Published in final edited form as:

Cold Spring Harb Protoc. ; 2015(3): 276–283. doi:10.1101/pdb.prot085092.

Evaluating the Activity of the Filamentous Growth MAPK Pathway in Yeast

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Abstract

Mitogen-activated protein kinase (MAPK) pathways are evolutionarily conserved signaling pathways that regulate diverse processes in eukaryotes. One such pathway regulates filamentous growth, a nutrient limitation response in budding yeast and other fungal species. This protocol describes three assays used to measure the activity of the filamentous growth pathway. First, western blotting for phosphorylated (activated) MAPKs (P~MAPKs; Slt2p, Kss1p, Fus3p, and Hog1p) provides a measure of MAPK activity in yeast and other fungal species. Second, the *PGU1* gene is a transcriptional target of the filamentous growth pathway. Cells that undergo filamentous growth secrete Pgu1, an endopolygalacturonase that degrades the plant-specific polysaccharide pectin. We describe an assay that measures secreted pectinase activity, which reflects an active filamentous growth pathway. Finally, in yeast, two mucin-like glycoproteins, Msb2 and Flo11, regulate filamentous growth. Secretion of the processed and shed glycodomain of Msb2 is an indicator of MAPK activity. Flo11, the major adhesion molecule that controls filamentous growth and biofilm/mat formation, is also shed from cells. Detecting shed mucins with epitope-tagged versions of the proteins (secretion profiling) provides information about the regulation of filamentous growth across fungal species.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at <http://cshprotocols.cshlp.org/site/recipes>.

Reagents

α -Factor mating pheromone

Anti-HA antibody (Roche 12CA5)

β -Mercaptoethanol

Bovine serum albumin (BSA)

Distilled H₂O, sterile

Fus3p antibody (Santa Cruz Sc-6773)

Goat α -mouse IgG-HRP (Bio-Rad 170-6516)

Goat α -rabbit IgG-HRP (Jackson ImmunoResearch Laboratories 111-035-144)

HCl (1 N)

P.J. Cullen

Immunoblot detection kit

KCl (5 M)

Kss1p antibody (Santa Cruz Sc-6775-R)

Liquid nitrogen (optional; see Step 3)

Nonfat dry milk

Pectinase agar plates <R>

Pgk1p antibody (Life Technologies 459250)

Phospho-p38 MAPK antibody (Cell Signaling Technology 9211)

This antibody recognizes Hog1, the high osmolarity glycerol response (HOG) MAPK.

Phospho-p44/42 MAPK antibody (Erk1/2) (D13.14.4E) (Cell Signaling Technology 4370)

This antibody recognizes the phosphorylated forms of the MAPKs Kss1 (filamentous growth), Fus3 (mating or pheromone response), and Slt2 (protein kinase C [PKC]).

Resuspension buffer <R>

Ruthenium red (1 mg/mL; Sigma-Aldrich)

SDS-PAGE gel (12%)

SDS gel-loading buffer (2 $\times$), prepared with freshly added 200 mM β -mercaptoethanol <R>

TBST <R>

TCA buffer <R>

Yeast extract-peptone-dextrose growth medium (YEPD) <R>

Yeast strains of interest

The Σ 1278b background undergoes filamentous growth (Gimeno et al. 1992). Commonly used laboratory strains have lost the ability to undergo filamentous growth (Liu et al. 1996). Use appropriate negative controls (for western blotting, see

Step 2; for the pectinase assay, see Step 13). Strains with epitope-tagged versions of mucins can be used for secretion profiling (see Step 18).

YEPD agar plates <R>

YEP-GAL medium <R>

Equipment

Digital camera

Forceps, sterile

Glass beads (Sigma-Aldrich G9143)

Wash beads in 1 N HCl for 5 min, rinse in saturating H₂O to pH 5, and dry.

Glass tubes with metal tops, sterile

Glassware, sterile

Heat block set to 100°C

ImageJ software (<http://imagej.nih.gov>; Schneider et al. 2012)

Incubator set at 30°C

Innoculation loop or long wooden toothpick, sterile

Microcentrifuge

Microcentrifuge tubes, sterile

Microscope

Multi-tube vortexer

Nitrocellulose filters, round

Nitrocellulose membrane (Protran BA85)

Protein transfer blot apparatus

SDS-PAGE apparatus

Shaking incubator or a shaker in a 30°C room and in a 37°C room (see Step 2)

Spectrophotometer

Toothpicks, sterile

METHOD

Western Blotting to Detect P-MAPKs

- 1 Using a long wooden toothpick or inoculation loop, inoculate yeast (from a fresh plate) into 5 mL of YEPD medium in a glass tube with a metal top and grow until saturation is reached.

We typically grow cultures for 16 h (overnight) at 30°C with shaking at 225 rpm.

- 2 Dilute aliquots of the saturated culture in the control and inducing conditions listed below.
 - Add 400 μ L of saturated cell culture to 10 mL of YEPD. Incubate cells at 30°C with shaking for 6 h or until they reach mid-log phase (1×10^8 cells/mL; determined by spectrophotometry, $A_{600} = 1.0$).
 This is the control condition. Grow control cells and induced cells to the same cell number, not necessarily for the same amount of time.
 - Add 750 μ L of saturated cell culture to 10 mL of YEP-GAL. Incubate cells at 30°C with shaking for 6 h or until they reach mid-log phase (1×10^8 cells/mL; determined by spectrophotometry, $A_{600} = 1.0$).
 $\Sigma 1278b$ cells undergo filamentous growth in YEP-GAL liquid medium, which can be confirmed by morphological examination (remove an aliquot and examine by microscopy at 100 $\times$). This condition can be used to assess P~Kss1.
 - Add 400 μ L of saturated cell culture to 10 mL of YEPD. Grow cells to mid-log phase as described above. Wash cells twice in sterile distilled H₂O and resuspend in YEPD ± 100 nM α -factor mating pheromone. Incubate at 30°C with shaking for 30 min.
 Shmoo response can be confirmed by microscopy (at 100 $\times$). This condition can be used to assess P~Fus3.
 - Add 400 μ L of saturated cell culture to 10 mL of YEPD. Grow cells to mid-log phase as described above. Transfer cells to 37°C with shaking for 30 min.
 This condition can be used to assess P~Slt2.
 - Add 400 μ L of saturated cell culture to 10 mL of YEPD. Grow cells to mid-log phase as described above. Add KCl to the medium to a final concentration of 0.4 M. Incubate at 30°C with shaking for 5 min.
 This condition can be used to assess P~Hog1.
 MAPK mutant yeast strains can be used as negative controls. Basal levels of P~Fus3, P~Kss1, and P~Slt2 are visible under noninducing conditions (Fig. 1). Basal P~Hog1 is not detectable under non-inducing conditions.
- 3 Centrifuge the cells at 16,000g for 2 min at 20°C. Discard the supernatant. Snap-freeze the cell pellets in liquid nitrogen or in an ultracold freezer (at -80°C).
- 4 Thaw the cell pellets by adding 300 μ L of TCA buffer.

- 5 Add ~0.2 mL of acid-washed glass beads to each sample. Apply five 1-min pulses at full speed in a multitube vortexer at 25°C. Place samples on ice for 3 min between each cycle.
- 6 Transfer cell lysates to sterile 1.5-mL tubes and centrifuge at 16,000g for 10 min at 4°C.
- 7 Discard the supernatant. Resuspend each pellet in 150 µL of resuspension buffer.
- 8 Boil the samples for 5 min at 100°C and centrifuge 16,000g for 30 sec at 25°C. Collect the supernatant. Add an equal volume of 2X SDS-PAGE loading dye.
- 9 Load the samples onto a 12% SDS-PAGE gel and run the gel using standard SDS gel techniques.

We generally load 40 µL (10 µg of total protein) per lane.

Load two SDS-PAGE gels, one to assess phosphorylation and one as a control for overall protein levels (using antibodies to a control protein, such as Pgk1).

- 10 Transfer the proteins to nitrocellulose membranes using standard techniques.
- 11 Perform standard immunoblot analysis with the antibodies of interest.

We use the following antibodies at the concentrations indicated.

- Fus3p antibody: 1:5000 dilution in 5% nonfat dried milk in TBST
- Kss1p antibody: 1:5000 dilution in 5% nonfat dried milk in TBST
- Pgk1p antibody: 1:20,000 dilution in 5% nonfat dried milk in TBST
- Phospho-p38 MAPK antibody: 1:5000 dilution in 5% BSA in TBST
- Phospho-p44/42 MAPK antibody: 1:1000 dilution in 5% BSA in TBST
- Goat α-mouse IgG-HRP: 1:3000 dilution in 5% nonfat dried milk in TBST

We typically perform primary antibody incubations for 16 h at 4°C and secondary antibody incubations for 1 h at 25°C with rocking.

- 12 Detect antibodies using an immunoblot detection kit.

See Figure 1 for a representative analysis and Lee and Dohlman (2008) and Roman et al. (2005) for sample experiments.

Pectinase Assay

- 13 Using a sterile toothpick, take cells of the yeast strain of interest from a fresh stock plate (1–2 d old) and make a circular patch on a pectinase plate.

The *pgu1* mutant strain can be used as a negative control.

- 14 Incubate the plates at 30°C for 2 d.

- 15 Pour freshly prepared ruthenium red over the plate containing the patched cells. Incubate at 25°C for 8 h.
- 16 Remove excess ruthenium red with a paper towel and rinse the plate under a stream of water.
- 17 Examine the stained pectin haloes by photography. Quantitate haloes with ImageJ software.

Haloes are indicators of secreted pectinase (see Fig. 2). For more information, see Gainvors et al. (1994), Blanco et al. (1998), Gognies et al. (1999), and Madhani et al. (1999).

Secretion Profiling of Yeast Mucins

- 18 Using a long wooden toothpick or inoculation loop, inoculate yeast (from a fresh plate) into 5 mL of YEPD medium and grow until saturation is reached.

We typically grow our cultures for 16 h (overnight) at 30°C with shaking at 225 rpm. Strains carrying functional epitope-tagged versions of mucins (e.g., Msb2 [Cullen et al. 2004; Vadaie et al. 2008; Chavel et al. 2010] and Flo11 [Karunanithi et al. 2010]) are required for this procedure. Alternatively, untagged strains can be used if there is an antibody available to the protein of interest.

- 19 Remove 0.5 mL of cells from the overnight culture. Pellet the cells by centrifugation at 16,000g for 1 min at 20°C. Wash the cells twice by adding 0.5 mL of sterile distilled H₂O and centrifuging as above. Resuspend the cells in 0.5 mL of sterile distilled H₂O.
- 20 Using sterile forceps, place a round nitrocellulose filter on a YEPD plate.
- 21 Spot 10 µL of the resuspended cells onto the nitrocellulose filter.
- 22 Incubate the plate for 48 h at 30°C.
- 23 Photograph the colonies with a digital camera.
- 24 Rub the cells off of the filter in a stream of water.
- 25 Remove the filter from the YEPD plate.
- 26 Allow the filter to air dry for several minutes.
- 27 Perform standard immunoblot analysis with the nitrocellulose membrane containing shed epitope-tagged proteins.

We use the following antibodies at the concentrations indicated.

- Anti-HA antibody: 1:2000 dilution in 5% nonfat dry milk in TBST
- Goat α-rabbit IgG-HRP: 1:5000 dilution in 5% nonfat dry milk in TBST

We typically perform primary antibody incubations for 16 h at 4°C and secondary antibody incubations for 1 h at 25°C with rocking.

28 Detect antibodies using an immunoblot detection kit.

See Figure 3 for a representative assay. This approach can be adapted for use in genomic collections (e.g., Fig. 3, right panel). Secretion profiling in various fungal species has been described by Perez-Nadales and Di Pietro (2011), Puri et al. (2012), and Szafranski-Schneider et al. (2012).

RECIPES**Pectinase Agar Plates**

- 1% polygalacturonic acid (Fluka)
- 6.7 g/L yeast nitrogen base without amino acids
- 2 g/L ammonium sulfate
- 2% glucose (or 2% galactose; see below)
- 2% agar
- Amino acids
 - 20 mg/L histidine
 - 120 mg/L leucine
 - 60 mg/L lysine
 - 20 mg/L arginine
 - 20 mg/L tryptophan
 - 20 mg/L tyrosine
 - 40 mg/L threonine
 - 20 mg/L methionine
 - 50 mg/L phenylalanine
- 20 mg/L uracil
- 20 mg/L adenine
- 50 mM potassium phosphate buffered solution (pH 8) <R>

Use 2% galactose instead of 2% glucose to measure the induction of the filamentous growth pathway in response to glucose limitation. Include any additional amino acids that may be required for the growth of an auxotrophic strain. Prepare in H₂O. Heat the solution to 70°C with stirring for 30 min before autoclaving (polygalacturonic acid has low solubility in H₂O). Fill sterile Petri dishes with ~25 mL of autoclaved medium.

Potassium Phosphate-buffered Solution (1 M, pH 8)

- 94 mL K₂HPO₄ (1 M, pH 8)
- 6 mL KH₂PO₄ (1 M, pH 8)

Resuspension Buffer

0.1 M Tris-HCl (pH 11.0)

3% SDS

SDS Gel-Loading Buffer (2×)

100 mM Tris-Cl (pH 6.8)

4% (W/V) SDS (sodium dodecyl sulfate; electrophoresis grade)

0.2% (W/V) bromophenol blue

20% (W/V) glycerol

200 mM DTT (dithiothreitol)

Store the SDS gel-loading buffer without DTT at room temperature. Add DTT from a 1 M stock just before the buffer is used.

200 mM β -mercaptoethanol can be used instead of DTT.

TBST

10 mM Tris-HCl (pH 8)

150 mM NaCl

0.05% Tween 20

TCA Buffer

10 mM Tris-HCl (pH 8.0)

10% trichloroacetic acid

25 mM NH_4OAC 1 mM Na_2EDTA **Yeast Extract-Peptone-Dextrose Growth Medium (YEPD)**

Reagent	Quantity (for 1 L)	Final concentration (W/V)
Bacto peptone	20 g	2%
Yeast extract	10 g	1%
Galactose	20 g	2%
H ₂ O	to 1 L	

Sterilize by autoclaving.

YEPD Agar Plates

Reagent	Quantity
Bacto-agar (2%)	20 g
YEPD liquid medium	1L

Add Bacto-agar to YEPD liquid medium in a 2-L flask and autoclave. Fill sterile Petri dishes with 30–40 mL of autoclaved medium.

YEP-GAL Medium

Reagent	Quantity (for 1 L)	Final concentration (W/V)
Bacto peptone	20 g	2%
Yeast extract	10 g	1%
Dextrose	20 g	2%
H ₂ O	to 1 L	

Sterilize by autoclaving.

Acknowledgments

Thanks to P. Pryciak and H. Dohlman for suggestions about the phosphoblot analysis. Thanks to G. Fink and T. Reynolds for discussions about mucin shedding. P.J.C. is supported from a U.S. Public Health Service grant (GM098629).

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FIGURE 1.

Detecting phosphorylated (active) MAP kinases in yeast. Immunoblot analysis of phosphorylated MAP kinases. P~Slt2p, P~Kss1p, and P~Fus3p are detected by p42/p44 (ERK-type MAP kinase) antibodies. P~Hog1p is detected by anti-phospho-p38 antibodies. Blots also show total MAPKs and Pgk1 as a control for protein levels.

FIGURE 2.

Detecting halos (an indicator of secreted pectinase) in the pectinase assay. The size of the halo is reduced in certain mutants (mutant 1 and mutant 2, *middle*) and absent in other mutants (e.g., *pgu1* mutant, *bottom*). Bar, 1 cm.

FIGURE 3.

Secretion profiling of yeast mucins. Colony immunoblot of hemagglutinin (HA)-tagged Flo11 shed from a biofilm in wild-type cells (*left*) compared to a MAPK pathway mutant (*middle*) defective for *FLO11* expression. (*Right*) Secretion profiling of yeast deletion mutant transformed with a plasmid carrying HA-Msb2 (*top panel*, colonies; *bottom panel*, anti-HA immunoblot). The mutant at position A2 is defective for Msb2 shedding. Bar, 1 cm.