Alkaline phosphatase (ALP) ASSAY

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METHOD

Construction of a yeast strain expressing Pho8D60

1. Order the plasmid set (A versatile toolbox for PCR-based tagging of yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes) (Janke et al. (2004)) at EUROSCARF (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/data/Knop.html).
2. Run PCR Using pYM-N14 or pYM-N15 plasmid as a template, and PCR primer set, pho8S1 (TATCAGCATACGGGACATTATTTGAACGCGCATTAGCAGCcgtacgctgcaggtcgac) and pho8S4 (TCACGAAGAATATGACATTCTTCTTCTTGTGTGATGCAGAcatcgatgaattctctgtcg). The resulting PCR fragment will contain the constitutive GPD1 promoter and selection markers kanMX4 (pYM-N14; G418 resistance) or clonNAT (pYM-N15; nourseothricin resistance) flanked by 40 nucleotides just upstream of the initiation codon of the PHO8 gene and 40 nucleotides downstream of amino acid 60.
3. Transform your favorite yeast strains which contain wild-type PHO8 gene with this PCR fragment, and select the colonies resistant to each antibiotics (Gietz and Woods, 2002).
4. Confirm if the PHO8 gene was correctly replaced with antibiotic marker, GPD1 promoter and pho8D60, which lacks first 60 amino acid of PHO8. Extract the genome of each clone and run PCR using the pho8S4 primer and the pho8 check primer (TTGCCAGCAAGTGGCTACATAAACATTTAC). If the correct replacement occurred, you will detect a band at about 0.6-kb.

Activity measurement

1. Your strains harboring Pho8D60 gene and appropriate positive and negative control strains are inoculated in liquid growth medium such as YPD or Yeast nitrogen Base with Glucose and grown to mid-log phase (O.D.600 = 0.5 to 1) for

at least over 3 doubling time. The culture size needs at least 10 ml for each assay point. For induction of autophagy, change the corresponding medium with appropriate wash step such as centrifugation at 1500 x g or 3 min with the same medium. The cells are incubated in each inducing conditions at the concentration for the desired time periods. At each point, collect 2 to 4 O.D.600 units of cells by centrifugation at 1500 g for 3 min and discard the medium completely.

2. Suspend the cells pellet in 0.2 ml of ice-cold assay buffer (100 mM of Tris-HCl, pH 9.0; 10 mM of $MgSO_4$, and 10 μM of $ZnSO_4$) and transfer the suspension to a 1.5-ml microcentrifuge tube. Add acid- washed glass beads (425–600 microns), to the level of the interface. Place the tube on ice for at least 5 min, then mix the tubes vigorously with a vortex mixer for 6 x 30 seconds, placing for at least 30-s intervals on ice in between the mixing. An automatic vortex mixer in refregirator chamber may be employed in this step.

3. Add an additional 0.2 ml of assay buffer, mix well, and Centrifuge at 14,000 g for 1 min. Transfer 0.3 ml of the supernatant fraction to new 1.5-ml microcentrifuge tube.

4. Add 0.05 ml of the cell lysate solution to the volume to a 0.45 ml with assay buffer in new tube. Triplicate would be desirable. Place each tubes into a 30 ^{o}C water bath and pre-warm at least 1 min.

5. Initiate the assay reaction by addition of 0.05 ml of 55 mM α-naphthyl phosphate disodium salt dissolved in assay buffer and mixing well. If you have multiple tube for the assay, stagger the initiation so as to allow totally same performance. Incubate the tubes at 30 ^{o}C for 20 min or appropriate time.

6. Stop the reaction by addition of 0.5 ml of stop buffer (2 M glycine-NaOH (pH 11.0)) and mixing well. Place the tubes on ice until all of the tubes are finished.

7. Measure the fluorescence in the reaction tube using a wavelength of 345 nm for excitation and 472 nm for emission. It is important to check if all the measurement is in the range that is not saturated.

8. Measure the protein concentration of the cell lysate using either the BCA or Bradford method.

9. The ALP activity is presented as emission per the amount of protein in the reaction (mg) and the reaction time (min).

MATERIALS

REAGENTS

• Add acid- washed glass beads (425–600 microns)(SIGMA G8772)
• α-naphthyl phosphate disodium salt (Sigma N7255)

EQUIPMENT

• Fluorescence spectrometer Twinkle LB970 (Belthold technologies)

REAGENT PREPARATION

Assay buffer (100 mM of Tris-HCl, pH 9.0; 10 mM of $MgSO_4$, and 10 μM of $ZnSO_4$)

Dilute 10× stock of each constitutents and mess up. This reagent can be stored at room temperature.

Stop buffer (2 M glycine-NaOH (pH 11.0))

pH of Conc Glycine solution is adjusted with 10 N or 1 N NaOH and mess up. This reagent can be stored at room temperature.

REFERENCES

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