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Parallel High-Throughput Automated Assays to Measure Cell Growth and Beta Galactosidase Reporter Gene Expression in the Yeast *Saccharomyces cerevisiae*

Andrew D. Napper[1,3], **Nuzhat Motlekar**[1], **Rogerio Alves de Almeida**[2], and **Graham D. Pavitt**[2]

[1]Penn Center for Molecular Discovery, Institute for Medicine and Engineering, and Department of Chemical and Biomolecular Engineering, University of Pennsylvania, 1160 Vagelos Research Laboratories, 3340 Smith Walk, Philadelphia, PA 19104

[2]Faculty of Life Sciences, University of Manchester, Manchester M13 9PT, UK

Abstract

Parallel high-throughput automated assays are described for the measurement of cell growth and β-galactosidase reporter gene expression from a single culture of the yeast *S. cerevisiae*. The dual assay measures the effect of test compounds on expression of a specific gene of interest linked to the β-galactosidase reporter gene, and simultaneously tests for compound toxicity and other effects on cell growth. Examples of assay development and validation results are used to illustrate how this protocol may be used to screen two yeast cell lines in parallel. Yeast cells are grown overnight in V-bottom polypropylene 384-well plates, after which portions of the cell suspension are transferred to clear and to white flat-bottom 384-well plates for measurement of cell growth and reporter gene expression, respectively. Cell growth is determined by measurement of absorbance at 595 nm, and β-galactosidase expression is quantified by Beta-Glo, a commercially-available luminescent β-galactosidase substrate.

Key terms for indexing

cell growth; yeast; reporter gene; luciferase; cell-based assay; model organism; mutant gene; B-galatosidase; luminescence

INTRODUCTION

Here we describe an HTS assay to assess reporter gene activity in the yeast *Saccharomyces cerevisiae*. Yeasts have been used extensively to study the effect of specific genes and genetic changes on cellular phenotypes. These studies have provided valuable insight into human disease processes and novel approaches for therapeutic intervention due to the similarity of yeast cellular control mechanisms to those in mammalian cells and the relative ease with which yeast genes can be manipulated.

Corresponding author: Andrew D. Napper, Nemours Center for Childhood Cancer Research, 1701 Rockland Road, Wilmington, DE 19803, Tel: 302-298-7209, napper@medsci.udel.edu.
[3]Current address: Nemours Center for Childhood Cancer Research, 1701 Rockland Road, Wilmington, DE 19803

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Two assays are described here:

Assay #1, Yeast growth assay: Growth is quantified by turbidity of a yeast cell suspension, as measured by absorbance.

Assay #2, Gene expression assay: The specific gene of interest is linked to the expression of the reporter gene β-galactosidase, quantified using a luminescent detection reagent.

The optimization of these assays is exemplified by their validation for a high throughput screen designed to discover chemical modulators of stress-response pathways in yeast (Alves de Almeida et al., 2008; Motlekar et al., 2009). Both cell growth and expression of a chromosomally integrated *GCN4*-dependent reporter gene fusion to β-galactosidase were measured in two yeast strains, wild type *Saccharomyces cerevisiae* and an eIF2B mutant. *GCN4* is a central regulator of general amino acid control stress response (GAAC), many effects of which are mediated through the translation factor eIF2B. Mutant and wild type yeast strains were screened in parallel to identify compounds acting specifically on either strain: wild type–specific compounds affecting GAAC through functional eIF2B, and mutant-specific compounds that restore normal function to mutant eIF2B. Assay #2 was optimized for high-throughput screening (HTS) by adaptation of a commercially available coupled β-galactosidase–firefly luciferase system for use with yeast cells (Alves de Almeida et al., 2008; Motlekar et al., 2009). In this assay β-galactosidase present in the yeast cells reacts with 6-O-β-galactopyranosyl-luciferin substrate to generate D-luciferin. This in turn reacts with ATP, O_2, and firefly luciferase (all provided in the commercially available assay reagent kit) to form oxyluciferin and emit light. We describe the assay protocols in detail and in the discussion that follows we highlight technical challenges encountered in miniaturizing to a robot-compatible 384-well format.

PROTOCOL

Protocol Contents

Materials (as needed for sections A-I)

Equipment required

Software required

Procedure

A. Preparation of frozen yeast stocks (matched pair, e.g., wild type and mutant gene)

B. Optimization of growth conditions

C. End-point growth assay: absorbance at 595 nm (Assay #1)

D. β-galactosidase reporter expression timecourse

E. End-point β-galactosidase expression assay: luminescence (Assay #2)

F. Single concentration compound screening

G. Data analysis and hit selection

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H. Dose-response testing

I. Curve fitting, IC_{50} determination, and hit confirmation

Materials

1. *Matched pair of yeast strains*. For the study used as an example here, wild type yeast strain GP4213 (*MATα leu2-3 leu2-112 ura3-52::HIS4–lacZ ino1 gcd6Δ gcn2Δ::hisG* pAV1265[GCD6 CEN6 LEU2]) and an isogenic *gcd6* mutant strain GP4198 (*MATα leu2-3 leu2-112 ura3-52::HIS4–lacZ ino1 gcd6Δ gcn2Δ::hisG* pAV1744[gcd6-R284H CEN6 LEU2]) (Alves de Almeida et al., 2008). Store at −80°C as single-use frozen stocks, prepared as described below. Generation of yeast strains is outside the scope of this protocol. Therefore, it is assumed that the reader planning to replicate the steps described below has obtained the desired strains.
2. *Growth media*. YPD media (10 g/l yeast extract; 20 g/l bacto peptone; 20 g/l glucose).Available from Sigma.
3. *Plate sealing films*. Breathe-Easy sealing films (Sigma cat# Z380059).
4. *Growth plates*. Polypropylene V-bottom 384-well plates (Greiner Bio-One cat# 781280).
5. *Assay plates*. Clear 384-well plates (Cat# 3702) and white 384-well plates (Cat# 3652) from Corning Life Sciences (Lowell, MA).
6. *Protease inhibitor tablets*. Cat# 1873580 from Roche Applied Science (Indianapolis, IN).
7. *β-galactosidase detection*. Beta-Glo assay system (Cat# E4780) from Promega (Madison, WI).
8. *Freezing media*. YPD with 15% v/v glycerol.
9. *Freezer vials for yeast stocks*. Snap-cap PCR tubes (0.2 ml) from any supplier of standard lab accessories.

Equipment Required

1. Plate reader capable of reading absorbance and luminescence in 384-well plates. E.g., EnVision multimode plate reader from Perkin Elmer.
2. Pipetting workstation equipped with 384-tip pipetting head. E.g., JANUS from Perkin Elmer or equivalent.
3. For compound screening: Pipetting workstation equipped with pintool consisting of 384 pins with nominal transfer volume of 100 nl. E.g., JANUS MDT from Perkin Elmer or equivalent.
4. Reagent dispenser. E.g., Multidrop-384 reagent dispenser from Thermo Scientific.

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Software required

1. Microsoft Excel: percent inhibition or activation calculation (if screening database is not available). Comparison of data sets and selection of hits.
2. GraphPad Prism or equivalent: graphing data and curve fitting for IC50 and EC50 calculation (if screening database is not available).
3. OpenHTS (CeuticalSoft), ActivityBase (IDBS), or equivalent screening database: percent inhibition or activation calculation. Comparison of data sets and selection of hits.

Procedure

A. Preparation of frozen yeast stocks

1. Store yeast strains in a −80°C freezer in YPD containing 15% v/v glycerol.
2. Remove strains from freezer, streak onto YPD plate, grow colonies by incubation of the plate for 3 days at 30°C.
3. Prepare cell stocks of the two yeast strains by inoculating a single colony into 50 ml of YPD media in a 250 ml Erlenmeyer flask and growing the culture overnight at 30°C with vigorous shaking (200 rpm).
4. Determine cell concentration after overnight growth by measurement of absorbance at 595 nm (A_{595}). Note that dilution of the culture might be necessary in order to obtain a reading in the linear range of the plate reader. Centrifuge cell culture at 3000 g for 5 min and resuspend the pelleted cells into YPD medium containing 15% v/v glycerol to give a cell suspension with A_{595} = 5.0.
5. Aliquot cells for single use. A volume of 0.5 ml contains sufficient cell stock for screening of 20 plates, taking into account dead volume and wastage of the liquid dispenser. Freeze on dry ice, and store at −80°C.

B. Optimization of growth conditions

6 *Linear range of absorbance values as a measure of cell density*. Thaw yeast cell stock frozen at −80°C (from **Section A, Step 5**) and serially dilute in a clear 384-well plate in YPD media to give 50 μl per well of dilutions ranging from 1:5 to 1:500. Measure A595 in the plate reader. See **Step 7**(d) and the Critical Parameters and Anticipated Results section (below) for more detailed notes on determining the linear range of absorbance values useful for monitoring yeast growth.

7 *Optimal time to incubate yeast suspensions prior to recording growth*. Select optimal time for end-point reading in growth assay by determining a growth time course over 24 hr as follows:

   a. For each time point fill a clear polystyrene 384-well plate with 25 μl per well of YPD medium.

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b. Dilute thawed wild type and mutant yeast cells 1:500 in YPD medium and inoculate 25 μl of each of the diluted stocks to specific wells as follows: Diluted wild type cells were added to wells A–P in columns 2 and 3-12, diluted mutant cells were added to wells A-P in columns 13-22 and 24, wells designated as blanks (wells A-P in columns 1 and 23) should be filled with an additional 25 μl of medium in place of yeast cells. Seal plates with Breathe-Easy membranes and incubate the yeast cultures at 30°C for 4, 8, 16, or 24 hr.

c. After incubation, remove the Breathe-Easy membranes and measure A_{595} in the plate reader. The optimal incubation time prior to recording growth will be one in which the strain is still growing in exponential phase and the cell density is in the linear range of absorbance values. See the Critical Parameters and Anticipated Results section (below) for more details.

8 *Effect of DMSO on growth*. Grow both wild type and mutant yeast cells for 16 hr in the presence of 0–1.6% (v/v) final DMSO concentration. Perform growth assay as described above (**Step 7a-c**), except that DMSO is mixed with medium in the assay plate prior to the addition of yeast cells. Wells without added DMSO are included as controls. To calculate the percent growth inhibition caused by addition of DMSO, see **Section G** (Data analysis and hit selection). Also, see the Critical Parameters and Anticipated Results section (below) for more details on the effects of DMSO on the growth of S. cerevisiae.

C. End-point growth assay: absorbance at 595 nm (Assay #1)—If two cell lines are tested in parallel (e.g., mutant and wild type), a control column of the other cell line should be included in each test plate, as described in 3(a) and (b) below. The assay plate layouts are shown in Figure 1 Only negative control wells (yeast grown in the absence of test compounds) are included in the plate layout described here. Positive control wells (containing compounds known to affect the growth of the yeast strains in the assay) could be included if desired in columns 2 or 24.

9 *Addition of growth medium*. Fill V-bottom polypropylene 384-well plates with 25 μl of YPD medium using reagent dispenser, and add an additional 25 μl of YPD medium to column 1 (for blanks).

10 *Compound addition*. Add compounds by pintool transfer to give a final concentration not to exceed 25 μM of each compound in 0.25% DMSO. Reserve wells in columns 1, 2, 23, and 24 for controls and blanks containing 0.25% DMSO but no compound. In the example assay here, a 384-pin pintool was used to transfer 120 nl of a 10 mM solution in DMSO into the medium-containing V-bottom growth plates, giving a final concentration of 24 μM of each compound in 0.24% DMSO in columns 3-22 once yeast cells are added. Columns 1, 2, 23, and 24 of the library plates contain DMSO in this case, so 0.24% DMSO (final concentration) but no compound is transferred to wells in those columns.

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11 *Yeast cell addition*. Thaw frozen stocks of wild type and mutant yeast cells and dilute 1:500 in YPD medium. Mix by vortexing to ensure even resuspension.

a. *Wild type yeast test plates*. Add 25 μl of diluted wild type stock to all columns except 1 and 23 in V-bottom growth plates using reagent dispenser. Add 25 μl of diluted mutant stock to column 23.

b. *Mutant strain test plates*. Add 25 μl of diluted mutant stock to all columns except 1 and 23 in V-bottom growth plates using reagent dispenser. Add 25 μl of diluted wild type stock to column 23.

12 *Cell growth*. Seal plates with Breathe-Easy membranes and incubate yeast cultures for 16 hr at 30°C. (Note: This incubation time was selected based on earlier determinations of growth curves in Section B, Steps 6-7. The optimal time may vary between different strains and needs to be determined empirically in each case.)

13 *Mixing and plate reading*. Mix the cell suspension by repeated aspirate-dispense cycles using 30 μl disposable tips on the 384-well pipetting head in the pipetting workstation. Transfer 5 μl to a white 384-well plate containing Z-buffer for Assay #2, the β-galactosidase reporter expression assay (**Section D, Step 15**). Transfer 30 μl from each well of the portion that remains to wells in a clear 384-well flat-bottom plate, taking care to avoid formation of bubbles in the destination plate. Record A_{595} using the plate reader.

D. β-galactosidase reporter expression timecourse (Assay #2)—Assay #2 measures expression of the gene of interest linked to the expression of the reporter gene β-galactosidase. Enzymatic conversion of the Beta-Glo reagent by β-galactosidase results in long-lived glow luminescence. To obtain consistent results, the light output should be allowed to reach a steady state prior to recording luminescence. The time to reach a steady state is determined from a luminescence timecourse.

14 Add 20 μl per well of Z-buffer (82 mM disodium hydrogen phosphate, 9 mM sodium dihydrogen phosphate, 0.1% SDS, 1 mM DTT, protease inhibitor tablets) to white, flat-bottom 384-well plates using reagent dispenser.

15 Add 5 μl of yeast cell suspension by tip transfer from V-bottom yeast growth plates using the 384-well pipetting head (see **Section C, Step 13** above).

16 Mix cells and Z-buffer by pipetting, using the pipettes on the pipetting head on the workstation from **Step 15**, and incubate for 20 min at room temperature to permeabilize the cells.

17 Add 25 μl of Beta-Glo reagent using reagent dispenser.

18 Measure luminescence at 2 min intervals for 4 hr on the Envision plate reader. For data workup see **Section G** (Data analysis and hit selection). See the Critical Parameters and Anticipated results section below for sample data from a luminescence timecourse.

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E. End-point β-galactosidase expression assay: luminescence (Assay #2)— Protocol identical to **Section D, Steps 14-18**, except in **Step 18**, luminescence is read once after light output has reached steady state (using the strain and protocol described here, that time is 90 minutes after Beta-Glo reagent is added).

F. Single concentration compound screening—Add test compounds to yeast growth plates as described in **Section C**. Measure cell growth (Assay #1, **Section C**) and β-galactosidase reporter gene expression at a single timepoint (Assay #2, **Section E**).

G. Data analysis and hit selection

19 Calculate percent inhibition of growth or percent inhibition of luminescence for each test compound using the signal in absorbance units (OD) or luminescence units (LU) read out for each well, and the mean of the plate negative controls (Columns 2 and 24), and the mean of the plate blanks (Column 1) [see plate maps in Figure 1]:

$$\%\,Inhibition = 100 \times \{1 - [(signal - blank\ mean)\,/\,(negative\ control\ mean - blank\ mean)]\} \quad \text{Eqn. 1}$$

[The reference yeast strain (column 23) is not used in the calculation.]

20 Correct percent inhibition of luminescence for cell growth:

$$Corrected\,\%\,inhibition = 100 \times \{1 - [(100 - \%\,reporter\ gene\ inhibition)\,/\,(100 - \%\,inhibition\ of\ growth)]\} \quad \text{Eqn. 2}$$

21 Eqns 1 and 2 (**Steps 19 and 20**) may also be used if the goal of the compound screening is to identify activators of growth or luminescence. A negative value of percent inhibition indicates activation, in which growth or luminescence in the presence of test compound is increased relative to the negative control mean. These negative percent inhibition values may be converted to percent activation as follows:

$$Percent\ activation = 1 \times percent\ inhibition \quad \text{Eqn. 3}$$

22 Select hits based on percent inhibition (or activation) exceeding a defined threshold. For the hits to be statistically significant (i.e., not just due to random scatter in the data), the threshold should be below (or above for activators) the mean of the plate negative controls by a minimum of 3x standard deviation of the controls. The standard deviation of the plate controls in the yeast growth and luminescence assays typically ranges from 10-15%; thus the minimal hit threshold would be set at 30-45% inhibition. For assays with a high hit rate (>1%), the threshold may be set as high as 80% inhibition to keep the number of hits to a manageable number for follow up testing.

H. Dose-response testing—The assay protocol is identical to single compound screening (**Section F**), except that compounds are tested at multiple concentrations obtained by serial dilution. A suitable layout is 16 two-fold dilutions of each compound giving an

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assay concentration range of 100 μM to 3 nM. In this case, compounds may be serially diluted vertically down each plate such that columns 3-22 each contain the dilutions of one compound. Protocol for serial dilution in DMSO:

19 Add 20 μl of each compound stock (10 mM in DMSO) arranged 20 per plate in wells A3 to A22 of 384-well V-bottom polypropylene plates.

20 Add 20 μl of DMSO to wells A1, A2, A23, and A24

21 Add 10 μl per well of DMSO to the entire plate except Row A using reagent dispenser.

22 Two-fold serial dilute compounds by transfer of 10 μl row-by-row from Row A to Row P using a single row of disposable tips, then remove and discard 10 μl from Row P.

The resulting dose-response plates contain 16 two-fold dilutions of each compound, ranging from 10 mM to 305 nM, arranged one compound per column in columns 3–22. Pintool transfer into growth plates (**Section C**) gives a final range of 100 μM to 3 nM.

I. Curve fitting, IC_{50} determination, and hit confirmation—Following dose-response testing (**Section H**), fit the yeast growth and luminescence data to a dose-response curve to calculate IC_{50} (for inhibitors) or EC_{50} (for activators).

1. Calculate percent growth and β-galactosidase activity for each dilution of each compound from A595 and luminescence, respectively, and the means of the plate controls and plate blanks [see plate maps in Figure 1]:

$$\%\,Activity = 100 \times [(signal - blank\ mean)\,/\,(control\ mean - blank\ mean)] \quad \text{Eqn. 4}$$

2. If there is a dose-dependent effect on growth (as revealed by a decrease in percent growth as the test compound concentration increases), correct luminescence assay percent activity values at each compound dose for cell growth:

$$Corrected\,\%\,activity = \%\,activity / \%\,growth\ (relative\ to\ growth\ controls) \quad \text{Eqn. 5}$$

3. Fit percent activity data to a dose-response curve using non-linear regression. A screening database or a curve-fitting and graphing program such as GraphFit Prism will perform a four parameter logistic fit and calculate IC_{50} or EC_{50} as appropriate.

COMMENTARY

Background Information

Reporter gene fusions have been widely employed in biological research for decades. They provide a simple experimental means to study the control of expression of genes. Ease and sensitivity of measurement and whether the host system naturally expresses the reporter gene are important considerations that govern the usefulness and choice of reporter gene fusion to use. Expression of commonly employed reporter genes may be detected by specific substrates that produce colored chemical products or emit light, easily detected with a

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spectrophotometer or luminometer (Sambrook and Russell, 2001). The *Escherichia coli LacZ* gene is one such reporter. The β-galactosidase enzyme was studied at least 60 years ago (Lederberg, 1950) and is encoded by *LacZ*. β-galactosidase naturally cleaves lactose into glucose and galactose. The development of colorimetric substrates including O-nitrophenyl-β-D-galactopyranoside (ONPG) greatly enhanced its widespread utilization from the 1960s onwards. ONPG is colorless, but when cleaved releases O-nitrophenol which is yellow and absorbs at A_{420}. Thus in the presence of excess ONPG, the yellow color develops at a rate proportional to the level of LacZ protein present in a cell lysate (Miller, 1972). In recent years, parallel development of instrumentation, data analysis, and substrates with enhanced sensitivity has made it possible for reporter gene studies to be performed in a variety of high-throughput screening (HTS) formats. Luminescent reagents have been especially useful in facilitating low-μl volume "mix-and-read" assays in 384-and 1536-well plates amenable to high-throughput robotic liquid handling (Inglese et al., 2007)

Critical Parameters and Anticipated Results

The yeast growth and B-galactosidase expression assays require careful and extensive optimization. Given the multitude of parameters that can affect data quality and reproducibility, careful analysis of the results is required. These issues are discussed here.

A. Preparation of frozen yeast stocks—Yeast strains are grown once to generate cell suspension sufficient for all assay development, screening and hit confirmation. Advantages:

- **i.** Consistency: A portion of the same cell suspension is used for each experiment.
- **ii.** Ease of assay set-up: Avoids the need to grow yeast strains before each experiment.

Cell suspension samples are stored in 0.2 ml PCR tubes as these allow for rapid freeze-thaw.

B. Optimization of growth conditions—To allow sufficient time to discern test compound effects, the yeast growth assay (Assay #1) was designed to measure cell density after overnight incubation, provided the yeast cells remain in an exponential growth phase. Yeast growth is determined by measurement of absorbance at 595 nm (A_{595}). Points to note:

- **i.** In this assay, absorbance is an indirect measure of turbidity. Turbidity of the yeast suspension increases in proportion to cell number during growth.
- **ii.** Increase in A_{595} is due to light scattering by the turbid suspension and not absorbance. Measurement at a long wavelength (595 nm or above) ensures that there is no absorbance due to the yeast cells or most test compounds.
- **iii.** At low cell density, there is a direct linear relationship between A_{595} and yeast cell number determined by turbidity of the suspension. Above a certain cell density, the relationship between absorbance and cell number deviates from linearity, and A_{595} understates the true cell number (Warringer and Blomberg, 2003).
- **iv.** To ensure that A_{595} provides a true measure of cell number, it is necessary to obtain a standard curve of A_{595} against yeast cell density to determine the linear range of the growth assay (see iii above).

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v. DMSO is known to affect yeast cells (Murata et al., 2003), so sensitivity of yeast cell growth to DMSO should be assessed.

Example results

1. *Linear range of absorbance values as a measure of cell density*. Figure 2: A standard curve of serially diluted yeast stock showed that absorbance values up to 0.4 are directly proportional to cell density.
2. *Optimal time to incubate yeast suspensions prior to recording growth*. Figure 3: Overnight growth curves confirmed that 16 hr growth of a 1:1000 dilution of the frozen yeast stocks gave absorbance values within the linear range of detection.
3. *Effect of DMSO on growth*. Figure 4: There was no significant effect on growth at 0.25% DMSO, but a reduction of almost 25% in the presence of 1% DMSO. Therefore, DMSO should be limited to 0.25% during compound screening for these particular strains, and for most other S. cerevisiae strains as well. Note that in this case, growth of the mutant yeast was repressed more strongly than that of wild type yeast.

C. End-point growth assay: absorbance at 595 nm—Yeast cells are grown overnight, after which growth is measured (Assay #1) and a portion of cells are removed for testing in the β-galactosidase reporter gene assay (Assay #2). Points to note:

i. Incubation time for the growth assay should be selected based on optimization of growth conditions (**Section B** above). A 16 hr incubation time was selected for the yeast cell lines described here.

ii. IMPORTANT: Overnight growth in V-bottom polypropylene plates instead of flat-bottom plates was necessary for consistent mixing of the suspension with pipette tips and precise cell transfer by automated liquid handling for the β-galactosidase reporter gene assay (see also **Section E** below).

iii. Concentration of test compounds should be no more than 25 μM to avoid non-specific toxicity. Likewise, DMSO concentration should be below the level at which effects on growth were observed (**Section B, Step 3**).

D. β-galactosidase reporter expression timecourse

Example results: Figure 5. A timecourse of luminescence resulting from *GCN4*-dependent expression of the β-galactosidase reporter gene showed that light output reached a steady state after 90 mins. Parallel testing of wild type yeast and a strain containing mutant eIF2B showed that the mutant eIF2B strain gave >2-fold higher luminescence. This difference provided an assay signal window sufficient for parallel screening of both wild type and eIF2B mutant yeast to identify test compounds that restore the function of mutant eIF2B (Motlekar et al., 2009).

E. End-point β-galactosidase expression assay: luminescence (Assay #2)— Yeast cells are grown overnight in V-bottom polypropylene plates, after which a portion of

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cells is removed for testing in the β-galactosidase reporter gene assay (Assay #2). Points to note:

- **i.** IMPORTANT: Mixing and transfer of the cell suspensions from the yeast growth plate to the β-galactosidase assay plate must be carefully optimized. After overnight growth the yeast cells needed to be resuspended to ensure reproducible transfer of a small volume of cells for the luminescence assay. Mixing in a flat-bottom plate tends to concentrate the cells to the rims of the well; therefore, cells should be grown in V-bottom polypropylene plates. Efficient mixing necessitates slow aspiration of the cell suspension with each pipette tip a fraction of a millimeter above the well bottom.
- **ii.** Luminescence should be read after light output has reached a steady state (see **Section D**).

Example results: Figure 6. Reporter gene assay validation plate. Cell suspension to which DMSO had been added by pintool (no test compounds) was grown overnight in a V-bottom 384-well plate, after which 5 μl was transferred to a white 384-well plate and mixed with Z-buffer and Beta-Glo. Luminescence results shown provide a measure of well-to-well variability and confirm the 2-fold difference between wild type and eIF2B mutant expression of the β-galactosidase reporter gene.

NOTE: Depending on the hit threshold chosen for screening (**Section G, Step 22**), several outliers in Figure 6 might be selected as hits (known as false positives as the deviation from the plate control mean is not due to compound activity). Luminescence values from wells 120, 240, and 255 in particular deviate significantly from the remainder of the data. If outliers such as these are observed, a repeat of the experiment will reveal whether there is a problem associated with a specific pin in the pintool or a specific channel in the pipetting head. If the outliers are not replicated at the same well locations between experiments, then they may be attributed to random data scatter. If further refinement of the liquid handling steps fails to reduce the occurrence of such outliers, screening may nonetheless proceed. False positive outliers will be eliminated during dose-response testing (**Section H**).

G. Data analysis and hit selection—The selection of "hits" following compound screening is a critical step. It is important that the data is analyzed in such a way that compounds with the desired activity are selected. Points to note:

- **i.** Luminescence values obtained in Assay #2 will be a true measure of inhibition (or activation) of β-galactosidase gene expression only if the data are corrected to take account of each compound's effect on cell growth in Assay #1. In the absence of this growth correction (**Section G, Step 2**), luminescence values are decreased or increased in proportion to the cell density following overnight yeast growth.
- **ii.** If the primary screening data are highly scattered (standard deviation of plate controls >15%), the correction described in (i) above may not be reliable. In this case the primary screening should be performed in duplicate, or hits should be selected based on uncorrected percent inhibition (or activation) of luminescence, and the correction made upon dose-response testing (**Sections H and I**).

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iii. If two cell lines are screened in parallel (see **Section C**), compounds selectively active against one of the cell lines may be identified after primary screening, provided the data quality is acceptable (standard deviation of plate controls <15%). Otherwise, determination of selectivity should be made after dose-response testing. For results of a screen against a library of 27,000 test compounds and in-depth discussion of hit selection, dose response testing, and data analysis, see Motlekar (2009).

H. Dose-response testing—Because DMSO affects yeast cell growth it is important that compounds are serially diluted in DMSO. Transfer of each dilution into the growth plate by pintool then yields a constant percent DMSO in each well. Serial dilution of compounds from a DMSO stock into buffer or growth media results in a different amount of DMSO in each well.

Time Considerations

A. Preparation of frozen yeast stocks—Total growth time is 4 days, with 1-2 hr of hands-on time on days 0, 3, and 4. Yeast stocks are stable at -80°C for at least one year, so this section may be required only once.

B. Optimization of growth conditions—Total growth time is 1 day, with 2-3 hr of hands-on time on days 0 and 1. Provided the experiment works according to the protocol, this section is required only once.

C. End-point growth assay: absorbance at 595 nm (Assay #1)—Total growth time is 16 hr, with 2-3 hr of hands-on time on days 0 and 1.

D. β-galactosidase reporter expression timecourse—Total growth time is 16 hr, followed by a 3 hr luminescence time. Allow 1-2 hr before and 2-4 hr after for set up and data analysis, respectively.

E. End-point β-galactosidase expression assay: luminescence (Assay #2)—Total growth time is 16 hr, followed by a 90 min luminescence assay. Allow 1-2 hr before and 2-4 hr after for set up and data collection, respectively.

F. Single concentration compound screening—Total growth time is 16 hr, followed by a 90 min luminescence assay. Up to 20 plates may be nested for batchwise testing. For a 20-plate screening run, allow 4-5 hr before starting yeast growth, and 8 hr the following day for luminescence assay set up and data collection.

G. Data analysis and hit selection—Allow 1 day per week during screening.

H. Dose-response testing—Allow 1 day to select hits and serial dilute. Testing time similar to F above.

I. Curve fitting, IC_{50} determination, and hit confirmation—Allow 1 day for data analysis per 100-200 compounds.

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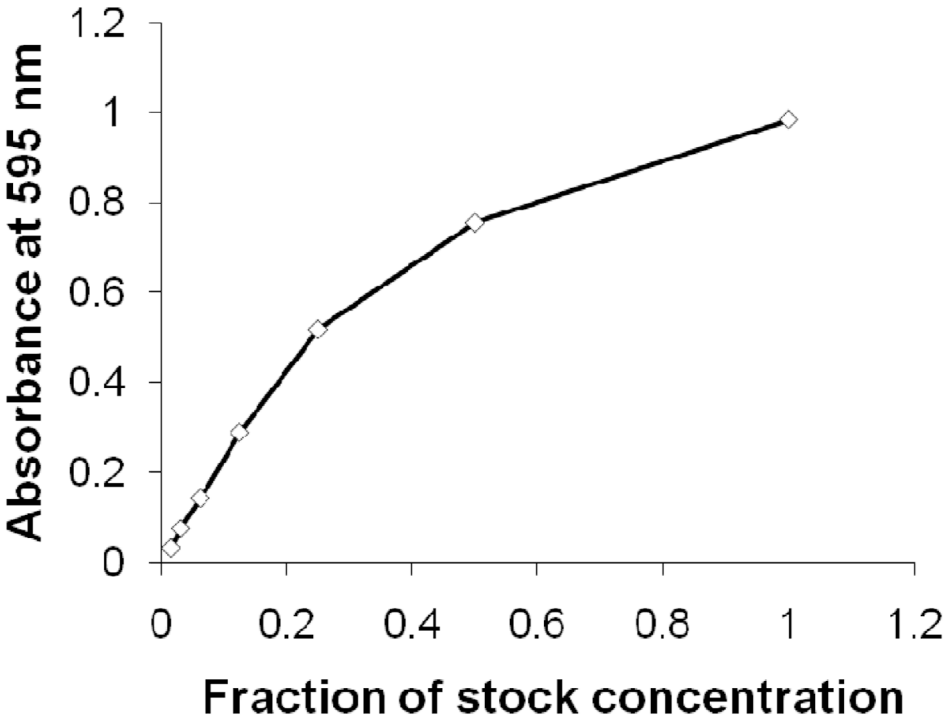

Figure 1.
Assay plate formats

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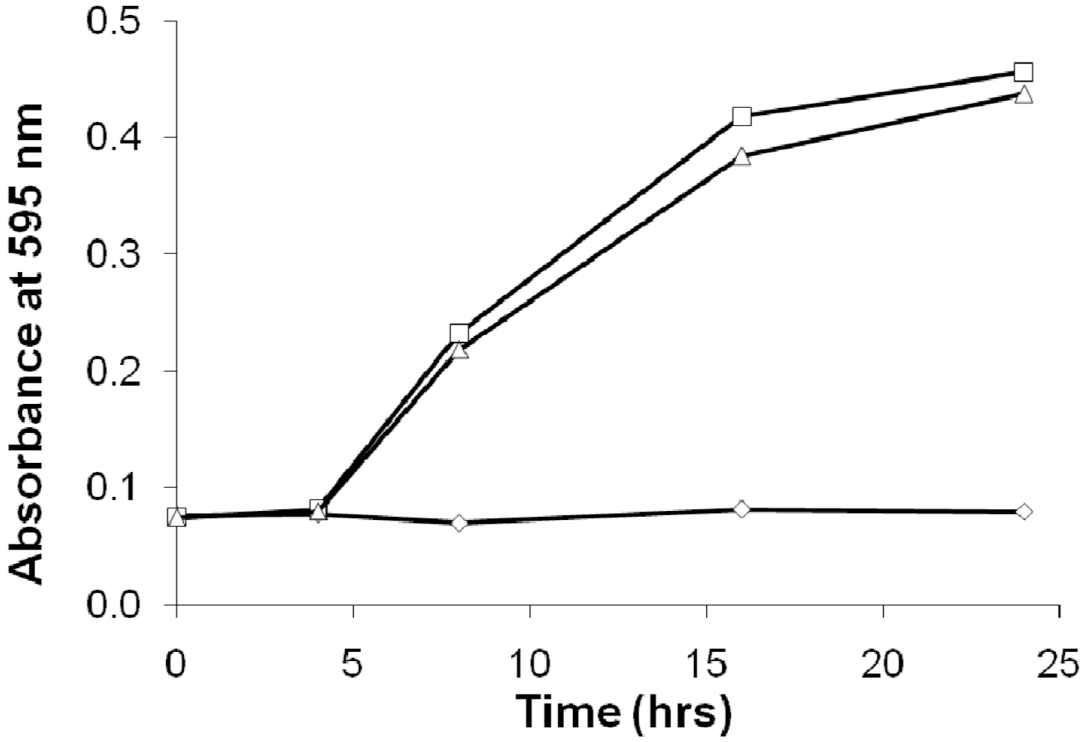

Figure 2.

Dilution of a suspension of yeast cell stock used to determine the linear range of absorbance determination at 595 nm (A_{595}). Previously published in Motlekar (2009).

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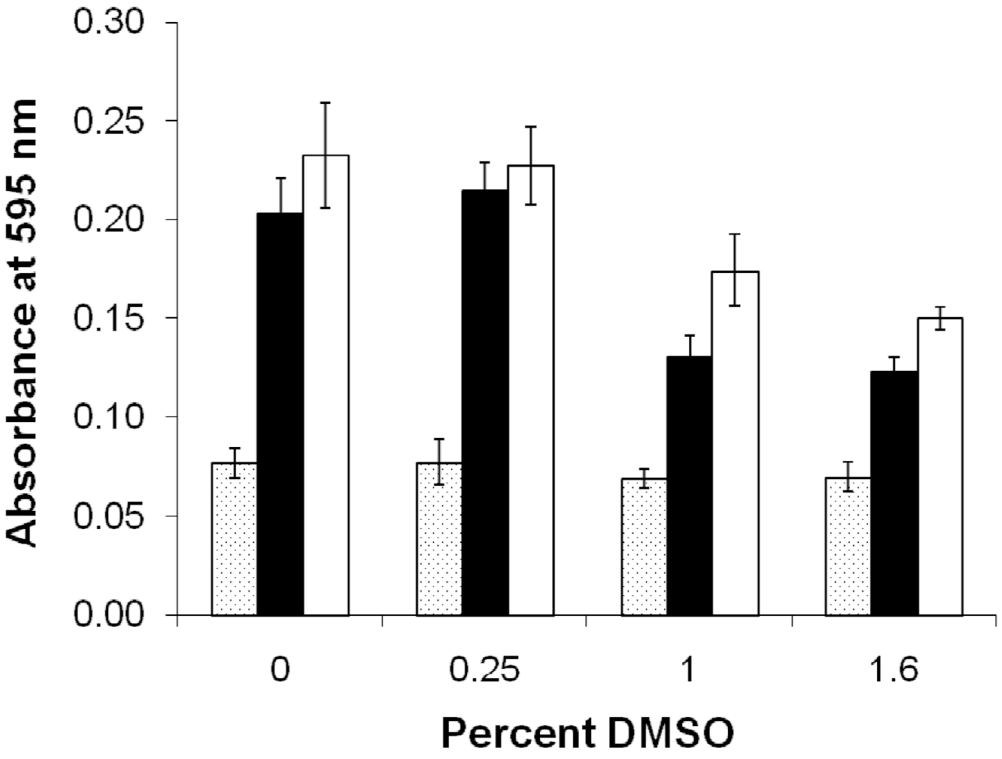

Figure 3.

Time course of yeast growth. Growth time course of wild type (□) and mutant (△) yeast determined by measurement of A_{595} at each time point. Blanks consisting of media alone (◇) were used to monitor A_{595} in the absence of cell growth. Previously published in Motlekar (2009).

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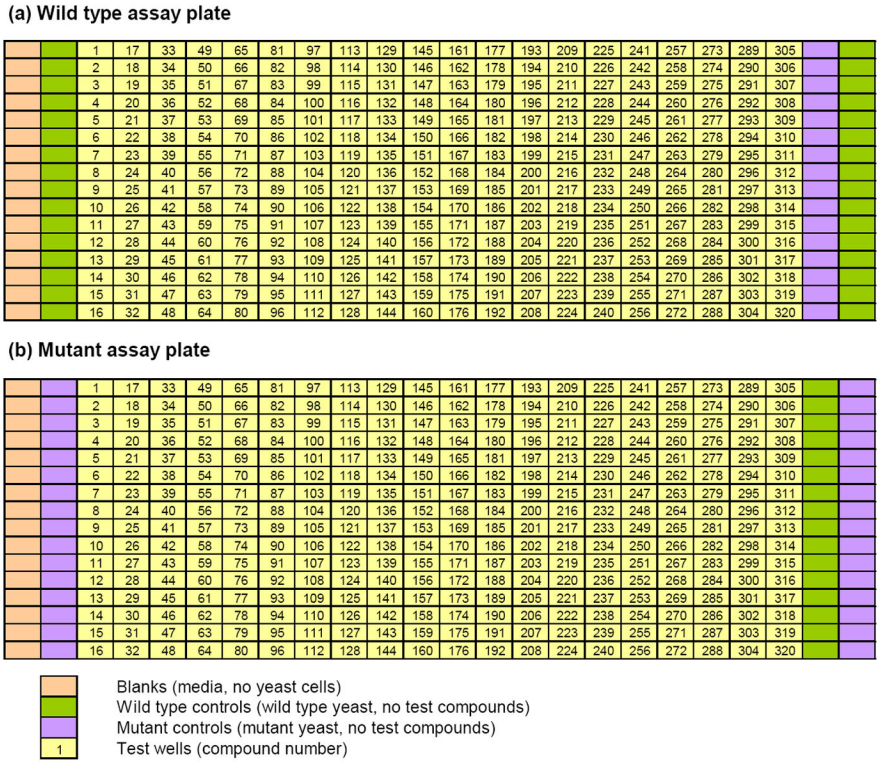

Figure 4.

Effect of DMSO on yeast growth. Cell growth after 16 hr was determined by measurement of A_{595} of a suspension of wild type cells (□), mutant cells (■), or media blank (pattern). Error bars represent mean ± standard deviation of 16 replicate wells. Previously published in Motlekar (2009).

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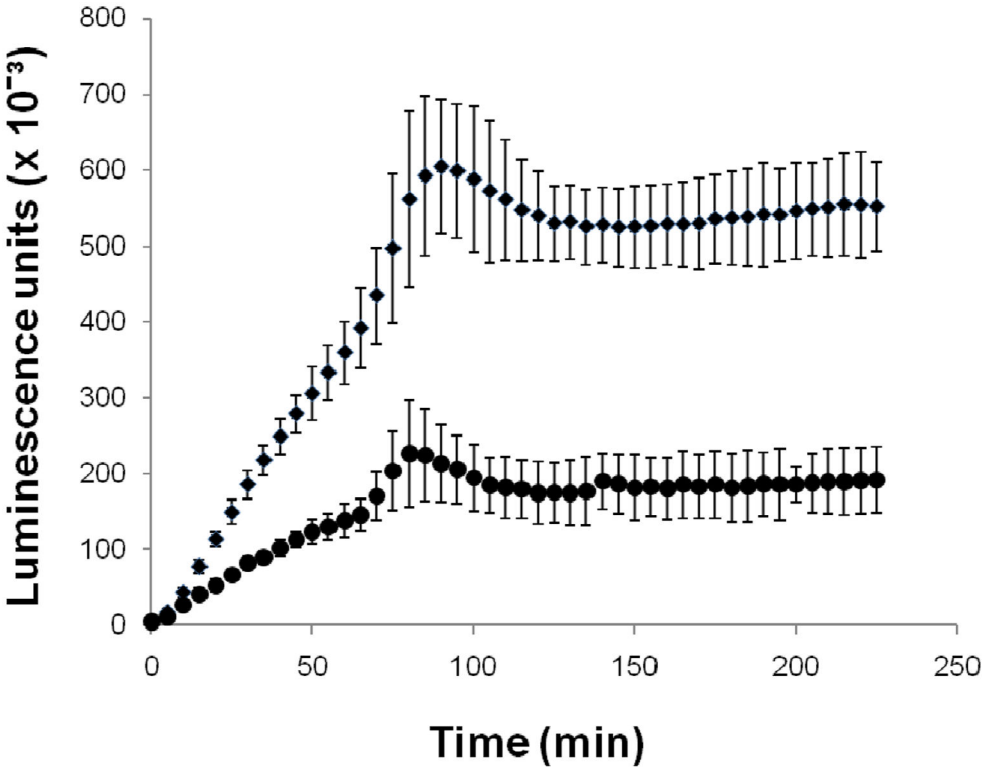

Figure 5.

Timecourse of luminescence resulting from *GCN4*-dependent expression of the β-galactosidase reporter gene. Wild type (●) and eIF2B mutant (◆) yeast strains were grown overnight and mixed with Beta-Glo reagent. Error bars represent mean ± standard deviation of 16 replicate wells. In this experiment there was no significant difference in growth rates between mutant and wild type strain and the data shown have not been corrected for cell growth. Previously published in Motlekar (2009).

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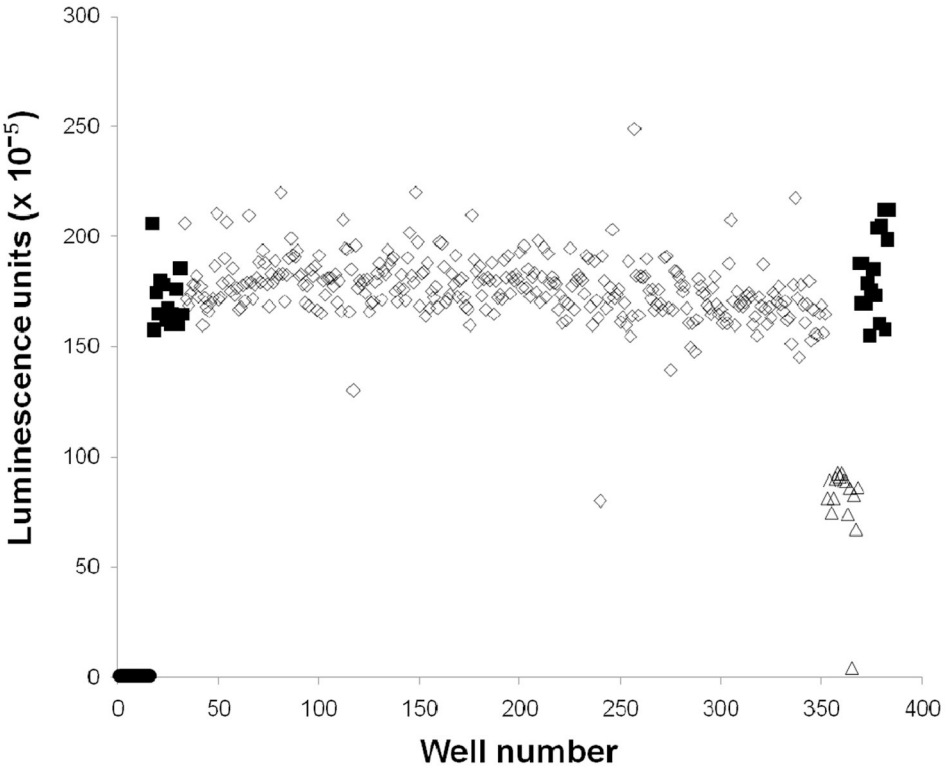

Figure 6.

Reporter gene assay validation plate. eIF2B mutant control (■), wild type control (△), blank (●), and test (◇) wells to which DMSO has been added to a final concentration of 0.24% were assayed for β-galactosidase expression. Previously published in Motlekar (2009).