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A rapid, inexpensive yeast-based dual-fluorescence assay of programmed−1 ribosomal frameshifting for high-throughput screening

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ABSTRACT

Programmed −1 ribosomal frameshifting (−1 PRF) is a mechanism that directs elongating ribosomes to shift-reading frame by 1 base in the 5′ direction that is utilized by many RNA viruses. Importantly, rates of −1 PRF are fine-tuned by viruses, including Retroviruses, Coronaviruses, Flavivriuses and in two endogenous viruses of the yeast *Saccharomyces cerevisiae*, to deliver the correct ratios of different viral proteins for efficient replication. Thus, −1 PRF presents a novel target for antiviral therapeutics. The underlying molecular mechanism of −1 PRF is conserved from yeast to mammals, enabling yeast to be used as a logical platform for high-throughput screens. Our understanding of the strengths and pitfalls of assays to monitor −1 PRF have evolved since the initial discovery of −1 PRF. These include controlling for the effects of drugs on protein expression and mRNA stability, as well as minimizing costs and the requirement for multiple processing steps. Here we describe the development of an automated yeast-based dual fluorescence assay of −1 PRF that provides a rapid, inexpensive automated pipeline to screen for compounds that alter rates of −1 PRF which will help to pave the way toward the discovery and development of novel antiviral therapeutics.

INTRODUCTION

Programmed −1 ribosomal frameshifting (−1 PRF) is a mechanism by which *cis*-acting elements in an mRNA can direct elongating ribosomes to shift reading frame by 1 base in the 5′ direction (1–3). Many RNA viruses utilize −1 PRF, including most Retroviruses, Coronaviruses, Flaviviruses and Totiviruses. In Retroviruses and Totiviruses, the open reading frame (ORF) encoding the major viral nucleocapsid proteins (e.g. Gag) is located at the 5′-end of the mRNA whereas the ORFs encoding proteins with enzymatic functions (typically Pro and Pol) are located 3′ of, and out of frame with, the Gag ORF. The mRNAs transcribed from these viral templates contain two overlapping ORFs. The enzymatic proteins are only translated as a result of a programmed ribosomal frameshift event that occurs with an efficiency of 1–40% depending on the specific virus and assay system employed (4). Thus, the majority of translational events result in the production of the Gag protein, while a minority of frameshifts yield viral enzymatic proteins. The ratio of Gag to Gag-pol synthesized in viruses as a consequence of programmed frameshifting varies between a narrow window of 20:1 to 60:1 (2). In Coronaviruses and Flaviviruses, frameshift events occur over a wider dynamic range to regulate the relative ratios of non-structural proteins (5–7).

The importance of maintaining precise ratios of viral proteins on virus propagation has been demonstrated in Retroviruses, Coronaviruses, Flavivriuses and in two endogenous viruses of the yeast *Saccharomyces cerevisiae* (5–8). Starting with the yeast 'killer' virus, we have extensively documented that small alterations in programmed frameshifting efficiencies promote rapid loss of the yeast dsRNA L-A 'killer' virus (9–20). In L-A, Gag-pol dimerization nucleates formation of the virus particle (21–24). Increasing the amount of Gag-pol protein synthesized may cause too many particles to initiate non-productively while producing too little may prevent efficient dimerization (9).

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Similarly, increasing or decreasing the efficiency of the +1 ribosomal frameshift in the Ty*1* retrotransposable element of yeast results in reduced retrotransposition frequencies (11,17,25–30). Proteolytic processing of the TyA-TyB (Gag-pol equivalent) polyprotein of Ty*1* is analogous to Gag-pol processing in retroviruses. In Ty*1*, increasing the amount of Gag-pol protein synthesized inhibited proteolytic processing of the polyprotein (25). As a consequence, formation of the mature forms of RNase H, integrase and reverse transcriptase is blocked (25). Similarly, changing the ratio of Gag to Gag-pol proteins in retroviruses like HIV or Moloney Murine Leukemia Virus interferes with virus-particle formation (31–36). In these cases, overexpression of the Gag-pol protein results in inefficient processing of the polyprotein and inhibition of virus production. Although the products produced by −1 PRF are different in Coronaviruses and Flaviviruses, the consequences of changes in −1 PRF efficiency are similar: in Coronaviruses, small changes in −1 PRF negatively impact on production of infectious virus (7), while in the Japanese encephalitis flavivirus, changes in −1 PRF are important for neuroinvasiveness (5). In sum, viral PRF efficiencies have been finely tuned to deliver the precise ratios of proteins required for efficient viral particle assembly: too much or too little frameshifting alters this ratio, with detrimental consequences.

Based on these studies, it has been proposed that −1 PRF presents a novel target for prevention of viral propagation (8). To this end, development of simple, cost effective assay systems would hasten identification of therapeutic agents that target this molecular mechanism. A number of quantitative assays have been employed to monitor −1 PRF. Cell free *in vitro* translation assays have the advantage of directly monitoring product formation, providing accurate readouts of the amounts and molecular weights of frameshift products. However, direct product monitoring presents numerous roadblocks to high-throughput screening (HTS) applications including the requirement for *in vitro* translation systems and radioactive labeling, the relatively small amount of products and the requirement for multiple secondary processing steps including SDS-PAGE, autoradiography, and/or immunoprecipitation. Mass spectrophotometric methods, while highly accurate, are also problematic for the same reasons. Enzymatic assays have been the preferred route, as they can be performed in either live cells or cell extracts, they do not require direct monitoring of the frameshift products, and they do not require use of radioactive materials or extensive handling. The first such assays employing monocistronic reporter systems were confounded by effects on reporter mRNA stability, producing false positive results; this was corrected by development of dual-reporter constructs which internally control for effects on mRNA stability and differences in rates of transcription (37–39). These second generation systems utilize *lacZ* and/or luciferases as reporter enzymes, which poses two drawbacks with regard to their utility in high-throughput screens: they require numerous manipulation steps, and they are not cost-effective. More recently, a mammalian cell *in vivo* dual-fluorescent protein-based system was described that enables rapid and accurate screening of compounds without the requirement for labor-intensive manipulations and expensive reagents (40). However the costs associated with producing and maintaining reporter cells using this system remain prohibitive. Here, we describe the development of a yeast-based dual-fluorescence system that can accurately detect even very slight changes in −1 PRF caused by mutations and drugs. This single plate assay is rapid, inexpensive and amenable to HTS applications.

MATERIALS AND METHODS

Yeast strains and plasmids

JD932 (*MATa ade 2-1 trp1-1 ura3-1 leu2-3*, *112 his3-11*, *15 can1-100*) was used for anisomycin studies. The wild-type L11 yeast strain was JD1381 (*MATα ura3-52 leu2Δ1 trp1Δ63 his3Δ200 rpl11a::HIS3 rpl11b::HIS3* + YCpRPL11B-TRP1). Isogenic *rpl11* mutant strains were based on JD1381, but contained low copy *TRP1-CEN6*-based plasmids expressing mutant versions of ribosomal protein L11. These were: JD1382 (*rpl11*-Y52Δ), and JD1433 (*rpl11*-92-6A). All dual fluorescence plasmids used in this study were based on pRS426 (41), a high copy 2μ, *URA3* vector. Transcription was directed from a yeast G6PD promoter into the monomeric Ds-Red ORF followed by the EGFP ORF reporter mRNAs. In pJD0980, the Ds-Red ORF was followed by the yeast L-A virus derived −1 PRF signal, which was in turn followed by the EGFP ORF, which was in the −1 reading frame relative to Ds-Red, so that synthesis of EGFP protein was contingent upon a −1 PRF event. pJD0981 was the same as pJD0980 except that one nucleotide was inserted immediately 5′ of the L-A −1 PRF signal, inactivating the −1 PRF signal and placing the EGFP ORF in the same frame as Ds-Red: this was used as the 0-frame control). Similarly, pJD1197 harbored the HIV-1 M type -1 PRF signal with EGFP in the −1 frame, while and pJD1198 was used as the HIV-1 M type 0-frame control. pJD0395 (a.k.a. pRS426) was used as the empty vector control.

Dual fluorescence assay

Yeast cells were transformed with empty vector plasmid (vector without fluorescence protein-coding sequences or frameshifting signal), control reporter plasmid (0-frame) and test reporter plasmid (−1 frame). Transformants were selected for on H-Ura plates for 3 days at 30°C. Colonies were transferred to 2 ml of liquid H-Ura medium and grown overnight in a 30°C shaker. Cultures were diluted 50-fold in H-Ura medium with or without 60 μg/ml anisomycin, mixed and aliquots of 150 μl were placed in 96-well plates. In the assays presented here, each reporter was represented by six replicates per plate per strain. The outer wells of each plate did not contain samples, but were filled instead with 150 μl of water to evenly account for evaporation and condensation rates among sample wells. Plates were covered with lids, sealed with parafilm and incubated in a Synergy HT microplate reader at 30°C for 46 h without shaking. Cell density was monitored by absorbance at 595 nm every 20 min over a period of 46 h.

Simultaneously, green (excitation 485 nm, emission 530 nm) and red (excitation 550 nm, emission 640 nm) fluorescence readings were taken.

−1 PRF (FS%) was calculated as follows:

$$G^* = \frac{G}{\mathrm{OD}} - \mathrm{Average}\left(\frac{G_{\mathrm{ev}}}{\mathrm{OD}_{\mathrm{ev}}}\right)$$

$$R^* = \frac{R}{\mathrm{OD}} - \mathrm{Average}\left(\frac{R_{\mathrm{ev}}}{\mathrm{OD}_{\mathrm{ev}}}\right)$$

$$\mathrm{FS}\% = \frac{\mathrm{Average}\left(\frac{G^*}{R^*}\right)_{\mathrm{Test}}}{\mathrm{Average}\left(\frac{G^*}{R^*}\right)_{\mathrm{Control}}} \times 100\%$$

where G is the green fluorescence for yeast transformed with test or control vector; G_{ev} is green fluorescence for yeast transformed with empty vector; R is red fluorescence for yeast transformed with test or control vector; R_{ev} is red fluorescence for yeast transformed with empty vector; OD is the optical density of cells transformed with reporter plasmid, and OD_{ev} is optical density of cells transformed with empty vector.

Dual luciferase assays

Yeast were transformed with the *Renilla*-firefly dual luciferase control and test plasmids: pJD0419 (L-A 0 frame control), pJD420 (L-A −1 PRF tester), pJD0375 (HIV 0 frame control) and pJD0378 (HIV −1 PRF tester) (39). Yeast were plated onto selective media for 3 days and then transferred to 4 ml of selective media with or without 60 μg/ml anisomycin. Cells were incubated overnight in a 30°C shaker till mid-logarithmic growth at an OD_{595} of 0.6–1.5. Yeast were washed three times and lysed with PBS plus 1 mM PMSF using 0.5 mm glass beads and a bead beater followed by centrifugation to clarify the lysate. Each strain was measured seven times for each reporter by sequentially mixing 10 μl of lysate with 50 μl of Promega's LARII reagent followed by 50 μl of Stop and Glo dual luciferase reagent, quantifying each step using a 96-well plate-reading Turner BioSystems Modulus microplate luminometer. Assays of −1 PRF and statistical analyses were performed as previously described (42).

RESULTS

A simple and inexpensive assay of −1 PRF in yeast

While the dual luciferase reporters are excellent for monitoring −1 PRF in small numbers of samples, there are three reasons why it is not particularly amenable for HTS applications. The first is cost: each sample costs approximately $2 ($1 each for test and control). Second, it is labor and time intensive: cells have to be lysed and cleared, lysates have to be spectrophotometrically read and normalized for protein content and two sets of reagents have to be sequentially introduced into lysates and read. With these limitations in mind, we developed a dual-fluorescent −1 PRF assay system for use in live yeast cells. A control reporter, in which monomeric Ds-Red and EGFP are in frame, provides baseline data (Figure 1A). In the frameshift reporter plasmid, EGFP production is dependent on

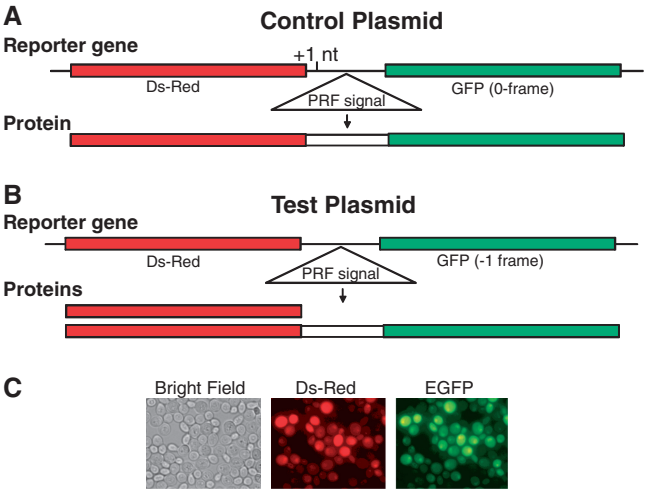

Figure 1. Dual-fluorescence reporter system. (**A**) Read-through control plasmid contains Ds-Red and EGFP ORFs. Note that insertion of one nucleotide 5′ of the −1 PRF signal inactivates frameshifting, and places the EGFP ORF in frame with the Ds-Red ORF to produce the Ds-Red/EGFP fusion protein. (**B**) −1 PRF test plasmid is the same as the control plasmid except that the EGFP ORF is in the −1 frame with respect to the Ds-Red ORF. No frameshift results in production of Ds-Red only, while a frameshift event results in production of the Ds-Red/EGFP fusion protein. (**C**) Microscopic images of cells transformed with the control plasmid.

−1 PRF, while Ds-Red serves as the internal control (Figure 1B). Figure 1C shows that high levels of Ds-Red and EGFP can be expressed using these vectors.

Figure 2A shows a timecourse experiment monitoring HIV-1 promoted −1 PRF monitored every 20 min for a total of 46 h in the presence (pink traces) or absence (blue traces) of 60 μg/ml anisomycin, a drug that inhibits −1 PRF (13). Note that the signals were extremely noisy during the first ~12 h. This is likely due to a combination of two factors: low cell densities and the time-dependent requirement for the two reporter proteins to mature, especially in the low-oxygen environment of this assay. As the proteins matured and densities increased, frameshift efficiencies began to become discernable, tending to stabilize at ~30 h. At the 46-h endpoint, HIV-1 promoted −1 PRF efficiency in untreated cells leveled off at ~13%, a value that is comparable to −1 PRF measured using the dual luciferase reporter in yeast cells (39). Notably, this assay was also able to recapitulate the inhibitory effect of anisomycin, which promoted ~8% −1 PRF at the 46-h timepoint. Figure 2B shows a plot of the effects of anisomycin on −1 PRF as fold wild-type levels, demonstrating that this drug inhibits −1 PRF by ~40%, consistent with previous findings (13).

Next, we tested the ability of this system to identify changes in −1 PRF encoded by the yeast L-A virus −1 PRF signal due to either the presence of 60 μg/ml anisomycin or consequent to cellular mutations known to affect −1 PRF. Specifically, anisomycin and the yeast *rpl11*-92-96A mutant were expected to promote decreased rates of −1 PRF, while the *rpl11*-Y52Δ mutant was expected to promote increased −1 PRF (43, M.H.J. Rhodin and J.D. Dinman, unpublished data). Figure 3A shows a

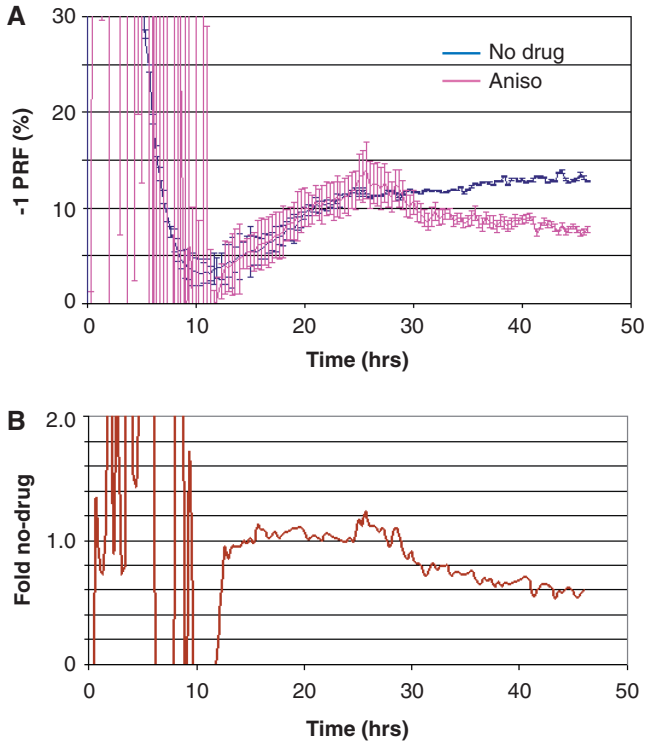

Figure 2. (**A**) Time course assay of HIV-1-promoted −1 PRF in yeast cells in the presence or absence of 60 μg/ml anisomycin. Error bars indicate standard error. (**B**) The effect of 60 μg/ml anisomycin on HIV-1 −1 PRF determined as fold untreated cells.

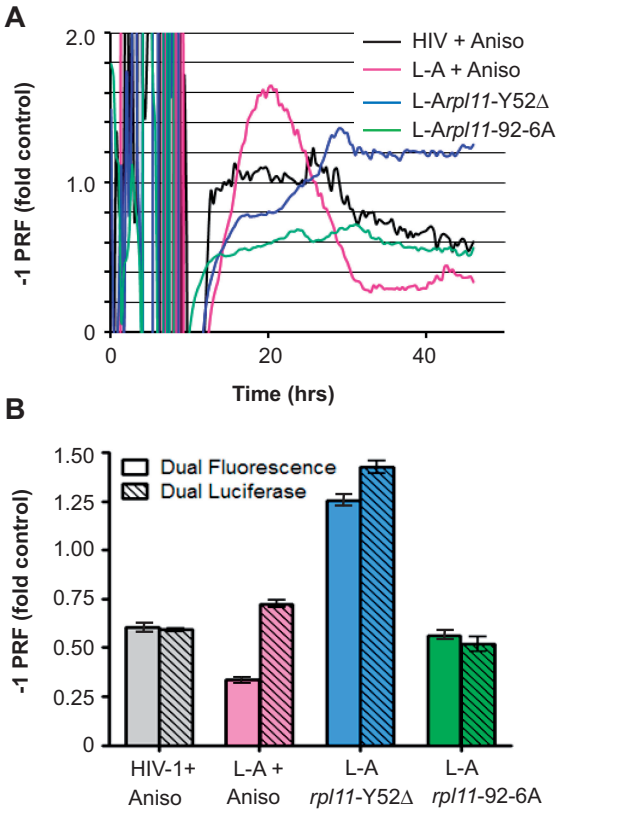

Figure 3. Effects of anisomycin and host cell mutants on HIV-1 and L-A promoted −1 PRF. (**A**) Time course assay. Wild-type cells harboring either HIV-1 or L-A −1 PRF reporters were treated with 60 μg/ml anisomycin. In parallel, isogenic *rpl11* mutant strains were assayed using the L-A −1 PRF reporter. Data are normalized to no-drug or isogenic wild-type *RPL11* cells. (**B**) Comparison of data obtained using dual fluorescence or dual luciferase reporters. Solid colored bars indicate 46-h endpoint measurements of −1 PRF expressed as fold wild-type or untreated cells. Striped bars represent fold wild-type results using dual luciferase reporter system for same cell types and testing conditions. Error bars indicate standard error.

plot of −1 PRF (as fold wild-type) monitored every 20 min for 46 h. Again, the system was noisy for the first ∼12 h and began to stabilize at ∼30 h. Taking 46 h as the endpoint, Figure 3B shows that the system produced the expected results. Specifically, anisomycin inhibited both HIV-1 (data duplicated from Figure 2B) and L-A-mediated −1 PRF. Further, L-A-mediated −1 PRF was decreased in the *rpl11*-92-96A mutant, and it was increased in the *rpl11*-Y52Δ strain.

The dual-fluorescent reporter system recapitulates data obtained using a dual-luciferase reporter system

The experiments were repeated using our previously described dual luciferase assay system (39) to compare the results obtained using the dual fluorescent reporter system. Side by side comparisons shown in Figure 3B demonstrate strong agreement between the two assay systems. These results match our previous findings for these conditions where 60 μg/ml anisomycin inhibited −1 PRF, *rpl11*-Y52Δ promoted increased −1 PRF and *rpl11*-92-6A promoted decreased −1 PRF. This independent validation underscores the potential for the less expensive and scalable dual fluorescence methodology to accurately detect changes in frameshifting.

DISCUSSION

The findings described here demonstrate the production of a robust and inexpensive assay that faithfully reproduces changes in −1 PRF due to cellular mutations and addition of drugs. An advantage to using the multiple well format is that it enables the data collected by the reader to be directly transferred into an excel spreadsheet, where −1 PRF rates are automatically calculated (42). Importantly, this software also determines standard errors, and flags samples with statistically significant *Z*-scores, rendering it ideal for HTS applications. Features inherent in the calculation of −1 PRF efficiency using the yeast-based *in vivo* dual-fluorescence assay also have two critical built in advantages with respect to counter screening. First, since the assay requires normalization of cell density by measuring OD_{595}, this parameter can be used to monitor the effects of compounds on cell growth, that is, to flag potential cell toxicity. Second, since it requires normalization to an in-frame control, these measurements can be used to identify deleterious effects on overall protein synthesis.

While we have only monitored −1 PRF from the L-A and HIV-1 frameshift signals, the modular nature of the reporter vectors renders insertion of −1 PRF signals from other human pathogenic viruses e.g. HTLV-I (Retrovirus

family), SARS-CoV (Coronavirus family), the Semliki Forest virus (Alphavirus family) and the NS2A-coding sequences of Flaviruses such as Japanese encephalitis virus, Eastern equine encephalitis virus and West Nile virus quite simple. As we and others have empirically determined that even very small changes in −1 PRF have strongly deleterious effects on virus propagation [reviewed in (8)], we suggest that initial cut-offs be set at >1.2-fold and <0.8-fold of wild type. These liberal cut-off values allow for a higher rate of false positives rather than false negatives, thus allowing false positives to be identified later with secondary assays. In addition, this basic format should be amenable for assays of other translational recoding mechanisms, for example, +1 PRF and termination codon suppression.

Once candidates are identified using this platform, secondary assays will be required to validate hits. Here we suggest two such assays. (i) Maintenance of the yeast 'killer' virus. We have shown that maintenance of this virus is critically dependent on −1 PRF, and have extensively used this property to validate changes in −1 PRF due to cellular mutations and drugs (8,44). Importantly, this assay provides an independent means to screen for antiviral effects of compounds that can be correlated with effects due to changes in −1 PRF. The killer assay involves replica-plating test cells onto a lawn of uninfected indicator yeast cells seeded onto 4.7 MB plates (yeast rich medium containing 0.5% methylene blue, pH 4.7). Cells are grown at 20°C for 4 days, and then visually inspected for the presence of a zone of growth inhibition due to production of the virus-encoded killer toxin. These assays are very simple and inexpensive, requiring only yeast cells, media and petri dishes. Currently, the dual fluorescence assay can screen up to 20 samples per 96-well plate if forgoing multiple replicates. Assuming initial identification of 500 compounds, these assays could be performed on as few as 25 plates when controls are included. The killer assay can be either performed manually (in the case of a few hits), or can be scaled up for high-throughput robotic screening. We believe that robotic technology currently used for high-throughput yeast 2-hybrid screens can be adapted to the killer assay. A potential disadvantage of this assay is that it may require serial passage of yeast cells to multiple rounds of drug exposure. For example, we have found that curing of killer by anisomycin requires at least two serial passages in the presence of this drug. Loss of the killer virus is further validated by extracting total RNA from cells, followed by non-denaturing 1.2% agarose gel electrophoresis, which detects the presence or absence of the viral dsRNA genome. (ii) Dual luciferase assays. In order to validate potential hits, −1 PRF values can be determined using both *in vivo* (live yeast cells and in HeLa cells), *in vitro* (rabbit reticulocyte lysates) dual-luciferase assays as previously described (39,45). These can be used to confirm the −1 PRF data obtained using dual-fluorescence reporters. The dual-luminescence assay as a secondary assay is also advantageous in that it enables identification of compounds that truly affect −1 PRF, as opposed to those that affect production or activity of the Ds-Red or EGFP reporter proteins. A further advantage of the *in vitro* assay is that it can identify compounds that directly affect −1 PRF as opposed to compounds that affect transcription of the reporter mRNAs.

There are a number of parameters that remain to be refined. One is automation, specifically plate seeding. In an industrial setting, this can easily be addressed using a robotic pipettor. Robotic pipetting can also solve the problem of interference of OD and fluorescence readings due to the presence of condensation on the plate lid at the end of the incubation period. Robotic transfer of cells to new plates just prior to reading will solve this problem. Data integrity is another problem that can be addressed by automation coupled with plate bar-coding, which will also assure that the worklist downloaded to the robot has 100% integrity. The data also have to be organized into a query-able format. However, the software that we developed to flag statistically significant *Z*-scores address this issue, and can also address the issue of operator bias. Process bottlenecks are also a common problem with HTS. In this case, the major bottleneck is the time required for maturation of the reporter proteins, hence the decision to take the 46 h endpoint. However, growth conditions may be further optimizable by incubating in a more humid and oxygenated environment, potentially reducing the time required for signal stabilization. Additionally, parallel processing of multiple plates can speed this process, rendering this bottleneck insignificant. For HTS, each plate must contain internal controls. We suggest that *rpl11*-Y52Δ mutant cells and *rpl11*-92-6A mutant cells or a sample containing anisomycin can be used as quality control for up- and down-frameshifting, respectively. Pooling is also commonly used in HTS. Although the idea is attractive, our experience is that this leads to mistakes, and we would recommend against this approach. Cell wall permeability is a common issue associated with yeast-based HTS because yeast cell walls are impermeable to many small molecules. However, yeast cells easily tolerate DMSO to concentrations of up to 0.8%. Thus, this reagent could be used to rectify this potential problem. Finally, most HTS technologies utilize 384 or 1536 well plates, and thus there are questions about ability to scale up this assay. However, there is *a priori* no reason for why scaling up to more wells will not work, although it is possible that scaling-up to higher density formats may present issues with oxygen transfer needed to support cell growth for this *in vivo* assay. Assuming this oxygen limitation is a problem, the incubator could easily be designed to increase the ambient oxygen content, thus mitigating any such problem.

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Conflict of interest statement. Preliminary disclosure documents have been filed with the University of Maryland and University of Delaware in pursuit of a patent application for this assay system.

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