MCM chromatin-binding assay

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Stephen Kearsey
Dept Zoology
South Parks Rd
Oxford

stephen.kearsey@zoo.ox.ac.uk

Buffers:

1. EMM sorb pH 7.0

KH phallate	3g	15 mM
Na_2HPO_4	2.2 g	15 mM
NH_4Cl	5 g	90 mM
Sorbitol	218.4 g	(1.2M)

pH with NaOH to 7.0

water to 1l

2. Zymolyase 20-T (ICN 320921 20000U/g)

20mg/ml in EMM sorb buffer - store 50 µl aliquots at -70°C

source of zymolyase is important when digesting –N cells

3. Extraction buffer – depends on protein being examined. For Mcm2-7 use buffer 1 or buffer For Mcm2-7 in meiosis experiments use buffer 2 (no magnesium).

Extraction buffers for CBA assay

Extraction buffer 1 (normal +Mg2+)

As per buffer 2 but omit ETDA and add MgAc to 2 mM
Also, do not add spermine and spermidine

Extraction buffer 2 (normal)

	for 500 ml
20 mM Pipes-KOH pH 6.8	3.02 g
0.4 M sorbitol	36.4
1 mM EDTA	1 ml of 0.5 M
150 mM KAc	7.35 g

After autoclaving add to **50 ml lots** in falcon tube

0.5 mM spermidine HCl	25 µl 1 M
0.15 mM spermine HCl	7.5 µl 1M

store at –20°C

Extraction buffer 3 (high pH, low salt)

	for 500 ml
20 mM HEPES-KOH pH 7.7	
0.4 M sorbitol	36.4
1 mM EDTA	1 ml of 0.5 M
50 mM KAc	2.45 g
Autoclave	

After autoclaving add to **50 ml lots** in falcon tube

0.5 mM spermidine HCl	25 µl 1 M
0.15 mM spermine HCl	7.5 µl 1M

store at –20°C

Extraction buffer 4 (ELB salts)

	for 500 ml
2.5 mM MgCl2	
50 mM KCl	
10 mM HEPES-KOH ph 7.7	

Extraction buffer 5 (normal, low salt, for DNA polymerase alpha)

	for 500 ml
20 mM Pipes-KOH pH 6.8	3.02 g
0.4 M sorbitol	36.4

to 50 ml aliquots add

0.1 ml 5 M KAc	(final conc 10 mM)
0.5 mM spermidine HCl	25 µl 1 M
0.15 mM spermine HCl	7.5 µl 1M
0.1 ml 0.5 M EDTA pH 8.0	(final conc 1 mM)

1. Basic procedure

1. Grow gfp-tagged strain strain into log phase in YE or EMM (EMM is recommended) (typically 20 ml @ OD_{595} =0.2-0.5).
2. Label and place all tubes and solutions on ice.
3. Transfer around 20ml to Falcon tube. (*Add 500µl Na Azide(HIGHLY TOXIC!- gloves, labcoat) (10%) (for eg time course where it is important to stop the cells in DNA replication*).
4. Spin tubes (3k, 3 min, 4°C).
5. Resuspend in 1.8ml EMM sorb buffer and transfer to 2ml eppendorf. (can include 10 mM DTT in this step for cells that may be difficult to digest eg after -n2 starvation).
6. Spin tubes (4k, 1 min, 4°C).
7. Resuspend cells in 450µl EMM sorb buffer containing DTT (10 mM, add 1/100 1M stock).
8. Add 50 µl of 20 mg/ml zymolyase 20-T (ICN 320921 20000U/g).

Digest at 32°C for 10-20 minutes. May need to use a much higher concentration of zymolyase or longer time of digestion, if cells have been nitrogen starved eg in meiosis release experiments.

9. Test for adequate digestion by adding SDS to aliquot - cells should go dark under phase (aim for >95% phase dark).
10. Add 1.5 ml EMM sorb buffer (at 0°C).
11. Spin tubes (4k, 1 min, 4°C). (Spin1)
12. Resuspend in 2 ml EMM sorb buffer (at 0°C).
13. Spin tubes (4k, 1 min, 4°C). (Spin2)
14. Resuspend in 2 ml extraction buffer (at 0°C).**
15. Spin tubes (4k, 1 min, 4°C). (Spin3)
16. Resuspend in 0.9 ml extraction buffer **containing protease inhibitor** (Roche 1 836 153 "complete mini)*.
17. Split into 2 x 450µl.
18. To one 450µl add 50 µl extraction buffer plus 10% Triton X-100(made in extraction buffer) = +Triton. Remaining 0.45 ml serves as minus Triton control).
19. Mix both tubes by inverting. Transfer +Triton to 20°C water bath. Keep -Triton on ice.
20. Incubate for 5 min, inverting periodically. Spin tubes (4k, 1 min, 4°C).
21. Take off supernatant, flick tubes so cells come off of tube wall and add 1.5 ml methanol.
22. Leave on ice for at least 6 mins.
23. Spin tubes (4k, 1 min, 4°C).
24. Resuspend in 1 ml acetone.

25. Store tubes at -20°C. Cells are okay for a few days.

*add one tablet to 10 ml extraction buffer, store at –20°C in 2 ml aliquots

** can also include protease inhibitors at this stage if desired.

Notes:

1. Cells in the –T control may lose soluble nucleoplasmic proteins even without detergent. It is useful to fix an aliquot of cells directly at step 1 to avoid this problem.

2. Using 100% ethanol for fixation, rather than using methanol/acetone works fine for some GFP tagged strains.

3. To carry out nuclease digestion control, to establish if chromatin integrity is required for the retention of a specific protein, add micrococcal nuclease to 2.5 units/ml and $CaCl_2$ to 2 mM at step 20 (+Triton). As a control add EGTA to 10mM as well.

2. Mounting cells for fluorescence microscopy for visualization of GFP fluorescence

1. Resuspend cells (from step 27) and transfer ca. 0.2 ml to 1.5 ml eppendorf tube. Spin down (4k, 1 min) and take off most acetone - leave about 20-50 µl.

2. Resuspend cells by vortexing and mildly sonicate (optional) to break up cell clumps. Spread about 10 µl of cell suspension onto a polylysine coated slide (eg BDH 406/0178/00). Allow acetone to dry, and add ca. 10 µl mountant. Use agarose mountant for YFP strains.

Agarose mountant (which seems to preserve YFP fluorescence of Pol1-YFP)

25/5/05

1 g DABCO (antifade*)
10 ml 0.1 M Tris-acetate pH 8.5

Dissolve, re pH to 8.5 with acetic acid
Make up to 100 ml

Add 0.15 g LMT agarose to 25ml and melt in microwave

Add DAPI to 50-125 ng/ml
Store in dark (4°C?)

To use

1. Melt agarose and cool to 37°C
2. Spread cells in acetone in normal way (use ordinary slide – no need to use polylysine coated)
3. Add 10 ul agarose moutant
4. Drop on coverslip – leave 5 min before viewing to allow time to set.

* doesn't actually seem to make a lot of difference to the rate of fading

DAPI/PBS mountant

50% glycerol
50% PBS
0.005-0.1 µg/ml DAPI (use low conc for CFP filters otherwise can get bleed through)

3. Staining extracted cells with antibody

This is for use with Triton-extracted cells after methanol and acetone fixation (i.e. from step 7).

1. Resuspend Triton-extracted cells and transfer ca. 0.2 ml to 1.5 ml eppendorf tube. Spin down (4k, 2 min) and take off most acetone - leave about 20-50 µl.

2. Resuspend cells by vortexing and mildly sonicate to break up cell clumps. Spread about 10 µl of cell suspension on to a polylysine coated 13 mm coverslip.

3. Rinse coverslip in PBS, then incubate in PBSBAL for 30 minutes.

4. Remove PBSBAL, add 20 µl primary antibody to coverslip. Incubate in a 'wet box' for at least 1h.

5. Wash in PBSBAL (3 x 5mins).

6. Add 20 µl secondary antibody (eg Texas Red conjugated). Incubate in dark 'wet box' for at least 1h.

7. Wash in PBSBAL (3 x 5mins).

8. Drain coverslip well. Mount in DAPI/PBS.

(we have stained extracted cells with anti tubulin (TAT1) antibody - gives good staining of mitotic spindles, although interphase microtubules are not preserved).

PBSBAL

1 X PBS containing:

100 mM lysine hydrochloride, pH 6.9
0.01% sodium azide
1% essentially fatty acid free BSA (Sigma A0281)

Cover slips

Soak in 0.1 % poly L lysine (5-15 mins). Drain, dry, rinse in water, dry.

Useful stocks

Spermine 1M stock

0.348 g for 1ml of 1M stock

Spermidine 1M stock

0.254g for 1ml of 1M stock

HU

1.388 g for 20 ml 1M stock

Trouble shooting problems

1. DAPI false signal problem with cfp – use dapi at low conc eg 5 ng/ml and integrate to get decent signal

2. when doing hu expts can add hu to zym to arrest

3. essential to get good digestion >95% - use at least 50 ul of zym per digest and prewash in DTT

4. use emm for temp shifts – gives lower bg with mcm4 at least?

5. use emm not yes for temp shifts and do a minimal shift!