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A Library Approach for the Discovery of Customized Yeast Three-Hybrid Counter Selections

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The rise of synthetic biology is heightening the demand for means to effectively connect complex in vivo circuitry to a readily assayable cellular phenotype, such as cell growth.[1,2] To date, cell engineers have almost exclusively borrowed geneticists' standard reporter genes that confer antibiotic resistance or complement auxotrophies. Since these traditional reporter genes were historically employed as digital "on/off" markers for purposes such as plasmid maintenance or gene knockouts, they can fail to provide the desired readout when simply grafted into more sophisticated systems. Recent advances in genomics and systems biology have provided a glimpse of nature's extensive arsenal of genetic components and presented us with the opportunity to search this natural diversity for components that match the needs of individual systems. In this work, we demonstrate that we can readily develop a robust counter selection for the yeast three-hybrid (Y3H) assay by directly screening libraries of genes not traditionally used as reporters, rather than by adapting classic reporter genes.

Our laboratory's Y3H assay uses the chemical dimerizer dexamethasone–methotrexate (Dex–Mtx) to reconstitute a transcriptional activator from LexA–dihydrofolate reductase (DHFR) and B42–glucocorticoid receptor (GR) fusion proteins.[3] We recently modified our Y3H system to provide a growth selection against chemical dimerizer-activated transcription by using the classic yeast counter-selection reporter *URA3*.[4] The *URA3* counter selection,[5] in which the gene product orotidine 5′-phosphate decarboxylase converts 5-fluoroorotic acid (5-FOA) to the toxic compound 5-fluorouracil, is the most widely used of the limited number of established yeast counter selections. However, adapting the *URA3* counter selection to provide the desired growth phenotype in the Y3H system was nontrivial, requiring a multistep strain construction, extensive optimization of growth conditions, and the screening of numerous strains.[4]

One explanation of why we encountered difficulties in optimizing the "gold standard" of known yeast counter selections for the Y3H assay is that there are fundamental differences between typical counter-selection applications and the Y3H assay. The *URA3* and other existing counter selections have primarily been used for purposes such as curing cells of plasmids or knocking out genes, where the reporter gene is either "on" (and being expressed from its endogenous promoter) or "off" (and completely deleted from the cell). In the Y3H assay, however, the functional reporter gene is present in all cells, and the "on" and "off" states reflect activated and basal transcription of the gene, respectively. If the expression level at which the reporter gene begins to inhibit cell growth happens to fall outside this window, the counter selection will be unable to discern between activated and basal transcription, and low levels of basal transcription of the gene could inhibit cell growth even in the absence of the reconstituted transcription factor.

As it would be difficult to rationally redesign the *URA3* Y3H system to appropriately modulate the reporter gene's expression, we hypothesized that the most straightforward route to improve the Y3H counter selection would be to screen a library of novel candidate reporter genes in the context of the desired application and thereby empirically identify one whose threshold for toxicity corresponds to the expression levels achieved in our existing system. Since reporter expression is not activated in the absence of the chemical dimerizer, a conditionally lethal reporter (e.g., only toxic in the presence of a compound such as 5-FOA) is unnecessary. This broadens the pool of candidate counter-selection reporters greatly, as a wealth of genetics studies has identified numerous yeast genes that inhibit growth or affect the cell cycle when simply overexpressed.[6–11] Interestingly, in spite of the paucity of effective yeast counter selections, this information has seldom been exploited to develop new counter selections.[11–13] We thought that an endogenous yeast gene could be a particularly suitable Y3H counter-selection reporter, as the gene's mere presence in the genome implies that the cell is able to tolerate some basal level of expression. These considerations led us to draw a list of eleven potential candidate counter-selection reporter genes (Table S1 in the Supporting Information) from overexpression studies in *S. cerevisiae*. Since each previous study used different expression conditions, we focused on genes that had been identified in multiple screens to increase the likelihood that the gene product would be toxic under the specific conditions of our assay.

We then put together a system to quickly and efficiently evaluate candidate reporters by constructing libraries of reporters directly in a Y3H strain and screening for growth-inhibiting effects under Y3H counter-selection conditions (Figure 1 A). We elected to put the reporter genes on low-copy centromeric plasmids, thereby allowing us to use plasmid gap-repair techniques to generate large libraries of reporter constructs in vivo[14,15] while minimizing cell-to-cell variation in expression levels.[16] To coarsely adjust the levels of basal and activated reporter expression in the Y3H assay, we built a family of six parental plasmids containing LexA operator–promoter constructs that should provide varying expression. Two or eight LexA binding sites were placed upstream of three differ-

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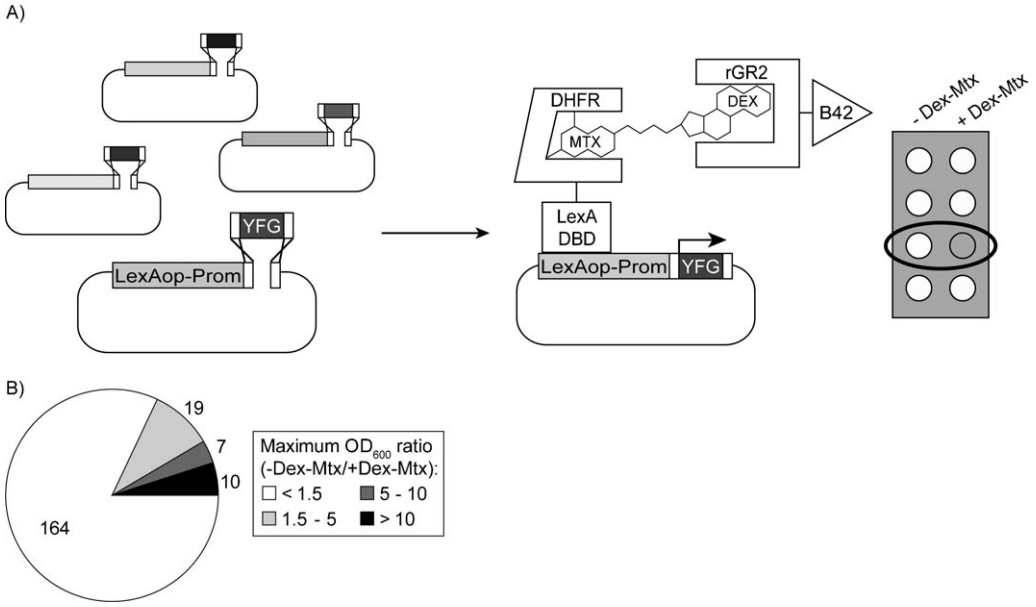

Figure 1. Screen for alternative reporters for the Y3H counter selection. A) Library construction and screening strategy. Yeast three-hybrid strains harboring a library of candidate reporters are constructed in vivo in one step by co-transforming pools of candidate reporter genes (dark gray) and plasmids containing various LexA operator–promoter constructs (light gray). Hundreds of transformants can then be screened in parallel for growth in the presence and absence of Dex–Mtx to identify strains whose growth is inhibited by the chemical dimerizer. B) Representation of 200 colonies' performance in the counter-selection reporter screen. Since colonies exhibited a variety of growth patterns, Dex–Mtx-dependent growth inhibition was scored by the maximum observed ratios of the cell densities (OD_{600}) of the −Dex–Mtx culture to the +Dex–Mtx culture for each colony, and the number of colonies that fell within each range is shown. Reporters from the ten best colonies (black) were further characterized.

ent parental promoters, p*KEX2*, p*CYC1*, and p*TEF1*, which have been shown to provide a range of gene expression of over three orders of magnitude.[17] Candidate reporter genes, PCR amplified with appropriate homology regions, can be readily inserted into these plasmids downstream of the promoter by homologous recombination.

We constructed our counter-selection reporter library by co-transforming the promoter library plasmids and the candidate reporter genes as a pool into our Y3H strain, thus conveniently generating 66 potential reporter plasmids in vivo by plasmid gap repair. With the exception of the reporter gene, we used a strain containing our previously reported Y3H framework, which has been optimized to ensure a consistent reporter readout.[18] To ensure full coverage of the reporter library, we picked 200 transformants and monitored their growth in the presence and absence of Dex–Mtx. As shown in Figure 1B, almost 20% of the colonies exhibited chemical dimerizer-dependent growth inhibition. Five unique constructs were identified from the ten best colonies (Table S2), and four of these continued to provide some degree of growth inhibition reproducibly upon retransformation into the Y3H strain (Figure S1).

The most promising reporter construct, 8LexAop-p*TEF1-GIS1*, was selected for further characterization. After re-transformation of the reporter plasmid into the Y3H strain, 26 colonies were individually assayed for growth in the presence and absence of Dex–Mtx (Figure 2A). Significantly, when using our unoptimized selection conditions, several *GIS1* reporter clones demonstrated growth inhibition superior to our extensively optimized *URA3* counter-selection strain[4] as tested under analogous conditions (Figure 2A).

Finally, we explicitly tested the ability of the *GIS1* reporter to provide enrichment in the Y3H counter selection by attempting to enrich an inactive three-hybrid strain that contained only the B42 activation domain from an excess of active three-hybrid strains that contained the requisite B42–GR fusion protein. To provide a convenient colorimetric assay for enrichment, we constructed *GIS1* counter-selection strains that bore either constitutively expressed *lacZ* or *gusA* on the plasmids containing the B42 constructs (Figure 2B). Cells were mixed to provide an initial mixture of 100:1 or 1000:1 active/inactive strains and subjected to Y3H selection conditions. After four days of growth, the inactive Y3H cells comprised the majority of the population for both selections (Figure 2C). Diluting the 1000:1 selection into fresh medium on the second day, or "seeding" the selection, was even more effective and allowed the inactive Y3H strain essentially to take over the culture.

In conclusion, a straightforward screen of only a small library of candidate genes yielded many alternative counter-selection reporter genes for the yeast three-hybrid assay. Furthermore, one of these proved to be as effective as the ubiquitous *URA3* counter selection in our system. Employing a library approach allowed us to define the conditions we wanted to use in our assay while still circumventing the laborious optimization process required to adapt the *URA3* reporter for the Y3H assay. As synthetic biologists endeavor to develop an arsenal of effective parts that will function in increasingly complex and diverse systems, our results underscore the importance of looking beyond the standard components historically used by geneticists, which were selected for their functionality in a different context. Rather, we should think broadly and creatively as we

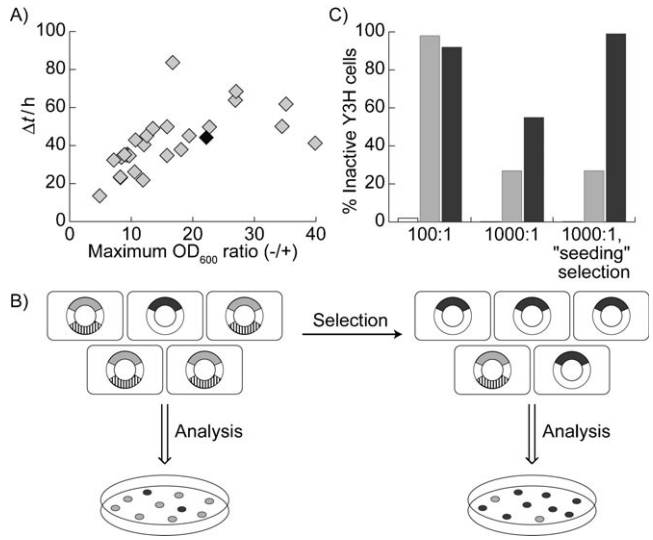

Figure 2. Characterization of 8LexAop-p*TEF1-GIS1* as a Y3H counter-selection reporter. A) Performance of 26 randomly selected Y3H colonies (◆) retransformed with the reporter plasmid in growth assays −/+1 μM Dex–Mtx. Two metrics were used to evaluate each colony's performance: the maximum observed ratio of the OD_{600} reading for the −Dex–Mtx culture and the +Dex–Mtx culture (maximum OD_{600} ratio (−/+)), and the difference in time required for the −Dex–Mtx and +Dex–Mtx cultures to reach an OD_{600} of 1 (Δt). The performance of our *URA3* Y3H counter-selection strain[4] in this assay is shown for comparison (◆). B) A colorimetric assay to easily monitor enrichment in the Y3H counter selection. Active Y3H cells have the necessary B42–GR fusion protein (striped) and constitutively express β-glucuronidase (*gusA*, light gray). Inactive Y3H cells have only the B42 domain, without the GR fusion, and constitutively express β-galactosidase (*lacZ*, dark gray). The percentage of active and inactive Y3H cells in the culture can be determined by plating cells on nonselective medium, assaying with X-Gluc and Magenta-Gal to turn colonies with β-glucuronidase and β-galactosidase blue and red, respectively, and counting the number of colonies of each color. C) Mock selection results. Active and inactive Y3H cells were mixed in 100:1 or 1000:1 ratios and grown in the presence of 1 μM Dex–Mtx. The percentage of inactive cells was determined after 0 (□), 2 (■), and 4 (■) days of selection by using the colorimetric assay described above. For the "seeding" selection, two rounds of two-day selections, rather than one longer four-day selection, were performed.

design the next generation of bioengineering tools. Furthermore, we should acknowledge that there will not always be "one-size-fits-all" solutions when designing sophisticated in vivo applications, and we should embrace the use of directed evolution and screening strategies to optimize systems' components and performance.

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