In vitro Protein Kinase Assay Using Yeast Sch9

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[Abstract] This protocol will describe experimental procedures for an *in vitro* kinase assay of the yeast protein kinase Sch9. This protocol can be tailored to detect kinase activity of other yeast protein kinase.

Materials and Reagents

1. W303a wild type yeast cells
2. Tris base ($C_4H_{11}NO_3$) (Thermo Fisher Scientific, catalog number: 77-86-1)
3. NaCl (Thermo Fisher Scientific, catalog number: 7647-14-5)
4. $Na_2EDTA{\cdot}2H_2O$ (EDTA) (Sigma-Aldrich, catalog number: ED2SS**)**
5. Triton X-100 (Thermo Fisher Scientific, catalog number: 9002-93-1)
6. Phenylmethanesulfonyl fluoride (PMSF) ($C_7H_7FO_2S$) (Sigma-Aldrich, catalog number: P7626)
7. Complete protease inhibitor cocktail (F. Hoffmann-La Roche, catalog number: 04693159001)
8. PhosSTOP tablet (F. Hoffmann-La Roche, catalog number: 04906837001)
9. Glycerol (Thermo Fisher Scientific, catalog number: 56-81-5)
10. $MgCl_2$ (USB, catalog number: 18641 500 GM)
11. Dithiothreitol (DTT) (Thermo Fisher Scientific, Pierce Antibodies, catalog number: 20290)
12. Rapamycin (Santa Cruz Biotechnology, catalog number: sc-3504)
13. Glass beads (Sigma-Aldrich, catalog number: G-8772)
14. HA antibody (12CA5) (Abcam, catalog number: ab16918)
15. ATP (Sigma-Aldrich, catalog number: A2383-1G)
16. [γ-^{32}P]-ATP (PerkinElmer, catalog number: BLU002250UC)
17. Coomassie Blue R250 (National Diagnostics, catalog number: HS-605)
18. HCl
19. 2.5x SDS loading dye
20. IP buffer (see Recipes)
21. Kinase buffer (see Recipes)

22. PBS buffer (see Recipes)

Equipment

1. Standard bench-top centrifuge
2. Shaker
3. 1.5 ml Eppendorf tubes
4. Authoradiograph

Procedure

1. Inoculate W303a wild type or W303a cells containing vector, *pRS315-SCH9-HA3* and its kinase dead form *(K441A)* and hyperactive form *(2D3E)* overnight in 10 ml SC-His- medium. Shaking vigorously (300 rpm) at 30 °C.
2. Subculture yeast cells in 2 flasks containing 100 ml YPD each with starting OD_{600}=0.2.
3. Shake vigorously at 30 °C to OD_{600}=0.5 (important to use YPD rather than SD medium).
4. Add to one flask 200 nM of rapamycin and another vehicle control. Shake vigorously at 30 °C for 30 min.
5. Spin down at room temperature (RT) at 3,000 *x g* to collect cells (it is important to avoid freezing).
6. Discard most of the supernatant, then suspend yeast cells in the remaining medium and split into 5x 1.5 ml Eppendorf tubes (20 ml cell culture/tube, more cells in one tube do not breakdown sufficiently).
7. Collect cell pellets, immediately add 200 µl ice-cold IP buffer and the same amount of glass beads.
8. Immediately breakdown cells by beads beater at 4 °C for 1 min.
   Note: Do not exceed 3 min, otherwise kinase activity will begin to decrease due to overheating. 15 sec x 5 beating with 45 sec in between also decreases Sch9 kinase activity.
9. Collect cell lysate by spinning down at 20,000 *x g*, 10 min at 4 °C. Combine lysate.
10. Immediately add 1 µg HA antibody (12CA5) to 1 mg/500 µl cell lysate, gently rotate at 4 °C for 1 h.
11. Wash protein A/G beads 3x with IP buffer. Add 50 µl (100 µl 50% slurry) to cell lysate and further rotate for another 1 h.
12. Spin down and collect beads. Wash 3x with 0.5 ml ice-cold IP buffer.
13. Add 0.5 ml 1x with ice-cold kinase buffer. Save 50 µl in another 1.5 ml Eppendorf tube for western blot with HA antibody.

14. Spin down and add to the immunocomplex 100 µM ATP, 0.5 µg bacterially-expressed GST-Maf1 in 50 µl kinase.
15. Add to the reaction system 50 µCi [γ-^{32}P]-ATP. Vortex and incubate at 30 °C for 15-30 min.
16. Kinase reaction was stopped by heating at 100 °C for 5 min in 2.5x SDS loading dye.
17. Half of the sample should be subjected to SDS-PAGE. Substrate will be detected by Coomassie blue staining.
18. Dry the gel using gel dryer at 80 °C for 2 h. Phosphorylation of substrate is revealed by authoradiograph.

Recipes

1. IP buffer
   50 mM Tris-HCl (pH 7.5)
   150 mM NaCl
   0.5 mM EDTA
   0.5% Triton X-100
   Add 2 mM PMSF, Roche Complete protease inhibitor cocktail and phosSTOP tablet before use. Vary NaCl concentration or/and Triton X-100 level to obtain optimum condition for different kinase and different antibodies.
2. 1x PBS (pH 7.4)
3. Kinase buffer
   1x PBS (pH 7.4)
   20% glycerol
   4 mM $MgCl_2$
   10 mM DTT
   Protease inhibitor

Acknowledgments

This protocol was adapted from and used in Wei and Zheng (2009) and Wei *et al.* (2009).

References

1. Wei, Y. and Zheng, X. F. (2009). Sch9 partially mediates TORC1 signaling to control ribosomal RNA synthesis. *Cell Cycle* 8(24): 4085-4090.

2. Wei, Y., Tsang, C. K. and Zheng, X. F. (2009). Mechanisms of regulation of RNA polymerase III-dependent transcription by *TORC1*. *EMBO J* 28(15): 2220-2230.

