Technical Report

Transcriptional regulation improves the throughput of three-hybrid counter selections in *Saccharomyces cerevisiae*

Marie D. Harton, Laura M. Wingler, and Virginia W. Cornish

Department of Chemistry, Columbia University, New York, NY, USA

The yeast three-hybrid (Y3H) assay expands the fields of drug discovery and protein engineering by enabling the search of large variant libraries for targets that do not inherently produce a distinct, measurable phenotype. The Y3H assay links the DNA-binding and activation domains of a transcription factor via a chemically synthesized heterodimeric small molecule, thereby activating a downstream reporter gene. Although the Y3H assay has been successfully applied as a positive selection to discover novel drug targets and to evolve proteins with improved functions, its expansion into applications requiring a high-throughput, versatile selection against transcriptional activation has been hindered by its limited dynamic range as a counter selection. Here, we describe the development of a second-generation Y3H counter selection that uses the dual tetracycline (Tet) system to tighten transcriptional regulation of the reporter gene. The Tet Y3H counter selection has an improved dynamic range and provides enrichment from mock libraries of up to 10^6, a 10^4-fold improvement over our original Y3H counter selection. This enhanced dynamic range brings the Y3H counter selection to a standard that is suitable for real-world protein engineering applications.

Received	31 MAY 2013
Revised	26 AUG 2013
Accepted	02 OCT 2013
Accepted article online	08 OCT 2013

Supporting information available online

Keywords: Dual tetracycline system · High-throughput · In vivo assay · Synthetic biology · Yeast three-hybrid assay

1 Introduction

The yeast three-hybrid (Y3H) assay brings small-molecule chemistry to the powerful *n*-hybrid genetic assay, which links biomolecular interactions to the reconstitution of a transcriptional activator that drives a reporter gene. The Y3H assay has been used to identify the protein targets of small-molecule drugs [1, 2], evolve protein receptors for small molecules [3], and as the basis for high-throughput assays to detect enzyme catalysis in vivo [4, 5]. Because it can be linked to growth selections, the Y3H assay allows the search of large libraries on the order of 10^6 variants or more, which is not feasible with medium-throughput screens that must explicitly analyze every variant (e.g. microtiter plate-based screens). Advances in protein engineering have shown that fully exploring these large variant libraries is critical to discovering proteins with dramatically improved or altered functions [6–9]. In addition, the Y3H assay is modular and can be readily applied to diverse targets that do not inherently produce conveniently selectable phenotypes. In our laboratory, we extended the Y3H assay to develop "Chemical Complementation" [10], a reaction-independent assay that links enzyme catalysis to the transcription of a reporter gene. We have successfully used Chemical Complementation for in vivo directed evolution experiments aimed to evolve bond-forming and bond-cleaving enzymes with improved function and altered substrate specificities [11–13].

Our laboratory's Y3H assay uses the chemical dimerizer dexamethasone-methotrexate (Dex-Mtx) to bridge the DNA-binding and activation domains of a transcription factor to activate a downstream reporter gene [14].

Correspondence: Prof. Virginia Cornish, Department of Chemistry, Columbia University, 550 W. 120th St., Room 1209, New York, NY 10027, USA
E-mail: vc114@columbia.edu

Current address: Laura M. Wingler, Duke University Medical Center, Durham, NC, USA

Abbreviations: AD, activation domain; **DBD,** DNA-binding domain; **Dex-Mtx,** dexamethasone-methotrexate; **DHFR,** dihydrofolate reductase; **Dox,** doxycycline; **5-FOA,** 5-fluoroorotic acid; **GR,** glucocorticoid receptor; **LexAop,** LexA operators; **Y3H,** yeast three-hybrid; **Tet,** tetracycline

The LexA DNA-binding domain (DBD) and B42 activation domain (AD) are expressed as fusion proteins with dihydrofolate reductase (DHFR) and the glucocorticoid receptor (GR), respectively. Dex-Mtx links the LexA DBD-DHFR and B42 AD-GR fusion proteins to activate the transcription of an auxotrophic reporter gene. We have reported both positive and counter Y3H selections based on the Brent "Interaction Trap" [15] and the Boeke "reverse" [16] yeast two-hybrid systems, respectively.

Our Y3H counter selection is derived from the "gold standard" counter selection in yeast genetics that uses the *URA3* reporter gene. The *URA3* reporter gene can be applied in counter selections because it encodes orotidine 5′-phosphate decarboxylase, which converts 5-fluoroorotic acid (5-FOA) into the toxic 5-fluorouracil compound that inhibits cell growth. We integrated the *URA3* reporter gene into our Y3H assay to select for the disruption of chemical dimerizer-mediated activation. In the Y3H *URA3* counter selection, Dex-Mtx links the LexA DBD-DHFR and B42 AD-GR fusion proteins to inhibit cell growth in the presence of 5-FOA. Based on the reverse yeast two-hybrid system, we constructed our Y3H system to have the *URA3* reporter gene under the control of the tightly regulated LexAop-*pSPO13* promoter to reduce basal transcription of *URA3* and thus maintain the chemical dimerizer dependence of the assay.

Despite using components from the well-established reverse yeast two-hybrid system to create our Y3H counter selection, we observed that it had a low dynamic range. The dynamic range of the Y3H counter selection is assessed using mock selections that test its ability to enrich a single inactive Y3H cell that is incapable of reconstituting the transcriptional activator with the chemical dimerizer, Dex-Mtx, from a large excess of undesirable active Y3H cells. In these mock selections, which are designed to mimic our counter selections for directed evolution experiments, a library of cells is grown in liquid culture and cells with disrupted Y3H systems that cannot reconstitute the transcriptional activator are enriched. Following the selection, the enriched cells are plated and individually assayed. We discovered that our original Y3H counter selection was only effective when enriching the desired inactive Y3H cells from small mock libraries with only 10^2 variants, a dynamic range that is not ideal for demanding directed evolution experiments. Even with the extensive optimization of conditions and screening of numerous strains, our adapted Y3H *URA3* counter selection did not show significant growth inhibition when cells were treated with the chemical dimerizer. Given our difficulties with the *URA3* reporter gene, we attempted to improve the dynamic range of our Y3H counter selection using endogenous yeast genes known to inhibit growth as novel counter selection reporters [17]. However, we encountered the same low dynamic range.

Thus, we undertook the design and construction of an improved Y3H counter selection. First, we characterized our original Y3H *URA3* counter selection to develop a hypothesis as to why the system had a low dynamic range. Based on this hypothesis, we then designed a small, focused library of reporter gene constructs that added another layer of transcriptional regulation for the *URA3* reporter gene with the dual tetracycline (Tet) system. The Tet Y3H reporter gene constructs were screened and a superior Tet Y3H construct with a significant growth difference in the presence and absence of Dex-Mtx was isolated and further characterized by mock selections.

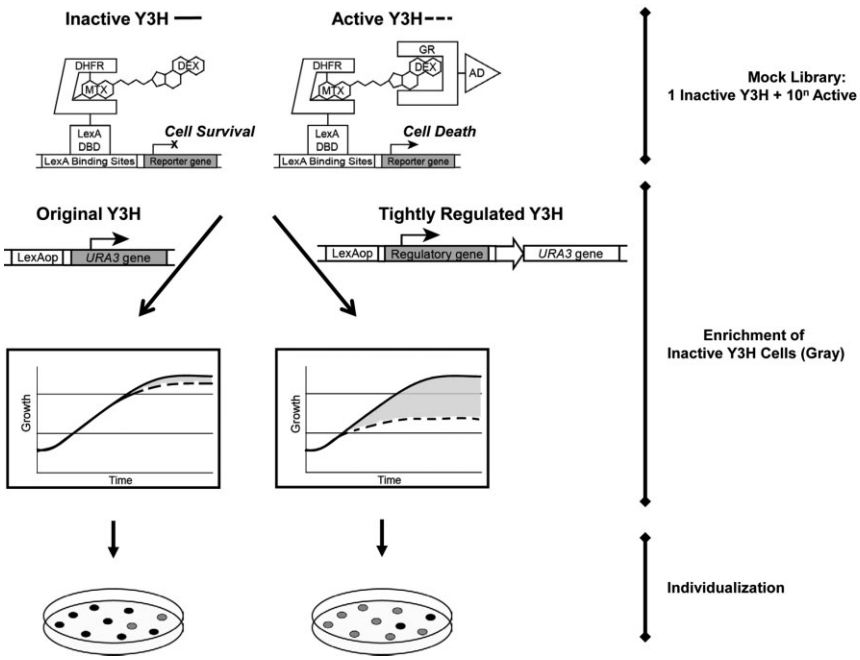

Figure 1. Improving the throughput of the Y3H assay with tighter transcriptional regulation of the reporter gene. The Y3H assay links the LexA DBD-DHFR and B42 AD-GR fusion proteins via the chemical dimerizer, Dex-Mtx, to reconstitute a transcriptional activator for a downstream reporter gene. We hypothesized that with tighter transcriptional control of the reporter gene we could increase the growth difference between cells treated with or without Dex-Mtx and therefore improve the throughput, or dynamic range, of the counter selection. The dynamic range of the counter selection is assessed using mock selections that test its ability to enrich 1 inactive Y3H cell from 10^n active Y3H cells. The selection includes mixing a mock library of inactive and active Y3H cells, enriching the desired inactive Y3H cells (gray) for 5 days, and plating the surviving cells for individualization.

2 Materials and methods

2.1 Growth assay for the tetracycline Y3H strains

Five colonies of each of the 16 Tet Y3H strains were inoculated into SC(HTL$^-$), 2% glucose media and shaken overnight at 30°C. Cells were pre-induced for 24 h in SC(HTL$^-$), 2% galactose, 2% raffinose media, and 2 μg/mL of doxycycline (Dox) was added if the strain contained the *tetR′*-Repressor. Pre-induced cells were added to SC(HTL$^-$), 2% galactose, 2% raffinose, 0.2%FOA, 2 μg/mL Dox, and ±5 μM Dex-Mtx media. Cells were shaken at 30°C and the OD_{600} was monitored. For the original Y3H growth curves, four colonies of the wild-type *URA3* (V2169Y), inactive *ura3-52* (FY251), active original Y3H (LW2635YActive), and inactive original Y3H (LW2635YInactive) strains were inoculated into SC(HT$^-$), 2% glucose media, and shaken overnight at 30°C. Growth curves were performed in SC(HT$^-$), 2% galactose, 2% raffinose, and 0.2%FOA media. We added 5 μM Dex-Mtx for the Y3H strains only. All growth curves were performed in duplicate.

2.2 Mock selection with the isolated Tet Y3H strain

Two colonies of the active Tet Y3H (MH24503_098) and inactive Tet Y3H (MH24503_082) strains were inoculated into 5 and 1 mL of SC(HTL$^-$), 2% glucose media, respectively and grown overnight at 30°C. Cells were pre-induced in SC(HTL$^-$), 2% galactose, 2% raffinose, and 2 μg/mL Dox media for 24 h. Pre-induced cells were harvested (5 min, 2000 rpm) and washed once with sterile water. Cells were resuspended in 500 μL of SC(HTL$^-$), 2% galactose, and 2% raffinose media. Based on the OD_{600}, cells were mixed to give initial ratios of $1{:}10^2$, $1{:}10^3$, $1{:}10^4$, $1{:}10^5$, and $1{:}10^6$ inactive Y3H to active Y3H cells in 800 μL of SC(HTL$^-$), 2% galactose, 2% raffinose, 0.2%FOA, 2 μg/mL Dox, and 5 μM Dex-Mtx media. Initial OD_{600} was equal to 1. Selections were shaken at 30°C and performed in triplicate. For reseeding, the selections were diluted to an OD_{600} of 0.1 two separate times. First, selections were allowed to grow for about 24 h to an OD_{600} of 3.0 and reseeded to an OD_{600} of 0.1 into fresh selective media. These diluted cultures were allowed to grow for another 24 h to an OD_{600} of 0.3 and reseeded again to OD_{600} of 0.1 into fresh selective media. Following the second reseeding, the selections were shaken at 30°C for 3 days. Selections were plated on SC(HTL$^-$), 2% glucose plates on Days 0 and 5. After 2 days of growth, colonies were assayed for *lacZ* and *gusA* expression using the Magenta-Gal/X-Gluc Overlay assay. Blue (active Y3H) and pink (inactive Y3H) colonies were counted to determine the percentage of inactive Y3H cells. For the original Y3H counter selection, two colonies from the active Y3H (LW2637Y) and inactive Y3H (LW2636Y) strains were used. Cells were pre-induced for 24 h in SC(HT$^-$), 2% galactose, and 2% raffinose media. Mock selections were setup in SC(HT$^-$), 2% galactose, 2% raffinose, 0.2% 5-FOA, and 5 μM Dex-Mtx media. Selections were performed in duplicate.

3 Results

3.1 Characterization of the dynamic range of the original Y3H URA3 counter selection

We characterized our original Y3H counter selection to determine whether the *URA3* reporter gene was impaired when placed under the control of the Y3H system. The performance of the *URA3* counter selection was compared for *URA3* under the control of its endogenous promoter or the Y3H system. When regulated by its endogenous promoter, the *URA3* counter selection exhibited a significant growth difference between cells with a wild-type copy of *URA3* versus cells with the inactive allele *ura3-52*, which produces no functional Ura3p. In mock selections, the *URA3* counter selection enriched cells with the inactive *ura3*-52 allele from an excess of 10^5 cells containing the wild-type *URA3* (Supporting information, Fig. S1). Under analogous conditions, the Y3H *URA3* counter selection showed a negligible growth difference between active and inactive Y3H cells, which both grew to a saturated cell density and had indistinguishable growth rates. The lack of a growth difference led to the enrichment of the inactive Y3H cells in mock selections with only an excess of 10^2 active Y3H cells (Supporting information, Fig. S2).

The low dynamic range of the Y3H counter selection could have been caused by (1) excessive basal expression of *URA3* in the absence of Dex-Mtx or (2) insufficient activation of *URA3* through the tightly regulated LexAop-*pSPO13* promoter in the presence of Dex-Mtx. The active Y3H strain grew comparably to the inactive *ura3-52* strain in the absence of Dex-Mtx (Supporting information, Fig. S3), which is inconsistent with high basal *URA3* expression. If basal *URA3* expression was occurring, then the active Y3H cells would exhibit impaired growth in the absence of Dex-Mtx. Therefore, we inferred that the minimal growth difference observed between active Y3H cells treated with or without Dex-Mtx was caused by insufficient activation of *URA3* when controlled by the LexAop-*pSPO13* promoter. The tightly regulated LexAop-*pSPO13* promoter effectively reduced basal expression of *URA3*. However, it hindered chemical dimerizer-dependent activation and resulted in low *URA3* expression that caused minimal growth inhibition of the active Y3H cells treated with Dex-Mtx.

We hypothesized that the key to improving the dynamic range of our Y3H counter selection was to rationally redesign the Y3H *URA3* system to maximize the growth difference between cells with activated and basal levels of *URA3* expression (Fig. 1). Our first attempt was to increase activation of *URA3* by replacing the LexAop-

pSPO13 promoter with the stronger LexAop-*pGAL1* promoter, which has been used successfully in yeast n-hybrid positive selections [15, 18]. This promoter provided sufficient maximal activation of *URA3* to inhibit the growth of many clones. However, this behavior was not chemical dimerizer-dependent, which suggested that the basal expression of *URA3* was too high (data not shown). To obtain high activation of *URA3* while reducing its basal expression, we turned to the dual Tet system to provide an additional level of transcriptional regulation.

3.2 Tetracycline Y3H URA3 counter selection design

The dual Tet system enables the conditional expression of genes through a *tetO* minimal promoter that is controlled by both a Tet-regulated activator and repressor. The activator and repressor are fused to a *tetR* component that controls their recognition of *tetO* binding sites within the *tetO* minimal promoter and response to tetracycline [19, 20]. There are two types of Tet systems: the Tet-repressible (direct) and Tet-inducible (reverse) systems. In the Tet-repressible (direct) system, the *tetR*-Activator enables *tetO*-driven transcription in the absence of tetracycline. In the Tet-inducible (reverse) system, the activator is fused to a mutated *tetR* molecule (*tetR′*) that is activated rather than inactivated upon tetracycline binding. Thus, the activator allows *tetO*-driven gene transcription in the presence of tetracycline. To achieve tighter regulation, the dual Tet system typically includes an activator and repressor that respond conversely to the presence of tetracycline (e.g. the reverse Tet system includes the *tetR′*-Activator and *tetR*-Repressor) [19, 20].

To achieve tighter regulation of *URA3*, the Tet Y3H counter selection was designed to have the Y3H system control the *tetR′*-Activator, which, along with the constitutively expressed *tetR*-Repressor, directly provides an additional level of modulation through the *tetO* minimal promoter (Fig. 2A). In addition, variations of the Tet Y3H counter selection fused the repressor to either the *tetR* or mutated *tetR′* components to test two ways to potentially counteract basal *URA3* expression. In one case, the *tetR*-Repressor is functional in the absence of tetracycline, and basal expression of *URA3* is controlled prior to *tetR′*-Activator expression. Alternatively, the *tetR′*-Repressor competes with the *tetR′*-Activator for regulation of *URA3* in the presence of tetracycline, and basal expression of *URA3* is repressed until a threshold level of the *tetR′*-Activator is produced by Y3H activation.

To maximize our chances of finding a Tet Y3H system with precisely the right balance of activated and basal *URA3* expression, we constructed a library of 16 Tet Y3H *URA3* counter selection strains that vary in components of both the Y3H and dual Tet systems that are known to modulate expression of the reporter gene. The variations included: the identity of the *tetR*$^{(\prime)}$-Repressor's constitutive promoter (p*CMV* or p*ADH*), the directionality of the *tetR*-Repressor (*tetR′* or *tetR*), the number of LexA operator (LexAop) binding sites upstream of the *tetR′*-Activator, and the number of *tetO* binding sites within the minimal *tetO* promoter upstream of *URA3* (Fig. 2B).This small, focused library represents all possible combinations of the available variable components from both the Y3H and dual Tet systems.

3.3 Growth assay to screen the tetracycline Y3H strains

Multiple clones of the 16 Tet Y3H strains were tested. First, we eliminated strains with high basal expression of *URA3* without Dex-Mtx – that is, those showing growth inhibition without *tetR′*-Activator expression. The initial growth assays excluded Dex-Mtx and were performed in media that contained 5-FOA and Dox, the more effective member of the tetracycline antibiotic family [18]. We expected strains to grow to a saturated cell density in these conditions as the *tetR′*-Activator was not expressed and the constitutively expressed *tetR*$^{(\prime)}$-Repressor turned off transcription of the toxic *URA3* reporter gene product. With Dox and 5-FOA present in the media, 75% of the strains showed the expected growth patterns (Supporting information, Fig. S4).

The Tet Y3H strains that grew well in the absence of Dex-Mtx were tested for chemical dimerizer-dependent growth inhibition in media with Dox, 5-FOA, and Dex-Mtx (Supporting information, Fig. S5). With Dex-Mtx, the *tetR′*-Activator is expressed and growth inhibition is expected. Multiple clones of the strain with the *pADH-tetR′*-Repressor, 7*tetO-URA3*, and 2LexAop-*tetR′*-Activator constructs showed the largest growth difference between cells treated with or without Dex-Mtx. The isolated Tet Y3H (gray) counter selection strain, MHTet24503, showed a maximum OD_{600} ratio (–Dex-Mtx/+Dex-Mtx) of 7, which was an improvement over the maximum OD_{600} ratio of 2 for the original Y3H (black) counter selection (Fig. 3C).

3.4 Mock selection with the isolated tetracycline Y3H strain

The MHTet24503 strain was further characterized by a mock selection [17] that directly tested its ability to enrich inactive Tet Y3H cells with only the B42 AD from an excess of active Tet Y3H cells with the requisite B42 AD-GR fusion protein. The inactive Tet Y3H cells cannot reconstitute the transcriptional activator for the *URA3* reporter gene, and are thus enriched in the culture over time. Following effective selections, we expect the majority of plated cells that are individually assayed to contain inactive Y3H systems. To determine the dynamic range of the MHTet24503 strain, inactive and active Tet Y3H cells were mixed to give ratios of $1{:}10^2$, $1{:}10^3$,

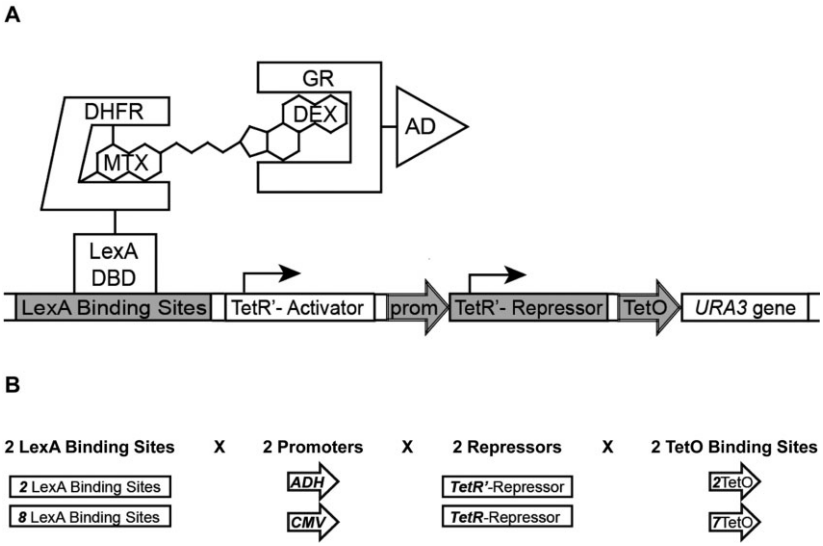

Figure 2. Sixteen variants of the Tet Y3H counter selection were screened for improved transcriptional regulation. (**A**) Tet Y3H counter selection design. The Y3H system directly controls the *tetR′*-Activator, which along with the constitutively expressed *tetR*-Repressor provides an additional level of regulation for *URA3*. (**B**) Because of the complexity of optimizing transcriptional regulation, a focused library of Tet Y3H strains was screened. The library consisted of: two or eight LexA operators (LexAop) placed upstream of the *tetR′*-Activator to vary the degree of Y3H activation; pADH or pCMV promoters to vary the expression levels of the Tet-Repressor; fusion of the Tet-Repressor to the *tetR* or *tetR′* components making it either repressed or activated by tetracycline, respectively; and finally two or seven *tetO* binding sites were placed upstream of *URA3* to enable varying levels of regulation. All 16 variants were constructed and screened (Supporting information, Figs. S4 and S5).

1:10^4, 1:10^5, and 1:10^6 with an initial OD_{600} of 1 and subjected to Y3H selection conditions with Dox, 5-FOA, and Dex-Mtx. To improve the Tet Y3H counter selection, the cell mixtures were "reseeded," or diluted into fresh selective media at lower cell densities. Reseeding enabled the inactive Tet Y3H cells to more effectively overtake the selection.

The Tet Y3H counter selection considerably outperformed our original Y3H counter selection. With reseeding conditions, the MHTet24503 strain (gray) enriched the inactive Y3H cells in populations as high as 10^6 after 5 days, while the original Y3H strain (black) was only capable of enrichment from an excess of 10^2 active Y3H cells under similar conditions (Fig. 3D). Based on separate control experiments, the enrichment of the inactive Y3H cells depended on the presence of Dox and Dex-Mtx (Supporting information, Fig. S6). With daily reseeding to lower cell densities (OD_{600} = 0.1) for the first 2 days, the percentage of the inactive Tet Y3H cells was improved by at least 28 ± 4% for the 10^4, 10^5, and 10^6 selections (Supporting information, Fig. S7).

4 Discussion

The dynamic range of the Y3H counter selection was considerably enhanced by increasing transcriptional regulation of the *URA3* reporter gene with the dual Tet system. Starting with our original Y3H system, there was little growth difference between cells treated with or without Dex-Mtx, and we could only enrich inactive Y3H cells from mock libraries with an excess of 10^2 active Y3H cells. With the improved Tet Y3H strain, a significant growth difference is observed and it can enrich the inactive Y3H cells from mock libraries with an excess of 10^6 active Y3H cells. This improvement should be impactful for many bioengineering applications where an increase in library size greatly improves the results.

With our Tet Y3H counter selection, the laborious effort to isolate desirable variants from large libraries with mostly unfit variants is eminently reduced. Because the undesirable variants do not survive the selection conditions, they are excluded from the time-intensive plate-based assays performed to individualize the variants and less time is wasted individually assaying unsatisfactory variants. Therefore, we envision that our Tet Y3H counter selection will be highly useful as a high-throughput assay to search large libraries and enrich desirable variants that can then be differentiated with specialized low-throughput screens for the function of interest. We plan to incorporate this improved Tet Y3H *URA3* counter selection into Chemical Complementation and apply it to the discovery of new bond-cleaving enzymes, particularly cellulases.

Critical to our success in discovering the appropriate Tet Y3H reporter gene construct were both characterization of the original Y3H strain and screening of a small focused library of Tet Y3H variants. This approach allowed us to screen for a Tet Y3H *URA3* construct with the precise activated and basal levels of *URA3* expression required to produce the proper growth phenotypes in our selections. Our best Tet Y3H strain, MHTet24503, included an arrangement of the variable components that was not apparent, such as more *tetO* boxes, fewer LexAop binding sites, and the non-standard combination of the Tet-inducible *tetR′*-Repressor and *tetR′*-Activator. By screening a small focused library, we discovered the most suitable Tet Y3H reporter gene construct and avoided the difficult optimization process often needed to appropriately adjust the transcriptional regulation of the reporter gene.

Not only is the improved Tet Y3H *URA3* counter selection useful for endeavors requiring a high-throughput

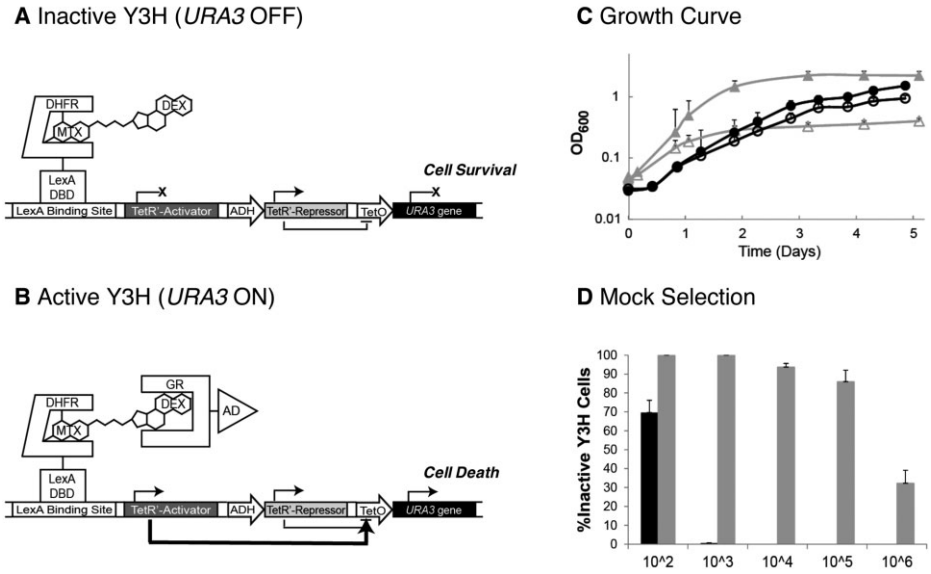

Figure 3. Characterization of the best Tet Y3H *URA3* counter selection strain (MH24503). (**A**) The inactive Tet Y3H strain does not contain the requisite B42-GR fusion protein and cannot activate the *tetR′*-Activator, thus the *tetR′*-Repressor represses *URA3* transcription and the cells survive. (**B**) The active Tet Y3H strain activates the *tetR′*-Activator upon binding to Dex-Mtx. The *tetR′*-Activator outcompetes the *tetR′*-Repressor and thus activates *URA3* transcription and the cells die in the presence of 5-FOA. (**C**) Growth curves for the Tet Y3H (▲) and Original Y3H (●) strains in selective media containing 2 μg/mL Dox, 0.2% 5-FOA, and 5 μM Dex-Mtx. The OD_{600} of five colonies was monitored in +Dex-Mtx (open symbols) and –Dex-Mtx (closed symbols) conditions. Shown is the mean growth and error bars represent the standard deviation from the mean. (**D**) Mock selection with active and inactive Tet Y3H strains in media containing 2 μg/mL Dox, 0.2% 5-FOA, and 5 μM Dex-Mtx. Performance of the Tet (■) and Original (■) Y3H counter selection with daily reseeding to an OD_{600} of 0.1 in the first 2 days of a 5-day selection. Error bars represent standard error in the percentage of inactive Y3H cells between duplicate selections performed in triplicate.

assay, but its development process should broadly inspire similar solutions in the field of synthetic biology. As the advance of bioengineering technology requires in vivo genetic circuits with sophisticated read-outs tailored to the application, our results emphasize the importance of pursuing solutions that do not solely rely on the intrinsic dynamic range of traditional auxotrophic markers. These markers have the appropriate dynamic range for common yeast genetic techniques, but when integrated into advanced in vivo genetic circuits, such as the Y3H system, they can sometimes provide a dynamic range that is too constricted. Thus, technologies based on these traditional auxotrophic reporter genes may not provide the proper regulation for all bioengineering applications. To improve and diversify these technologies, guided strategies based on a fusion of rational design and library approaches should be embraced. This method allows one to appropriately adjust the read-out of technologies based on traditional reporter genes and screen for a reporter gene construct with expression levels precisely fitted to refined in vivo applications.

This research was funded by NSF grant CHE 0957569 and NIH grant GM062867. M.D.H. is supported by the National Science Foundation (NSF) Integrative Graduate Education and Research Training (IGERT) grant 0801530. We are grateful to Z. Chen for the synthesis of Dexamethasone-Methotrexate. We also thank Dr. S. Billerbeck and A. Jobe for critical reading of the manuscript.

Conflict of interest: The authors declare the following competing financial interest(s): Columbia University is the assignee of U.S. Patent 7,419,780 on the Methotrexate Yeast 3-Hybrid Technology, which will be developed by HybrID Drug Discovery, LLC of La Jolla, California. V.W.C. will be the Chief Scientific Officer and a Member of HybrID Drug Discovery, LLC.

5 References

[1] Licitra, E. J., Liu, J. O., A three-hybrid system for detecting small ligand–protein receptor interactions. *Proc. Natl. Acad. Sci. USA* 1996, *93*, 12817–12821.

[2] Shepard, A. R., Cornrow, R. E, Pang, I. H., Jacobson, N. et al., Identification of PDE6D as a molecular target of anecortave acetate via a methotrexate-anchored yeast three-hybrid screen. *ACS Chem. Biol.* 2013, *8*, 549–558.

[3] Liberles, S. D., Diver, S. T., Austin, D. J., Schreiber, S. L., Inducible gene expression and protein translocation using nontoxic ligands identified by a mammalian three-hybrid screen. *Proc. Natl. Acad. Sci. USA* 1997, *94*, 7825–7830.

[4] Firestine, S. M., Salinas, F., Nixon, A. E., Baker, S. J. et al., Using an AraC-based three-hybrid system to detect biocatalysts in vivo. *Nat. Biotechnol.* 2000, *18*, 544–547.
[5] Clark, D. D., Peterson, B. R., Fluorescence-based cloning of a protein tyrosine kinase with a yeast tribrid system. *ChemBioChem* 2005, *6*, 1442–1448.
[6] Aharoni, A., Griffiths, A. D., Tawfik, D. S., High-throughput screens and selections of enzyme encoding genes. *Curr. Opin. Chem. Biol.* 2005, *9*, 210–216.
[7] Tao, H., Cornish, V. W., Milestones in directed enzyme evolution. *Curr. Opin. Chem. Biol.* 2002, *6,* 858–864.
[8] Wang, L., Brock, A., Herberich, B., Schultz, P. G., Expanding the genetic code of *Escherichia coli*. *Science.* 2001, *292*, 498–500.
[9] Yano, T., Oue, S., Kagamiyama, H., Directed evolution of an aspartate aminotransferase with new substrate specificities. *Proc. Natl. Acad. Sci. USA* 1998, *95*, 5511–5515.
[10] Baker, K., Bleczinski, C., Lin, H., Salazar-Jimenez, G. et al., Chemical complementation: A reaction-independent genetic assay for enzyme catalysis. *Proc. Natl. Acad. Sci. USA* 2002, *99*, 16537–16542.
[11] Lin, H., Tao, H., Cornish, V. W., Directed evolution of a glycosynthase via chemical complementation. *J. Am. Chem. Soc.* 2004, *126*, 15051–15059.
[12] Tao, H., Peralta-Yahya, P., Decatur, J., Cornish, V. W., Characterization of a new glycosynthase cloned by using chemical complementation. *ChemBioChem* 2008, *9*, 681–684.
[13] Peralta-Yahya, P., Carter, B. T., Lin, H., Tao, H. et al., High-throughput selection for cellulase catalysts using chemical complementation. *J. Am. Chem. Soc.* 2008, *130*, 17446–17452.
[14] Lin, H., Abida, W. M., Sauer, R. T., Cornish, V. W., Dexamethasone-methotrexate: An efficient chemical inducer of protein dimerization in vivo. *J. Am. Chem. Soc.* 2000, *122*, 4247–4248.
[15] Gyuris, J., Golemis, E., Chertkov, H., Brent, R., Cdi1, a human G1 and S phase protein phosphatase that associates with Cdk2. *Cell* 1993, *75*, 791–803.
[16] Vidal, M., Brachmann, R. K., Fattaey, A., Harlow, E. et al., Reverse two-hybrid and one-hybrid systems to detect dissociation of protein–protein and DNA–protein interactions. *Proc. Natl. Acad. Sci. USA* 1996, *93*, 10315–10320.
[17] Wingler, L. M., Cornish, V. W., A library approach for the discovery of customized yeast three-hybrid counter selections. *ChemBioChem* 2011, *12*, 715–717.
[18] Fields, S., Songs, O., A novel genetic system to detect protein–protein interactions. *Nature* 1989, *340*, 245–246.
[19] Bellí, G., Garí, E., Piedrafita, L., Aldea, M. et al., An activator/repressor dual system allows tight tetracycline-regulated gene expression in budding yeast. *Nucleic Acids Res* 1998, *26*, 942–947.
[20] Garí, E., Piedrafita, L., Aldea, M., Herrero, E., A set of vectors with a tetracycline-regulatable promoter system for modulated gene expression in *Saccharomyces cerevisiae*. *Yeast* 1997, *13*, 837–848.

Biotechnology Journal's latest Special Issue on "Biomolecular Engineering" is based peer-reviewed papers derived from some of the best presentations at the *4th International Conference on Biomolecular Engineering*. The cover is a snapshot of solvated HER4/ErbB4 kinase domain with juxtamembrane region; image provided by Ravi Radhakrishnan.

Biotechnology Journal – list of articles published in the December 2013 issue.