ChIP assay in *S. pombe*

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Adapted from protocol by Eishi Noguchi 4/9/03

Preparation of cells

- Use strain containing *cdc25-22* and FLAG tag so cells can be synchronized using temp sensitivity.
- Start a 50ml O/N culture at 25°C, one culture for control and one for HU treated cells.
- The next day, when OD~0.50, dilute into 200ml culture with a final OD of 0.1, incubate at 25°C for about 6hrs.
- When OD reaches ~0.2, then dilute into 600-700ml culture. Incubate O/N at 25°C.
- Final OD target the next day is ~ 0.3 in 600-700ml. If OD is higher, but still in log phase (0.4-0.8), can dilute to 0.3OD.
- Shift cells to 36°C shaker waterbath to start arrest. Incubate for 4 hours.

DAY 1

CROSS-LINK CELLS

- Prepare tubes for cross-linking: two 50ml conicals and one 1.5ml screw-cap tube per sample, per timepoint.
- When 4hr incubation at 36°C is done, take 45ml for 0hr timepoint (one tube). Add 10mM HU to one of the cultures.
- Shift cells back to 25°C to release from arrest. To cool down, place flask in ice bath and monitor temp. When temp reaches 27°C, move flask to 25°C waterbath.
- Begin "count up" on timer.
- Follow timetable for cross-linking: at each timepoint, transfer 45ml of cells from each culture to a 50ml conical tube.
- Remove 10µl sample to count cells and get septation index (count 200 cells total).
- Fix cells: add 1.25ml of 37% Formaldehyde (final conc. is 1%).
- Nutate tube for 20min at room temp.
- Quench cells: add 2.5ml of 2.5M Glycine (final conc. is 125mM).
- Return to nutator for 10min.
- Wash cells: Pour culture into filter apparatus and wash filter and walls with 20ml ice cold TBS.
- Remove filter and place in new 50ml conical tube.
- Add 20ml ice-cold TBS.
- Vortex well to release cells from membrane.
- Centrifuge at 2,500rpm for 4min. 4°C.
- Pour off supernatant and discard filter.
- Resuspend in 1ml ice-cold TBS.
- Transfer sample to a 1.5ml screw-cap tube.
- Spin down, remove supernatant, and immediately freeze pellet at -80°C (or using liquid nitrogen).

DAY 2

PREPARE TUBES

- Need one of these sets tubes (1.5ml) for each sample:
    1. for recovery from glass beads
    2. for supernatant after 5min centrifuge
    3. *siliconized tube* for supernatant after 10min centrifuge
    4. for input sample
    5. for protein assay
    6. *siliconized tube* for DOC buffer step

BREAK CELLS

- Remove cell pellets from freezer and place on ice. ***Keep everything on ice from this point.***
- Prepare Lysis Buffer L, Lysis Buffer H, DOC buffer, TE, and TES.
- Add 400µl of Lysis Buffer L.
- Add cold glass beads until they reach the surface of the buffer.
- Break cells by FastPrep: output 6, 20 seconds, 2 cycles, 2 minute interval between cycles.
- Recover cell lysate in a new eppendorf tube (tube #1).
- Pierce the bottom of the FastPrep-ed tube with a hot needle.
- Place in new eppendorf tube.
- Centrifuge- 3,000rpm for 30sec.
- Mix well by vortexing.

SONICATE CELLS

- Place all tubes in special float for sonicator.
- Set sonicator to "program 9":
    - Output- 10
    - Process time- 4min
    - On time- 20sec
    - Off time- 1min, 30sec
- Start sonication.

IMMUNOPRECIPITATION

- Pre-wash α-FLAG agarose beads, 40µl bead (50% slurry, 20µl bed volume) mixture per sample.
    - Centrifuge total amount at 7,000rpm for 10-15sec.
    - Aspirate supernatant and resuspend (by inverting) in 1ml Lysis Buffer L.
    - Wash in Lysis Buffer L two more times, then resuspend up to total amount needed (for example, bring up to the 500µl mark for a set of 12 samples).
    - Keep on ice until needed.
- Add 200µl Lysis Buffer L to sonicated samples.
- Centrifuge- 14,000rpm for 5min, 4°C.
- Transfer supernatant to a new tube (tube #2).
- Centrifuge- 14,000rpm for 10min, 4°C.
- Transfer 552µl of supernatant to a new siliconized tube (tube #3).

    - Use 2µl of supernatant for protein assay:
        - Dilute BioRad Protein Assay Dye Reagent Concentrate (at 4°C) 1:5 with water.
        - Put 1ml of diluted dye into each tube (tube #5)- one tube per sample.
        - Add 2µl of sample to each; use 2µl of Lysis Buffer L for the control tube.
        - Allow 5min for color change to occur.
        - Transfer samples to cuvettes and read at OD595.
    - Adjust protein concentration to lowest sample concentration:
    - Prepare input control:
        - Add 35µl TES to each tube #4.
        - Add 5µl of the adjusted sample to the TES.
        - Incubate in 65°C air incubator for 12-16hr.
- Add 40µl of the pre-washed α-FLAG agarose beads to the remaining adjusted sample.
- Rotate for 2hr at 4°C.
- Wash beads (spin down, add 1ml of buffer, invert 10 times, remove supernatant by aspirator)
    - Wash 3 times with Lysis Buffer L
    - Wash 2 times with Lysis Buffer H
    - Wash 2 times with DOC buffer- transfer to #6 tube
    - Wash 1 time with TE buffer, remove supernatant with pipette b/c pellet will be loose.
- Samples can now be at room temp.

<u>REMOVE CROSS-LINK</u>
- Add 52µl TES to pellet.
- Incubate for 30min in 65°C hot block, vortexing occasionally.
- Centrifuge 7,000rpm, 10-15sec.
- Take 10µl out for protein sample, transfer to a new 1.5ml tube.
    - Add 10µl of 2X SDS-PAGE loading buffer.
    - Incubate in 65°C air incubator for 12-16hr.
- Take 40µl out for ChIP sample, transfer to a new 1.5ml tube.
    - Incubate in 65°C air incubator for 12-16hr.

<u>DAY 3</u>
- Remove samples from 65°C air incubator.
- Freeze SDS-PAGE samples at -20°C until needed.
- Do DNA extraction with ChIP and input control samples.

<u>DNA EXTRACTION</u>
- Make master mix- multiply quantities by number of samples, make some excess:
    - 160µl 10mM Tris pH7.4
    - 1µl of 20mg/ml proteinase K

- 4µl of 10mg/ml yeast tRNA

- Add 165µl of master mix to each sample.
- Incubate 2hr in 50°C air incubator.
- Remove samples, purify DNA using Eppendorf Gel Cleanup Kit.
- For last step- add 30µl elution buffer to ChIP samples, and 50µl elution buffer to input control samples.
- Store purified samples at -20°C until needed for PCR.

PCR REACTION

Template	1µl
Primers (3pmol/µl)	1.5µl each primer
dNTP mix (2.5mM each)	2.4µl
10X EXtaq buffer	3µl
EXtaq	0.15µl
dH_2O	21.95µl
total:	30µl

cycles: 94°C 10min

94°C 30sec
50°C 30sec repeat 30-35xs
72°C 30sec

72°C 7min

electrophoresis:

gel- 1.5% agarose
load samples with loading buffer containing less dye.

REAGENTS NEEDED:

1. Ice-cold TBS
2. Chilled glass beads
3. 37% Formaldehyde- use fresh bottle, open less than one month

4. 2.5M Glycine
5. Lysis Buffer L
6. Lysis Buffer H
7. DOC buffer
8. TE
9. TES
10. 20mg/ml proteinase K (Invitrogen fungal proteinase K: 25530-031)
11. 10mg/ml yeast tRNA (Roche tRNA from baker's yeast: 109495)

Prepare buffers fresh just before use.

Lysis Buffer L (L= low sodium)

1M Hepes-KOH, pH7.5	2.5ml
5M NaCl	1.4ml
5M EDTA	100µl
20% Triton X-100	2.5ml
10% DOC	0.5ml
0.2M pAPMSF	50µl
complete EDTA free	1 big tablet
H_2O	42.95ml
Total volume	*50ml*

Lysis Buffer H (H= high sodium)

1M Hepes-KOH, ph7.5	1.5ml
5M NaCl	3ml
0.5M EDTA	60µl
20% Triton X-100	1.5ml
10% DOC	300µl
0.2M pAPMSF	30µl
complete EDTA free mini	3 tablets
H_2O	23.61ml
Total volume	*30ml*

DOC (sodium deoxycolate) Buffer

1M Tris-HCl, pH8.0	0.3ml
1M LiCl	7.5ml
10% NP-40	1.5ml
10% DOC	1.5ml
0.5M EDTA	60µl
H_2O	19.14ml
Total volume	*30ml*

TE

1M Tris-HCl, pH8	0.5ml
0.5M EDTA	100µl
H_2O	49.4ml
Total volume	*50ml*

TES

TE	20ml
20% SDS	1ml
H_2O	18.76ml
Total volume	*40ml*