Chapter 9

Screening for Chemical Inhibitors of Heterologous Proteins Expressed in Yeast Using a Simple Growth-Restoration Assay

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Summary

Overexpression of heterologous proteins in the yeast *Saccharomyces cerevisiae* often inhibits its growth, while inhibitors of the overexpressed proteins can restore growth. These simple observations form the basis of a technically easy, inexpensive, scalable, and widely applicable assay to identify inhibitors of such proteins. An expression plasmid for the inducible expression of a gene of interest is introduced into a yeast strain rendered more sensitive to chemicals by deletion of efflux pumps. Protein expression is induced, cells are exposed to test chemicals, and growth is measured by A_{600} reading. The chemicals that relieve growth inhibition are subjected to secondary assays to establish their selectivity toward the protein of interest. This assay has been used successfully to identify inhibitors of proteins of viral, microbial, and mammalian origin.

Key words: Chemical inhibitor, Drug screening, Growth restoration, *Saccharomyces cerevisiae*.

1. Introduction

Identifying chemical inhibitors of proteins can be a difficult task, requiring the development of in vitro or cell-based assays tailored to the specific activity of a protein target. The assays may be complex or necessitate expensive reagents, making it difficult to scale them up to the level of throughput needed to screen large chemical libraries. Traditional drug-discovery efforts have therefore tended to focus on enzymes and small-ligand receptors, which are amenable to in vitro high-throughput activity or ligand-binding assays. Many other protein classes playing major roles in disease have been overlooked in drug discovery because

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it is not obvious how to assay them in high-throughput screens. These include structural proteins, regulatory proteins, effector proteins, proteins with ill-defined functions, or proteins that are difficult to express in vitro.

It has been observed that overexpression of heterologous proteins in the yeast *Saccharomyces cerevisiae* often inhibits growth *(1)*. Inhibitors of these proteins can restore yeast growth, providing a simple generic drug-screening assay. This type of assay has been used to identify an inhibitor of the influenza virus M2 envelope protein, which is an ion channel *(2)*, inhibitors of human poly(ADP)ribose polymerase *(3)*, and inhibitors of human indoleamine dioxygenase *(4)*. Tugendreich et al. have observed growth inhibition for 30% of human proteins overexpressed in yeast *(1)*, an observation corroborated by our own studies. For proteins that do not inhibit growth in standard culture media, it may be possible to alter culture conditions to reveal a growth-inhibitory phenotype *(4)*.

The basic assay, represented diagrammatically in **Fig. 1**, consists of inserting the coding sequence of a gene of interest into a

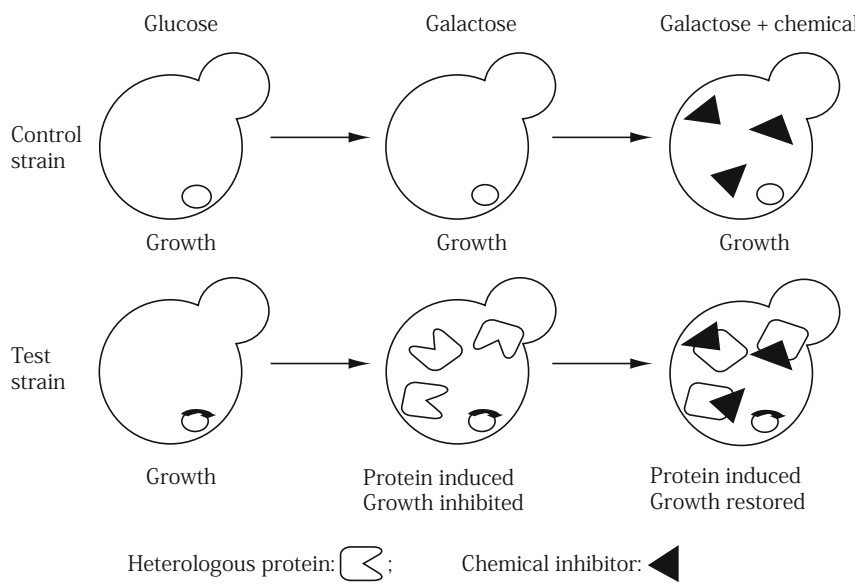

Fig. 1. Outline of the growth-restoration principle. Left column: a control strain containing an empty expression plasmid (*circle*) and a test strain containing an expression plasmid with the coding sequence of the gene of interest (*circle with curved arrow*) are grown in a medium containing glucose to repress expression. Middle column: the strains are transferred to a medium containing galactose to induce expression of the heterologous protein, resulting in inhibition of the growth of the test strain, but not of the control strain. Right column: the chemicals to be screened are added. Chemicals that inhibit the heterologous protein restore growth of the test strain and are scored as active.

plasmid containing the galactose-inducible *GAL1* promoter and introducing this construct into a yeast strain deleted of major drug-efflux pumps. The yeast strain bearing the plasmid is seeded at low density into multiwell plates in a medium containing galactose to induce protein expression; the chemicals to be screened are added, and yeast growth is measured by absorbance reading in a microplate reader. Active chemicals restore yeast growth. Simple controls can eliminate false-positive readings. Specialized secondary assays can then be used to verify the inhibitory activity of the active chemicals in the biological systems of interest. The screening assay is the same for different proteins. It is inexpensive, consists of very few steps, and its positive readout tends to exclude toxic compounds.

2. Materials

2.1. Yeast Strains and Expression Plasmids

1. Yeast strain EIS20-2B (*MAT**a**, ade2-1, his3-11, 15 leu2-3, 112 trp1-1, ura3-1, can1-100, pdr5*Δ*, snq2*Δ*)*, lacking the major efflux pumps Pdr5p and Snq2p *(1)* or strain FY1679-28C/TDEC (MAT***a***, *pdr1*Δ*2::TRP1, pdr3*Δ*::HIS3, ura3-52, leu2*Δ*, trp1-*Δ*63, his3*Δ*200, GAL2+*), lacking the drug efflux pump transcription factors Pdr1p and Pdr3p *(5)* (*see* **Note 1**).
2. Yeast expression plasmid: A multicopy plasmid for expression of proteins in yeast under the control of an inducible GAL1 promoter, such as pARC25B (LEU selection) *(1)*, or Gateway-compatible plasmids pJG482 (URA selection), pJG483 (LEU selection), or pJG514 (TRP selection) *(6)*. It is also possible to use plasmids for the expression of proteins fused to a V5-6xHis tag, such as pJG484 (LEU selection), pJG485 (URA selection), or pJG516 (TRP selection) *(6)* (*see* **Note 2**).

2.2. Yeast Transformation and Growth

1. Yeast extract peptone dextrose (YPD or YEPD) liquid medium: In a 200-mL glass bottle, weigh 1-g Bacto yeast extract (BioShop Canada, Inc., Burlington, ON) and 2-g Bactopeptone (BioShop), add 90-mL water, and autoclave the solution. When the autoclaved solution is cool, add 10 mL of 20% sterile glucose (*see* **step 10**). Store at room temperature.
2. 50% (w/v) polyethylene glycol 4000 (PEG): To a 150-mL beaker containing 30-mL water, add 50-g PEG (BDH, Inc., Toronto, ON). Stir with gentle heat to speed up dissolution of PEG, cool to room temperature, and adjust the volume to

100 mL with water. Transfer to a glass bottle. Autoclave and store at room temperature.

3. 1.0 M lithium acetate: Dissolve 10.2 g of lithium acetate (BDH) in 100-mL water. Autoclave the solution and store at room temperature.
4. 10 mg/mL sheared salmon sperm DNA (SSS DNA): Dissolve 10 mg of salmon sperm DNA (Sigma, St. Louis, MO; single-stranded carrier DNA) in 100 mL of TE (10 mM Tris–HCl, 1 mM disodium EDTA, pH 8.0) on a stir plate overnight at 4°C. Dispense 1.0 mL into 1.5-mL microcentrifuge screw cap tubes and store at –20°C. To denature the carrier DNA, place a tube in a boiling water bath for 5 min and chill immediately in an ice/water bath before use *(7)* (*see* **Note 3**).
5. Plasmid DNA or linear DNA, 1–2 μg: The DNA preparation should be quite pure.
6. Synthetic Complete (SC) mix: 0.6-g adenine, 0.6-g uracil, 0.6-g tryptophan, 0.6-g histidine, 0.6-g arginine, 0.6-g methionine, 0.9-g tyrosine, 0.9-g lysine, 1.5-g phenylalanine, 6.0-g threonine, 3.0-g aspartic acid, 1.8-g isoleucine, 4.5-g valine, 1.8-g leucine (Sigma). Weigh all ingredients, mix together, and use a mortar and pestle to grind into a homogeneous powder. Store in 50-mL Falcon tubes at room temperature.
7. SC dropout mix: Weigh all the ingredients except for leucine, tryptophan, or uracil, depending on the plasmid-selection marker used, and mix, grind, and store as in **step 6**.
8. SC selection medium: Yeast nitrogen base without amino acid (BD/Difco, Sparks, MD) 0.67%, SC dropout mix 0.067%, dissolve in water. Add 0.25 mL of 1N NaOH to every 100-mL medium to raise the pH of the solution to 6.5. Autoclave the solution and store at room temperature.
9. SC selection medium containing 2% agar and 2% glucose: Yeast nitrogen base without amino acid 0.67%, SC dropout mix 0.067%, dissolve in water. Add 0.25 mL of 1N NaOH and 2-g Bacto Agar (BioShop) to every 100 mL of medium. Autoclave the solution. When the autoclaved solution has cooled down to about 45°C, add 10 mL of 20% glucose (*see* **step 10**) to every 100 mL and pour 20 mL into sterile Petri dishes. Store the agar plates in a plastic bag at room temperature.
10. Glucose (BioShop): Dissolve 20 g in 100-mL warm water (20% solution), and filter sterilize using 0.22-μm filter into a sterile bottle. Store at room temperature.

11. Galactose (BioShop): Dissolve 20 g in 100-mL warm water (20% solution), and filter sterilize using 0.22-μm filter into a sterile bottle. Store at room temperature.
12. SC selection medium containing 2% glucose: Store in a sterile bottle at room temperature.
13. SC selection medium containing 2% galactose: Store in a sterile bottle at room temperature.
14. 50% Glycerol (glycerin; Fisher Scientific; Fairlawn, NJ). Dissolve 25-mL glycerol in 25-mL water. Autoclave the solution and store at room temperature.
15. Frozen stock of yeast strain: Mix 100 μL of 50% glycerol (v/v, autoclaved) sterile solution and 100 μL of yeast cell suspension in a sterile cryotube. Store the aliquot at –80°C.
16. Transformation mix: 240-μL PEG 4000 solution, 36-μL 1.0 M lithium acetate, 5-μL SSS DNA, 80-μL sterile water (total volume = 361 μL) for each transformation reaction.

2.3. Inhibitor Screening

1. Sterile nontissue culture 96-well plates with lids (Corning Costar, Lowell, MA).
2. Dispensing eight-channel pipettor (ePET, Biohit, Neptune, NJ).
3. Hand-pinning tool with 0.4- to 1.2-mm diameter pins (V&P Scientific, San Diego, CA) or automated pinning instrument such as TAS1 robot with a 0.7-mm diameter 96-pin tool (Bio-Robotics, Cambridge, England) (*see* **Note 4**).
4. 30°C incubator and temperature-maintained shaker incubator.
5. Humidifier box: Select a plastic box with lid that will hold the required number of plates and will fit into the incubator. Line the bottom of the plastic box with a sheet of paper towel and add sufficient water to wet the paper. Place the lid of a smaller plastic box on the paper towel to make a platform onto which you will put the 96-well plates so they do not touch the damp paper. Cover the humidifier box lightly with the lid to ensure air circulation.
6. Microplate reader and cuvette spectrophotometer with a 595- or 600-nm filter (e.g., Dynex Opsys MR, Chantilly, VA).
7. Chemical or natural product extract library: Compounds or extracts are dissolved in DMSO and stored in 96-well plates.

3. Methods

First, the coding sequence of the gene of interest is inserted into a yeast expression plasmid for galactose-inducible expression and introduced into yeast. The growth of this test strain in galactose is compared with the growth of a control strain containing the empty plasmid. If the heterologous protein causes growth inhibition, then a screen for chemicals that restore growth can be carried out. The chemicals showing most growth restoration are then tested in a concentration curve to confirm screening results, establish potency, and eliminate those also showing toxicity. The selected compounds can be further tested for selectivity using a strain expressing an unrelated protein that also causes growth inhibition. Follow-up assays must be used to confirm direct activity on the protein of interest.

3.1. Plasmid Preparation and Yeast Transformation [(7); see Note 5*)]*

1. Introduce the coding sequence of the gene of interest into the yeast expression plasmid using standard molecular biology techniques (*see* **Note 6**).
2. Grow the *S. cerevisiae* strain in 5-mL YPD liquid medium overnight in a shaker at 220 rpm and 30°C.
3. Measure the A_{600} of the overnight yeast suspension and dilute to an A_{600} of 0.5 in 20-mL YPD liquid medium. Incubate the cells for 4–5 h in an incubator shaker at 220 rpm and 30°C until the A_{600} reaches 2.0.
4. Transfer the cells to a centrifuge tube and centrifuge at 2000 × *g* for 8 min. Suspend the pellet with 20-mL sterile water and centrifuge again.
5. Resuspend the pellet in 1-mL sterile water and transfer to a 1.5-mL microfuge tube. Centrifuge at 16,000 × *g* for 2 min.
6. Resuspend the pellet in 0.5-mL sterile water. Transfer 100 µL into 1.5-mL microfuge tubes, one per transformation reaction.
7. Centrifuge at 16,000 × *g* for 2 min and discard the supernatant.
8. Prepare transformation mix.
9. Transfer 361-µL transformation mix to each 1.5-mL microfuge tube containing a yeast pellet.
10. Add 1–2 µL (0.5–1.0 µg) empty plasmid DNA or plasmid containing the gene of interest. If using recombinational cloning, add 100 µL of PCR-amplified gene-coding sequence containing the appropriate flanking DNA and 0.5–1.0 µg of linearized plasmid DNA *(1, 3)*. Gently suspend the yeast pellet by pipetting up and down (*see* **Note 6**).

11. Incubate the tubes in a water bath at 42°C for 40 min.
12. Centrifuge the tubes at 16,000 × *g* for 2 min and discard the supernatant.
13. Add 1-mL sterile water to each tube and mix the pellet gently by pipetting up and down.
14. Spread 50 or 100 µL of this suspension onto agar plates of appropriate SC selection medium containing 2% glucose but lacking uracil or tryptophan, depending on the plasmid used.
15. Incubate the plates at 30°C for 2–3 days until colonies appear.
16. Select 5–6 colonies per transformation to test for growth inhibition.

3.2. Test for Growth Inhibition

1. Inoculate the control strain with the empty plasmid and 5–6 colonies of the test strain bearing the plasmid with the gene of interest into tubes containing 2-mL SC selection medium with 2% glucose. Grow cells overnight at 30°C with shaking at 220 rpm.
2. The next day, transfer 1 mL of each overnight culture to microfuge tubes. Centrifuge at 4,700 × *g* for 5 min. Discard the supernatants, wash the pellets with sterile water, and centrifuge again at 4,700 × *g* for 5 min (*see* **Note 7**).
3. Resuspend the pellets in 1-mL sterile water, measure the A_{600}, and dilute the cells to A_{600} = 0.01 in appropriate SC liquid-selection medium containing 2% galactose.
4. Transfer 100 µL of yeast cells containing the control or test plasmid to wells of sterile 96-well plates, in several replicates.
5. Place the plates in the humidifier box and incubate them at 30°C for 40–42 h.
6. Shake the plate on a vortex at low speed (e.g., setting 4 of a Genie 2 Vortex mixer) for 90 s to resuspend the yeast cells and measure the A_{600} using a 96-well plate reader (*see* **Note 8**).
7. Calculate the percent growth inhibition by dividing the average A_{600} of transformants containing test plasmid by the A_{600} of transformants containing the empty plasmid, multiplying by 100, and subtracting this value from 100. A growth inhibition level of 50–80% is ideal for screening (*see* **Note 9**).
8. Prepare frozen stocks of selected strains.

3.3. Inhibitor Screening

1. The day before screening, inoculate the control strain containing the empty plasmid and the selected test strain bearing the plasmid with the gene of interest into 2 mL of SC selection medium containing 2% glucose. Grow cells overnight at 30°C with shaking at 220 rpm.
2. The next day, transfer 1 mL of overnight culture to a microfuge tube. Centrifuge at 4700 × *g* for 5 min. Discard the supernatant, wash the pellet with sterile water, and centrifuge at 4700 × *g* for 5 min, to eliminate traces of glucose.
3. Remove the plates containing chemicals to be tested from the freezer and thaw at room temperature for at least 30–60 min.
4. Suspend the pellet in 1-mL sterile water, measure the A_{600} and dilute the cells to A_{600} = 0.01 in appropriate SC liquid selection medium containing 2% galactose. Prepare ≥10 mL diluted test cells for each 96-well plate to be tested. A lower volume of control cells is required.
5. Transfer 100 µL of test cells to all but four wells of sterile 96-well plates using a dispensing eight-channel pipettor. Add 100-µL medium without yeast to two control wells and 100-µL medium with control cells to two wells (*see* **Note 10**).
6. Prepare a control 96-well plate. To columns 1–4 (32 wells) add 100-µL control cells diluted to A_{600} = 0.01. To columns 5–8 add 100-µL test cells diluted to A_{600} = 0.01, and to columns 9–12, add 100-µL medium without cells.
7. Transfer the chemicals from storage plates to plates containing yeast using a hand-held pinning tool or a robotic pinning tool. Clean and disinfect the pinning tool by dipping and shaking the pins in 10% bleach for 10 s, followed by dipping and shaking in 70% ethanol for 10 s, followed by air drying or drying over a flame. When pins are cool, dip the pinning tool into a chemical storage plate, remove the pinning tool carefully without touching the edges of the well, dip into the test plates without touching the edges of the wells, and remove in the same manner. Wash and disinfect the pins and repeat the process until all chemicals have been transferred to test plates (*see* **Note 11**).
8. Place the plates in the humidifier box and incubate them at 30°C for 40–42 h.
9. Shake stacks of five plates on a vortexer at low speed (e.g., setting 4 of a Genie 2 Vortex mixer) for 90 s to resuspend the yeast cells, and measure the A_{600} using a 96-well plate reader (*see* **Note 8**).

3.4. Growth Restoration Calculation

1. Control plate: Calculate the average A_{600} of test wells (columns 1–4), control wells (columns 5–8), and medium-only wells (columns 9–12).
2. Calculate the % growth restoration for each compound tested using the formula: % Growth restoration = (Test & chem – Test)/(Control – Test) × 100 *(3)*, where "Test & chem" is the A_{600} reading of a well containing the test strain treated with a chemical, "Test" is the average A_{600} of test cells not treated with chemicals determined from the control plate, and "Control" is the average A_{600} of control cells determined from the control plate.
3. Select as "actives" the wells showing highest levels of growth restoration (*see* **Note 12** and **Fig. 2**).

3.5. Active Chemical Confirmation

1. Visually inspect "active" wells in an inverted microscope to ensure that the increased A_{600} reading is indeed due to an increased number of yeast cells rather than being due to compound precipitation or contamination by other microorganisms (*see* **Note 13**).
2. To confirm the primary screening results, retest the activity of each active chemical at various concentrations against both the test and control strains. Establish the EC_{50} for each active compound and select compounds combining high potency and low toxicity (*see* **Note 14** and **Fig. 3**).
3. The active compounds can also be tested against a test strain for an unrelated gene that also causes growth inhibition when overexpressed in yeast. Chemicals that restore growth by general mechanisms, such as interference with the activity of the *GAL1* promoter, should also restore growth inhibited by any gene.

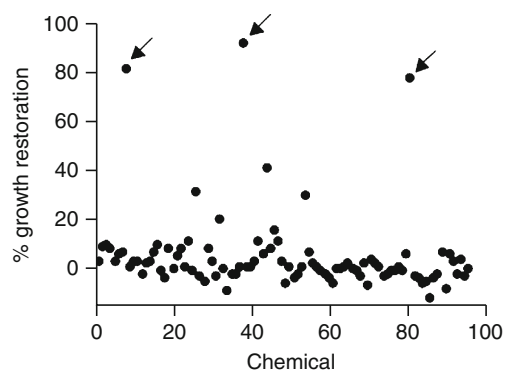

Fig. 2. Example of screening results obtained with one 96-well plate of chemicals. Most chemicals showed little or no growth restoration. Some caused growth restoration values below 0% because they are toxic to yeast. The three chemicals causing >50% growth restoration were scored as active (*arrows*).

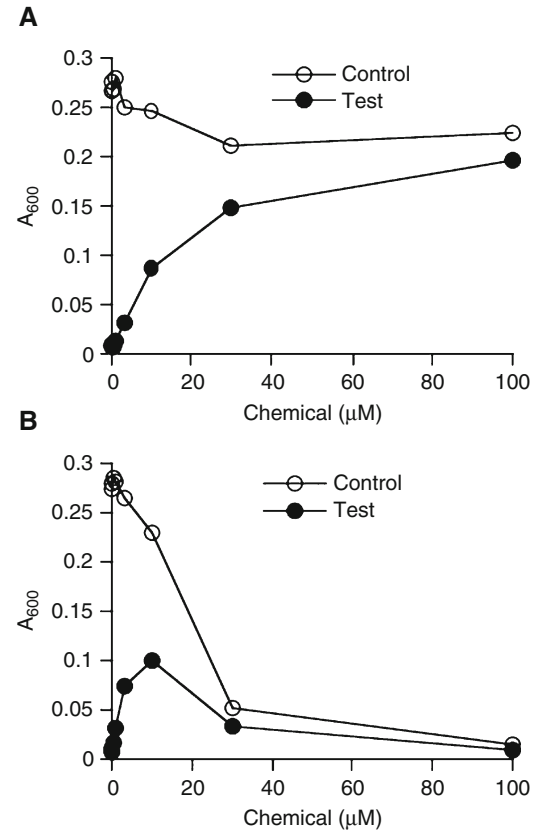

Fig. 3. Effects of two active chemicals on the growth of control and test strains. (**A**) The chemical causes concentration-dependent growth restoration of the test strain and shows little toxicity to the control strain. (**B**) The chemical causes growth restoration at low concentrations, but its activity is reduced at high concentrations. The chemical is toxic to the control strain at high concentrations. This chemical is less selective in yeast than the one shown in (**A**), and likely also in other biological systems.

4. Activity in the yeast growth restoration assay does not constitute a demonstration of direct inhibition of the protein of interest. Specific assays should be used to verify directly the inhibition of the protein in a suitable biological system.

4. Notes

1. Using a yeast strain deleted of one or more drug efflux pumps, instead of a wild-type strain, will increase the number of chemicals that can penetrate the yeast cell and increase the probability of finding inhibitors. A strain lacking *PDR5* and *SNQ2* has been used successfully in our laboratory. Strains deleted of

additional pumps are available, such as AD1-3-AD123 (*Matα, pdr1-3, ura3, his1, yor1Δ::hisG, snq2Δ::hisG, pdr5Δ::hisG*) lacking four major drug efflux pumps and strain AD1-9- (*Matα, ura3, his1, yor1Δ::hisG, snq2Δ::hisG, pdr5Δ::hisG, pdr10Δ::hisG, pdr11Δ::hisG, ycf1Δ::hisG, pdr3Δ::hisG, pdr15Δ::hisG, pdr1Δ::hisG*) *(5)*. However, such strains may be less suitable for inhibitor screening because of their slow growth.

2. A number of expression plasmids are available, including Gateway-compatible ones. Start with a multicopy plasmid for higher protein expression. If growth inhibition is too strong, use a plasmid that can be integrated in a single copy at a chromosomal locus. Plasmids for protein expression fused to a V5-xHis tag can be useful to verify expression of the protein upon incubation in galactose.
3. Denatured carrier DNA can be boiled 3 or 4 times without loss of activity.
4. The volume of chemical that will be transferred, and hence the chemical concentration used in screening, will depend on the pin diameter and geometry, the concentration of the chemical, the depth of the solution, as well as the solvent used, usually DMSO. For example, our chemical plates contain 100 µL of 5-mM solutions, and our 0.7-mm diameter pin tool transfers 0.34 µL, for a final chemical concentration of 17 µM in the test plates. We have had success testing pure chemicals at 5–20 µM.
5. All procedures with yeast and sterile media are carried out on a benchtop near a lighted Bunsen gas burner that creates an updraft to help prevent contamination of the media or the yeast strain. A sterile hood is not necessary.
6. As an alternative to using restriction fragments and ligations, the coding sequence can be introduced into the plasmid by recombinational cloning in yeast *(1, 3)*. In addition, many sequence-verified human and mouse gene-coding sequences can be purchased in Gateway entry plasmids and transferred into Gateway destination vectors for yeast expression by in vitro recombination (http://www.invitrogen.com/content.cfm?pageid = 4072).
7. It is not sufficient to dilute cells grown in a medium containing glucose to a medium containing galactose, because the cells will use glucose preferentially over galactose, even when glucose is present in small amounts. This washing step is important to eliminate all traces of glucose from the medium.
8. The yeast will settle to the bottom of the wells during growth. For accurate A_{600} measurements, it is of paramount importance to resuspend them thoroughly before reading

the plates. The plates can be checked in an inverted microscope to ensure that suspension is complete.

9. If growth inhibition is very high (>90%), determine whether the cells are dead by adding 2% glucose and incubating for an additional 48 h. If the cells still do not grow, they are dead, so there is no point in trying to rescue growth with inhibitors. If cells are dead or growth inhibition is greater than 90%, consider integrating the plasmid at a chromosomal locus to maintain it in single copy instead of multiple copies when present as an episome. This will result in lower protein expression levels and, hopefully, lower growth inhibition. If growth inhibition is too low, consider changing the growth conditions *(4)*.
10. Place controls according to where empty wells are positioned in your chemical plates. If the chemical plates contain no empty wells, place controls in a separate plate, but run a control plate for each screening experiment. Screens can be carried out in duplicate, but we prefer to screen single points in order to assay larger numbers of compounds.
11. It is very important to wash and disinfect the pins thoroughly to prevent contaminating your chemical plates with yeast or with other chemicals. Always check that you use the same plate orientation for chemical plates and test plates (e.g., position A1 at the top left).
12. To select active chemicals for secondary assays, it is useful to graph the percent growth restoration obtained for many chemicals. We generally select as active chemicals all compounds showing >50% growth restoration, or obvious outliers (**Fig. 2**).
13. False-positive readings can be caused by compound precipitation, which can obstruct the light path. Compound precipitation is easily seen in an inverted microscope as crystals or powdery deposits at the bottom of the well. Green-colored chemicals can also provide false-positive readings. Contamination by other microorganisms is possible, but we have never observed it in our experiments.
14. The shape of the concentration dependence of growth restoration provides both an indication of the potency of the compound for growth restoration and of its toxicity at higher concentrations. Compounds that cause a high extent of growth restoration at low concentrations and that do not inhibit the growth of the control or test strains (**Fig. 3A**) are more likely to show selectivity in your biological assay than compounds that inhibit growth at concentrations barely higher than active concentrations in the growth restoration assay (**Fig. 3B**).

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