Cds1 kinase assay by the use of MBP as a substrate

2006-0227

/// *Cell preparation*

Samples:

	HU 0 hr	HU 2 hr	HU 4 hr
Y1; WT [positive control]	1	2	3
Y1113; sap1-1	4	5	6
Y1119; sap1-27	7	8	9
Y1125; sap1-48	10	11	12
Y149; cds1Δ [negative control]	13	14	15
Y211; swi1Δ [shift down control]	16	17	18

Cell collection:

- Culture cells in YES, at 25˚C.
- 12 mM HU for 2 or 4 hours (from O.D. 0.2~0.35, depends of the cell growth)
- Collect cells; 50 ml of O.D. 0.5 /sample
  [Spin down, decant YES, resuspend in 1 ml of STOP buffer, transfer to a 1.5 ml screw cap tube, spin down, aspirate buffer and freeze cell pellet at -80˚C]

/// *IP*

Reagents: (for 18 samples) …Make right before you use

Lysis Buffer

Final Conc.	Ingredient	Stock Conc.	For 80ml	
50 mM	Tris-HCl (pH7.5)	1 M	4 ml	
250 mM	NaCl	5 M	4 ml	
80 mM	ß-glycerophosphate	1 M	6.4 ml	
15 mM	p-NPP*	1 M	1.2 ml	
50 mM	NaF	0.5 M	8 ml	
5 mM	EDTA	0.5 M	0.8 ml	
1 mM	DTT	1 M	80 μl	
0.1 %	NP-40	10 %	0.8 ml	
	Complete		1 + 3mini	
1 mM	pAPMSF	1 M	80 μl	
	H_2O		54.64 ml	

*p-NPP = p-Nitrophenyl Phosphate (in H_2O, in dark, -20˚C)

2x Kinase Buffer

Final Conc.	Ingredient	Stock Conc.	For 40ml	
20 mM	Hepes (pH7.5)	1 M	800 μl	
150 mM	KCl	4 M	1.5 ml	
10 mM	$MgCl_2$	1 M	400 μl	
1 mM	EDTA (pH8.0)	0.5 M	80 μl	
2 mM	DTT	1 M	80 μl	
	H_2O		37.14 ml	

1x Kinase Buffer

Final Conc.	Ingredient	Stock Conc.	For 60ml	
1x	2x Kinase Buffer	2x	30 ml	
	H_2O		30 ml	

1) Binding protein-A and anti-Cds1

For 1 sample:

- 10 μl [bed volume] of protein-A, pre-washed with Lysis Buffer
- 10 μl of Lysis Buffer
- 1 μl of anti-Cds1

Rotate for 1~2 hours at 4˚C

2) Protein extraction

- Add 200 μl of Lysis Buffer and glass beads to cell pellet.
- FastPrep; out put 6.0, 20 sec x 2 times with 2 min in between.
- Pierce a hole at the bottom of the tube.
- Place it onto a new 1.5 ml tube, and spin down to collect supernatant from beads.
- Add 400 μl of Lysis Buffer, and mix well.
- Centrifuge at 14 krpm for 5 min, 4˚C.
- Transfer 600 μl of supernatant to a new 1.5 ml tube.
- Centrifuge at 14 krpm for 10 min, 4˚C.
- Transfer 552 μl of supernatant to a new 1.5 ml tube.
    - Use 2µl of supernatant for protein assay:
        - Dilute BioRad Protein Assay Dye Reagent Concentrate (at 4°C) 1:5 with water. (4 ml + 16 ml water for 18 sample and one control)
        - Put 1ml of diluted dye into each tube - one tube per sample.
        - Add 2µl of sample to each; use 2µl of Lysis Buffer 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 OD:
        - y=(x/lowest OD595)x550-550
          x=sample OD595

- Add yμl of Lysis Buffer to the sample, mix well by inverting, then remove yμl from sample and discard. You now have the adjusted sample.

3) IP

- Add 20 μl of Cds1-antibody-conjugated-protein-A to each crude extract sample.
- Rotate for 1~2 hours at 4˚C.
- Wash 3 times with Lysis Buffer.
- Wash 3 times with 1x Kinase Buffer. (Keep buffer in the tube after the last centrifuge.)

4) Kinase reaction

- Make γ-32P cocktail for 20 samples. (Total 20 μl/sample)
  - 200 μl of 2x Kinase Buffer
  - 4 μl of [γ-32P] ATP (20 - 40 μCi)
  - 4 μl of 10 mM ATP
  - 10 μl of 10 mg/ml MBP (Myelin Basic Protein; substrate of Cds1)
  - 182 μl of H_2O (Total; 400 μl)
- Centrifuge samples of the last Kinase Buffer wash, and aspirate supernatant.
- Add 20 μl of 32P cocktail to each beads sample.
  - Take 20 μl of 32P cocktail as a blank control.
- Incubate for 15 min at 30˚C. Tap every 1~3 min.
- Add 25 μl of 2x SDS Sample Buffer to stop the reaction. (20 μl for control)
- Boil for 5 min. Store samples at -20˚C.

5) Running gel

- Apply 10 μl of sample on 15% SDS-PAGE.
- Apply samples in order of: #1 ~18 and control in one big gel.

6) Staining

- Soak gel in Coomassie Stain to fix for 30 min.
- Destain in Destain Buffer for 30 min.
- Dry in Cellophane for overnight.
- Wrap with Saran Wrap.
- Expose in Imaging Cassette for 30 ~ 60 min, and read.
- Cut the bands out, and measure 32P with Scintilation Counter.