler (with heated lid) using the following protocol: PROGRAM TA6 under ROS

37^{o} for 60 min
70^{o} for 10 min
4^{o} for 2-10 min

57. Store labeled reactions at -20^{o} until hybridization

58. To hybridize, take labeled samples and arrays to Alyson in the core. Make sure you tell her that the samples are already labeled and she only needs to hybridize (again just as she has done for Rebekka's tiling samples).

59. Alyson will give you a DVD with the raw data - .CEL files – these can be taken to Kunal to process so that you can browse the data in IGB.