Western blot analysis of proteins expressed in yeast

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Method Article

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Abstract

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Reagents

- 0.2M NaOH - 2x SDS-PAGE sample solvent: 4% SDS, 20% glycerol, 0.02% bromophenol blue, 0.1 M DTT, 0.125 M Tris-HCl pH 7.5 - Protein Assay Dye Reagent Concentrate \(500-0006 BIO-RAD) for Bradford Assay - Acrylamide/Bis-acrylamide 30% solution \(A3699-100ML, SIGMA-ALDRICH) - Resolving gel buffer: Tris-HCl 0.75 M pH 8.9, 0.2% SDS, 4 µM EDTA - Stacking gel buffer: 0.1 M Tris-phosphate pH 6.7, 0.2% SDS, 4 µM EDTA - Temed \(K37762132 741 MERCK) - PSA: ammonium persulfate 10% - Electrophoresis buffer: 0.025 M Tris, 0.2 M glycine, 0.1% SDS - Transfer buffer: 0.025 M Tris, 0.2 M glycine, 0.1% SDS, 20% methanol - Blocking solution: Skim milk powder \(LP0031 OXOID) 2% in TBS-T - TBS-T solution: 20 mM Tris-HCl pH 7.5, 0.137 M NaCl, 0.01% Tween-20 - Supersignal West Femto Maximum Sensitivity Substrate \(34096 Thermo Scientific)

Equipment

- Incubator - Corning Tubes Centrifuge - Eppendorf Tubes Centrifuge - Thermoblock - Colorimeter - Western blot equipment: BIORAD electrophoresis and membrane transfer system - Stirrer - Refrigerator and freezer - Chemiluminescence reader ImageQuant™ LAS 4000mini biomolecular imager \(GE Healthcare).

Procedure

Sample collection 1. Collect 5x107 cells from the exponentially growing yeast culture 2. Centrifuge the cells at 2300xg for 2 min. Discard the supernatant 3. Wash in 1ml of distilled H2O and pass into an eppendorf 4. Centrifuge at 2300xg for 2 min. Remove the supernatant with the vacuum. *Freeze the cells at -20°C if the protocol will be continued the next day. Protein extraction with NaOH 5. Add 100µL of distilled H2O to resuspend the cell pellet. Then add 100µL of 0.2M NaOH \(this treatment disrupts the cell wall) and vortex 6. Incubate the sample at room temperature for 5-10 min 7. Centrifuge at 13400xg for 1 min. Remove the supernatant with a vacuum pump 8. Add 50µL of the 2X SDS-PAGE sample solvent and vortex until to resuspend 9. Place the samples on the thermoblock at 95°C for 5 min 10. Centrifuge at 800xg for 10 min at 4°C 11. Carefully collect the supernatant \(protein extract) – Do not touch the pellet- and pass it into a new eppendorf. Keep these eppendorfs on ice or at -20°C if the protocol will be continued the next day Sample normalization 12. Normalization of the samples is performed based on a Bradford assay Each eppendorf should contain: 0.5µL of the protein sample to be quantified 800µL distilled H2O 200µL Protein Assay Dye Reagent 13. Leave the samples for 5 min at room temperature 14. Measure absorbance at A595. 15. Equivalent amount of each protein sample is loaded into PAGE- gel based on this measurement Electrophoresis 16. Prepare the SDS-PAGE polyacrylamide gels and place them in the electrophoresis system 17. Run the electrophoresis at 100V for 2 h approximately depending on the migration you need Membrane Transfer 18. Take a tray and pour Transfer Buffer 19. Place the

plastic holder required to make the membrane sandwich 20. For a wet-BioRad transfer system, place a nitrocellulose membrane in contact with the acrylamide gel between 2 filter papers on top and bottom making sure all have been bathed in transfer buffer. To avoid bubbles being trapped, you may press down with a glass rod. Close the sandwich 21. Place the sandwich on the correct direction in the sandwich support. Place the whole apparatus into the transfer system. Ensure to place some ice blocks to avoid over-heating. Fill the container with transfer buffer 22. Set the voltage at 100V and run for 45 min Protein Detection 23. Wash the membrane with TBS-T buffer \(and place it with agitation) 24. Incubate with the blocking agent for 1h 25. Discard the blocking agent and pour the primary antibody mixture onto the membrane. Incubate the membrane with the primary antibody overnight 26. Prepare the secondary antibody mixture with the corresponding dilution required 27. Discard the primary antibody mixture. Wash the membranes 3 x 15 min with TBS-T 28. Discard TBS-T and add the secondary antibody. Incubate the membranes with the secondary antibody for 1h 29. Discard the secondary antibody and again, wash the membranes 3 x 15 min with TBS-T 30. Blots are developed using the Supersignal West Femto Maximum Sensitivity Substrate \(Thermo Scientific). Bands are observed in a ImageQuant™ LAS 4000mini biomolecular imager \(GE Healthcare). PRIMARY ANTIBODY SECONDARY ANTIBODY Monoclonal anti-HA peroxidase 3F10 antibody \(Roche Diagnostics, 12013819001) Diluted 1:5000 Not required Monoclonal anti-c-myc 9E10 antibody \(Roche Diagnostics, 1667149) Diluted 1:5000 Goat Anti-mouse IgG \(H+L) Horseradish Peroxidase conjugate \(170-6516, Pierce Antibody, Thermo Scientific) Diluted 1:20000 Monoclonal anti-GFP \(Roche Diagnostics, 11814460001) Diluted 1:5000 Goat Anti-mouse IgG \(H+L) Horseradish Peroxidase conjugate \(170-6516, Pierce Antibody, Thermo Scientific) Diluted 1:20000 Monoclonal anti-FLAG M2 \(Sigma-Aldrich, F3165) Diluted 1:10000 Goat Anti-mouse IgG \(H+L) Horseradish Peroxidase conjugate \(170-6516, Pierce Antibody, Thermo Scientific) Diluted 1:20000 Monoclonal anti Cdc2 p34 \(PSTAIRE) \(Santa Cruz Biotechnology Inc. SC-53) Diluted 1:2000 Goat Anti-rabbit IgG \(H+L) Horseradish Peroxidase conjugate \(31460, Pierce Antibody, Thermo Scientific) Diluted 1:20000