

Video Article

Rapid Synthesis and Screening of Chemically Activated Transcription Factors with GFP-based Reporters

R. Scott McIsaac^{*1,3}, Benjamin L. Oakes^{*1}, David Botstein^{1,2}, Marcus B. Noyes¹

¹The Lewis-Sigler Institute for Integrative Genomics, Princeton University

²Department of Molecular Biology, Princeton University

³Division of Chemistry and Chemical Engineering, California Institute of Technology

*These authors contributed equally

Correspondence to: R. Scott McIsaac at r.scott.mcisaac@gmail.com, Marcus B. Noyes at mnoyes@princeton.edu

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Abstract

Synthetic biology aims to rationally design and build synthetic circuits with desired quantitative properties, as well as provide tools to interrogate the structure of native control circuits. In both cases, the ability to program gene expression in a rapid and tunable fashion, with no off-target effects, can be useful. We have constructed yeast strains containing the *ACT1* promoter upstream of a *URA3* cassette followed by the ligand-binding domain of the human estrogen receptor and VP16. By transforming this strain with a linear PCR product containing a DNA binding domain and selecting against the presence of *URA3*, a constitutively expressed artificial transcription factor (ATF) can be generated by homologous recombination. ATFs engineered in this fashion can activate a unique target gene in the presence of inducer, thereby eliminating both the off-target activation and nonphysiological growth conditions found with commonly used conditional gene expression systems. A simple method for the rapid construction of GFP reporter plasmids that respond specifically to a native or artificial transcription factor of interest is also provided.

Video Link

The video component of this article can be found at <https://www.jove.com/video/51153/>

Introduction

Developing genetic switches in yeast is of great interest in both academic and industrial research. In the ideal case, genetic switches can be used to activate a particular gene (or set of genes) only when desired by the experimenter. A gene's coding sequence placed downstream of a synthetic promoter is not expressed in the absence of an inducing molecule: upon inducer addition the gene should be rapidly expressed. It has only recently been demonstrated that such a switch can be engineered in yeast, where in the absence of inducer, the strain displays a deletion phenotype, but in the presence of inducer, expression is activated in proportion to the level of inducer in all cells^{1,2}. Historically, conditional expression systems in yeast have relied heavily upon nutrient-responsive DNA sequences, including the *MET*, *PHO*, and *GAL* promoters (whose activities are sensitive to extracellular levels of methionine, phosphate, and galactose, respectively). While conditional expression can be achieved, it comes at the cost of significant pleiotropic effects.

Hormone receptors such as the human estrogen receptors have proven to be effective switches in eukaryotes^{3,4}. In the absence of hormone, a protein fused to a hormone receptor can be sequestered in the cytoplasm where it interacts with the Hsp90 chaperone complex. Upon binding the appropriate ligand, the receptor undergoes a conformational change, causing it to be released from the Hsp90 chaperone complex and revealing a nuclear localization signal. To observe the localization dynamics of a particular hormone receptor-containing protein, we created a C-terminal fusion of Gal4dbd.ER.VP16 (GEV, a synthetic transcription factor containing the Gal4p DNA binding domain, the ligand binding domain of the human estrogen receptor, and VP16) with GFP². Nuclear localization was observed within 8 min following addition of saturating amounts of the inducer, β -estradiol, to which wild type yeast is completely inert. By this time, the majority of cells have clearly visible active transcription sites for GEV target genes (assayed via fluorescence *in situ* hybridization) as well as fully mature mRNAs^{2,5}. GEV target genes increased in expression >2-fold within 2.5 min^{2,6} (measured using microarrays), demonstrating that it takes <2.5 min for the chimeric activator to translocate to the nucleus, bind DNA, and activate transcription.

While several other on-switches have been applied in yeast that utilize various small molecules or drugs (including the classic doxycycline-based switches from *Escherichia coli*^{7,8} and an indigo-based switch⁹ from *Arabidopsis thaliana*), none have achieved the speed, specificity, or tightness of regulation exhibited by the hormone-based switches. It is worth mentioning that rapid off-switches have also been developed in yeast, and typically work by fusing a gene to a protease or ubiquitin ligase targeting sequence^{2,10,11,12}. The ability to rapidly remove a target protein from the cell facilitates the study of essential genes as well as genes that are prone to genetic suppression when deleted¹⁰.

The methods described in this protocol are for building and characterizing synthetic on-switches in yeast. First, we show how to rapidly generate genomically-integrated fusion proteins consisting of a DNA binding domain of interest, the ligand binding domain of the human estrogen receptor, and VP16 (DBD-EV's). Following our protocol, the fusion protein is expressed from an ACT1 promoter. We then show how to create a cognate reporter plasmid for the ATF and test its functionality using flow cytometry. Though we envision these reporter plasmids being used with new ATFs (**Figure 1**), they can be used as reporters for a native transcriptional activator as well. Finally, details for testing the effect of ATF activation on cell growth in 96-well plates and for engineering inducible genomic alleles are provided.

Protocol

Figure 2 provides a schematic of protocol sections 1-3.

1. Creation of ATFs

1. Create primers for PCR amplifying DNA binding domain of interest. Homology to the ACT1 promoter and hormone receptor (as described in **Figures 3** and **4**) is added through PCR to facilitate correct recombination into the strain yMN15.
2. PCR amplify DBD of interest using a high fidelity polymerase.
3. Transform >1 µg of purified PCR product into yeast using the lithium acetate method¹³.
4. Following transformation, spin cells at top speed for 15 sec in microcentrifuge and remove transformation mix.
5. Resuspend in ~200 µl of sterile water and plate on YPD.
6. The next day, replica plate from YPD onto 5-FOA plates (5-FOA plates are made as described in the Cold Spring Harbor Yeast Manual)¹³.
7. Allow growth for 2-4 days, and restreak at least 5-10 colonies onto YPD. Perform a colony PCR using the primers in **Table 1** and sequence to verify inclusion.

2. Creation of ATF Plasmid Reporter

1. Determine the binding site of the ATF (most likely to come from the literature).
2. Order desired binding region as oligos (or Ultramers, if needed). Order as top and bottom strands with correct overhangs for NotI & XbaI as shown in **Table 2**.
3. Dilute oligos to equimolar concentrations (100 µM).
4. Phosphorylate using T4 Polynucleotide Kinase and then anneal in a thermocycler. The reaction conditions are in **Table 3**.
5. Digest ~1-2 µg of pMN8 with NotI-HF & XbaI for 1 hr at 37 °C (reaction conditions in **Table 4**).
6. Gel purify the backbone band.
7. Dilute the purified backbone to 10 ng/µl.
8. Dilute the double-stranded, phosphorylated binding site to 5x the molar concentration of backbone per µl.
9. Using T4 DNA Ligase, ligate the binding sites into the digested backbone at room temperature for 30 min (**Table 5**).
10. For transformation, add half the ligation reaction to 50 µl of standard, chemically competent *Escherichia coli* such as XL1-Blue or DH5-Alpha.
11. Heat shock cells for 45 sec in a 42 °C water bath and then place on ice for 2 min.
12. Add 900 µl of SOC medium to transformation reaction.
13. Grow at 37 °C for 1 hr on a roller drum.
14. Spread 100-200 µl cells per LB+AMP (100 µg/ml) plate and incubate overnight.
15. Grow up *E. coli* in LB+AMP liquid and harvest plasmid DNA. Sequence verify new reporter plasmid from ligation colonies using the primer 5'-GTACCTTATATTGAATTTTC-3'.
16. Transform new reporter plasmid into ATF-containing yeast strain from Section 1 using lithium acetate transformation.

3. Quantify the Effect of ATF Induction on Gene Expression Using GFP Reporter

Preparing the samples:

1. Make 25 mM β-estradiol stock (10 ml of ethanol + 0.0681 g of β-estradiol). Keep refrigerated.
2. Grow ATF + Reporter-containing strain overnight in 5ml SC-URA.
3. Obtain nine 250 ml shake flasks and add 25 ml SC-URA to each.
4. Add 0 nM, 0.1 nM, 1 nM, 10 nM, 100 nM, 1 µM, or 10 µM β-estradiol. This is most easily done by making 10-fold serial dilutions of the 25 mM β-estradiol stock. For a final concentration of 10 µM β-estradiol, add 10 µl of the 25 mM β-estradiol stock to 25 ml of SC-URA.
5. Add 125 µl of overnight cultures to flasks (1:200 dilution).
6. For the 2 remaining flasks, inoculate with a constitutive GFP producing strain and a strain lacking GFP. These are needed for calibrating the flow cytometer.
7. Shake at 200 rpm at 30 °C for 12-18 hr.
Note: The optimal incubation time is determined by identifying when GFP induction no longer changes following addition of β-estradiol.
8. Take 500 µl of each culture and spin down in separate 1.7 ml microcentrifuge tubes.
9. Aspirate SC-URA.
10. Wash once in 1 ml PBS+0.1% Tween-20 and aspirate.
11. Add 1 ml of PBS+0.1% Tween-20 and resuspend.
12. Add 1 ml of PBS+0.1% Tween-20 to Falcon tubes.
13. Add the resuspended cells to 5 ml tubes (final volume = 2 ml) (can be stored at 4 °C for several days).
14. Optional: sonicate cells lightly to breakup any clumped cells.

Flow cytometry analysis: Overview: The fluorescence of 10,000-100,000 cells is typically analyzed per sample. Data can be processed either in FlowJo or using custom scripts in MATLAB or R. Make sure to calibrate voltage of the GFP channel using GFP positive and

negative controls. Once the FCS files are exported, a script such as the one in **Figure 6** along with the function `fca_readfcs` (<http://www.mathworks.com/matlabcentral/fileexchange/9608-fcs-data-reader>) can be used to process the data.

15. Perform flow cytometry.
 1. Use forward scatter (FSC) and side scatter (SSC) to identify yeast cells.
 2. Set the flow rate to ~3,000 cells/sec.
 3. Measure GFP (FITC channel).
 4. Once data are collected, export .FCS files
16. Load MATLAB.
17. Move the .FCS files, `fcs_readfcs.m`, and a script similar to that from **Figure 6** to the same folder.
18. Change the present working directory to the folder containing the data and scripts.
19. Run the script from **Figure 6** (note: the legend has been manually entered to produce what's seen in **Figure 7**).

4. Quantify the Effect of ATF Induction on Cell Growth

1. From frozen stocks, streak out both (1) an ATF-containing strain and (2) an ATF-lacking strain (such as yMN15) onto separate YPD plates.
2. After 2 days of growth, inoculate separate culture tubes containing 5 ml of YPD liquid with each strain and grow overnight at 30 °C.
3. Perform 10-fold serial dilutions of a 0.25 mM β -estradiol stock solution up to 10,000-fold (0.025 μ M β -estradiol) in 100% ethanol.
4. Add 40 μ l of overnight cultures to 10 ml of YPD liquid (1-250 dilution).
5. For each strain, add 96 μ l of diluted cells to 18 wells of a 96-well plate.
6. Add 4 μ l of β -estradiol to the cells. An essential point is to make sure each well has the same amount of total ethanol. (Alternatively, a more concentrated stock of β -estradiol can be used and subsequently diluted to the point the effect of ethanol on growth is not measurable).
7. Perform in triplicate. Three of the wells for each strain should be ethanol-only controls.
8. Cover the 96-well plate in gas permeable membrane.
9. Monitor growth (OD_{600}) in plate reader.
10. Quantify the growth rates using any number of standard methods such as finding the maximum slope.

Note: We've had good success with the open source software package *grofit*¹⁴, which runs in the R programming environment. It is worth mentioning that while growth rates computed from 96-well plate assays compared to shake-flasks (25 ml cultures) are not necessarily the same (due to both differences in physiology and in instrumentation), we've observed that the relative growth rates are similar (though this may not always be the case).

5. Create Inducible Genomic Alleles

1. Obtain pMN10, a noncentromeric plasmid with the ATF-responsive promoter from pMN8 fused to KanMX (in the opposite orientation).
2. Restriction digest the vector backbone and gel extract as in Section 2.
3. Anneal, phosphorylate, and ligate oligos containing appropriate binding sites as described in Section 2.
4. Sequence verify. This plasmid will now serve as a DNA template for creating the inducible allele.
5. Obtain the yeast strain expressing the corresponding ATF of interest.
6. PCR amplify the KanMX-Promoter cassette (~2 kb) using primers from **Table 6**.
7. Transform the PCR product into the ATF-expressing strain using the lithium acetate method.
8. Plate on YPD and replica plate onto YPD+ G418 the following day.
9. Confirm appropriate insertion of the promoter using the forward primer (5'-CTGCAGCGAGGAGCCGTAAT-3') and a reverse primer internal to the gene whose promoter has been replaced. The reverse primer is typically designed to be between 200-300 bp downstream of the first ATG¹⁵. The final PCR product is ~1.2 kb.

Representative Results

Figure 5 shows induction of GFP by Z₃EV, Z₄EV, or GEV over time. Note the increase in GFP production in response to the ATFs containing nonyeast DNA binding domains. We recently speculated that this results from these factors achieving single-gene specificity in the yeast genome¹. GEV induction alone leads to activation/repression of hundreds of genes, whereas Z₃EV and Z₄EV only activate expression of a target gene placed downstream of a synthetic promoter containing appropriate binding sites (5'-GCGTGCGGCG-3' and 5'-GCGGCGGAGGAG-3', respectively)¹. **Figure 7** demonstrates the tight regulation of the system (no inducer results in no detectable GFP) and that all cells get induced in response to inducer. The ability of Z₃EV to induce GFP across a range of β -estradiol concentrations is shown in **Figure 8**.

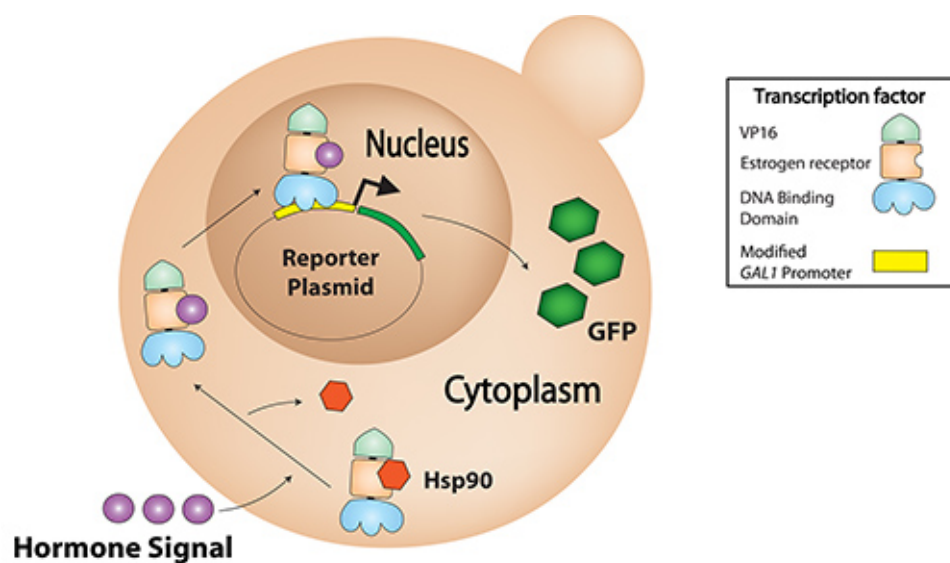


Figure 1. Artificial transcription factors interact with Hsp90 in the absence of β -estradiol. When β -estradiol binds to the ATF, Hsp90 dissociates, allowing the ATF to enter the nucleus and activate expression from the plasmid reporter.

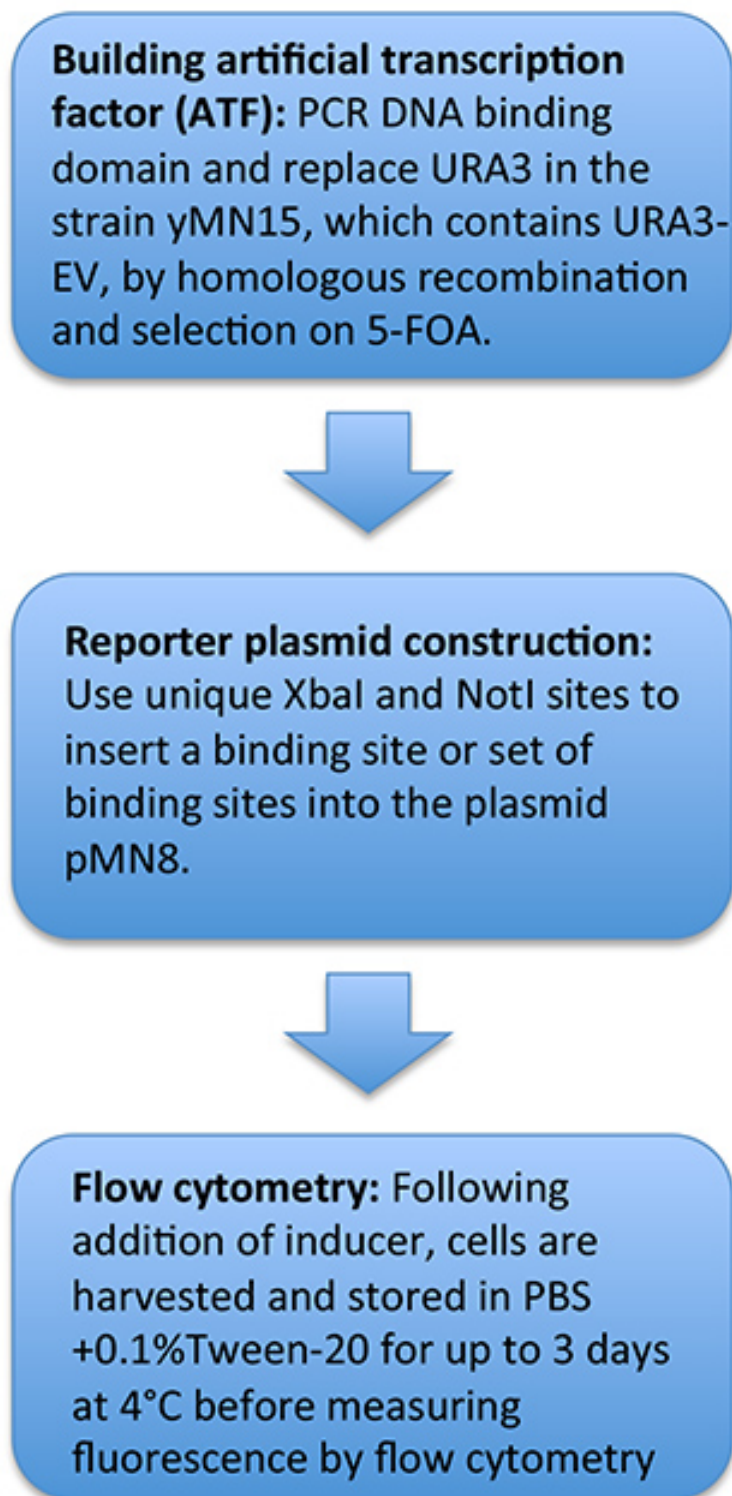


Figure 2. Schematic of protocol for engineering strains that contain artificial transcription factors and building/testing cognate GFP reporters.

GCCTCTACCTTGCAGACCCATATAATATAATACTAAATAAGTAAATAAGACACACGCG
 AGAACATATATACACAATTACAGTAACAATAACAAGAGGACAGATACTACCAAAATGTG
 TGGGGAAGCGGTAAGCTGCCACAGCAATTAATGCACAACATTTAACCTACATTCTTCC
 TTATCGGATCCTCAAAACCTTAAAAACATATGCCTCACCTAAACATATTTTCAATTAA
 CCCTCAATATTTCTCTGTCAACCGGCTCTATTTTCCATTTTCTCTTTACCCGCCACGC
 GTTTTTTCTTTCAAATTTTTTCTTCTTCTTCTTTTCTTCCACGTCCTCTTGCAATAA
 TAAATAAACCGTTTTGAAACCAAACTCGCCTCTCTCTCTCTTTTGAATATTTTTGGG
 TTTGTTTGATCCTTTCTTCCCAATCTCTTGTGTTAATATATATTCAATTTATACACGC
 TCTCTTTTATCTTCTTTTCTTCTCTCTGTATTTCTTCTTCCCTTTCTACTCAAA
 CCAAGAAGAAAAAGAAAAGGTCAATCTTTGTTAAAGAATAGGATCTTCTACTACATCAG
 CTTTATAGATTTTTCACGCTTACTGCTTTTTTCTTCCCAAGATCGAAAATTTACTGAATTA
 ACAGGGCCCCCTCGAGGTGACGGTATCGATAAGCTTGAAGCAAGCCTCCTGAAAG
 ATGTCGAAAGCTACATATAAGGAACGTGCTGCTACTCATCCTAGTCTGTTGCTGCCAA
 GCTATTTAATATCATGCACGAAAAGCAAACAACTTGTGTGCTTCATTGGATGTTTCGTA
 CCACCAAGGAATTACTGGAGTTAGTTGAAGCATTAGGTCCCAAAATTTGTTTACTAAAA
 ACACATGTGGATATCTTGACTGATTTTTCCATGGAGGGCACAGTTAAGCCGCTAAAGG
 CATTATCCGCAAGTACATTTTACTCTTTCGAAGACAGAAAATTTGCTGACATTGGT
 AATACAGTCAAATGCACTACTCTGCGGGTGTATACAGAATAGCAGAAATGGGCAGACA
 TTACGAATGCACACGGTGTGGTGGGCCAGGTATTGTTAGCGGTTTGAAGCAGGCGGC
 AGAAGAAGTAACAAAGGAACCTAGAGGCCCTTTGATGTTAGCAGAATTGTCATGCAAG
 GGCTCCCTATCTACTGGAGAAATATACTAAGGGTACTGTTGACATTGCGAAGAGCGACA
 AAGATTGTTTATCTGGCTTTATTGCTCAAAGAGACATGGGTGGAAGAGATGAAGGTTA
 CGATTGGTTGATTATGACACCCGGTGTGGGTTAGATGACAAGGGAGACGCATTGGGT
 CAACAGTATAGAACCCTGGATGATGTGGTCTCTACAGGATCTGACATTATTATTGTTGG
 AAGAGGACTATTTGCAAAAGGGAAGGGATGCTAAGGTAGAGGGTGAACGTTACAGAAAA
 GCAGGCTGGGAAGCATATTTGAGAAAGATGCGGCCAGCAAACTAATAGGGTACCCCTG
 CAGCTGCGTGCAGCTCTAGAGGATCCATCTGCTGGAGACATGAGAGCTGCCAACCTTTG
 GCCAAGCCCCGCTCATGATCAAACGCTCTAAGAAGAACAGCCTGGCCTTGTCCTGACG
 GCCGACCAGATGGTCACTGCTTGTGATGCTGAGCCCCCATACTCTATTCCGAGT
 ATGATCTTACCAGCCCTTCACTGAAGCTTCGATGATGGGCTTACTGACCAACCTGGC
 AGACAGGGAGCTGGTTCACATGATCAACTGGGCGAAGAGGGTGGCAGGCTTTGTGGAT
 TTGACCTCCATGATCAGGTCCACCTTCTAGAATGTGCTGGCTAGAGATCCTGATGAT
 TGGTCTCGTCTGGCGCTCCATGGAGCACCCAGTGAAGCTACTGTTTGTCTCAACTTGC
 TCTTGGACAGGAACCAGGGAATGTGTAGAGGGCATGGTGGAGATCTTCGACATGCT
 GCTGGCTACATCATCTCGGTTCGCTATGATGAATCTGCAGGGAGAGGAGTTTGTGTGC
 CTCAAATCTATTATTTGCTTAATTCTGGAGTGTACACATTTCTGTCCAGCACCTGAA
 GTCTCTGGAAGAGAAGGACCATATCCACCGAGTCTGGACAAGATCACAGACACTTTG
 ATCCACCTGATGGCCAAGGCAGGCTGACCCTGCAGCAGCAGCAGCAGCGGCTGGCCC
 AGCTCCTCCTCATCTCTCCACATCAGGCACATGAGTAACAAAGGCATGGAGCATCT
 GTACAGCATGAAGTGAAGAACGTGGTGCCCTCTATGACCTGCTGCTGGAGATGCTG
 GACGCCCACCGCTACATGCGCCCACTAGCCGTGGAGGGGCATCCGTGGAGGAGACG
 GACCAAAGCCACTTGGCCACTGCGGGCTCTACTTCATCGGAGCTCCACTTAGACGGCG
 AGGACGTGGCGATGGCGCATGCCGACGCGCTAGACGATTTCGATCTGGACATGTTGGG
 GGACGGGGATTCCCCGGGTCCGGGATTTACCCCCACGACTCCGCCCCCTACGGCGCT
 CTGGATATGGCCGACTTCGAGTTTGAGCAGATGTTTACCGATGCCCTTGGAAATTGACG
 AGTACGGTGGGTAG

ACT1 promoter

URA3

ER-VPI6

Underlined text = Homology used in PCR primers for recombination of new DBDs.

Figure 3. The ACT1pr-URA3-EV sequence in the strain yMN15 at the LEU2 locus.

PRIMERS FOR PCR AMPLIFYING DNA BINDING DOMAIN

Fwd:

5'~GAATTAACAGGGCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGAAGCAA
GCCTCCTGAAAGATG~DBD homology~3'

Rev:

5'~AAAGGTTGGCAGCTCTCATGTCTCCAGCAGATGGATCCTCTAGAGTCGACGC
AGCTGCAGGTGTGCCTCCGCC-DBD homology REV~3'

*Note: Make sure to include the start codon (bold, underlined).

To make Z₃EV, the Zif268 DNA binding domain was amplified using the following primers:

Fwd:

5'~GAATTAACAGGGCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGAAGCAA
GCCTCCTGAAAGATG**GGTACCCGCCCATATG**~3'

Rev:

5'~AAAGGTTGGCAGCTCTCATGTCTCCAGCAGATGGATCCTCTAGAGTCGACGC
AGCTGCAGGTGTGCCTCCGCC**ACCTGTATGGATTTTGGA**~3'

where the DBD homology is shown in red.

The Zif268 template sequence is from <http://www.addgene.org/12612/>:

ATGGGTACCCGCCCATATGCTTGCCCTGTCGAGTCCTGCGATCGCCGCTTTTCTC
GCTCGGATGAGCTTACCCGCCATATCCGCATCCATACCGGTCAGAAGCCCTTCCA
GTGTCGAATCTGCATGCGTAACTTCAGTCGTAGTGACCACCTTACCACCCACATC
CGCACCCACACCGGCGAGAAGCCTTTTGCCTGTGACATTTGTGGGAGGAAGTTTG
CCAGGAGTGATGAACGCAAGAGGCATACCAAAATCCATACAGGT

Figure 4. Design of PCR primers for amplifying a DNA binding domain of interest with additional sequence homology to generate a new fusion protein when successfully transformed into yMN15.

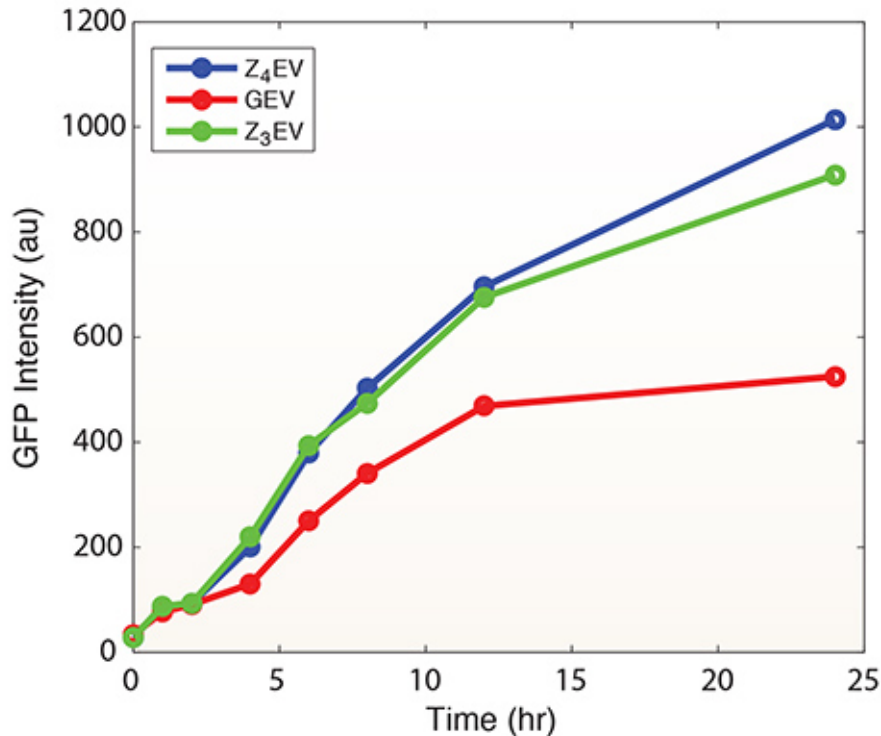


Figure 5. GFP induction by three different ATF-promoter pairs in response to 1 μ M β -estradiol.

```
clear all;
Bins=linspace(0,12,80); %Bins to use for plotting histograms
FinalData=[];

files = {'NoGFP.fcs',...
        'OnM.fcs',...
        '1uM.fcs'};

for i=1:length(files)
    [fcsdat, fcsHdr] = fca_readfcs(files{i});
    logx=log(fcsdat(:,3)); %log-transform column containing GFP data
    logx=real(logx);
    f=find(logx==Inf);
    logx(f)=-1000;
    n =hist(logx,Bins);
    FinalData=[FinalData; n];
end
close all;
hold on
for j=1:3
    plot(Bins, FinalData(j,:), 'LineWidth',2, 'Color',[rand rand rand]);
end
legend({'No GFP',...
        '0 \muM Estradiol',...
        '1 \muM Estradiol'});
xlabel('ln(Fluorescence)', 'FontSize',14);
ylabel('Counts', 'FontSize',14)
hold off;
```

Figure 6. A MATLAB script for processing flow cytometry data. This script was adapted from http://openwetware.org/wiki/McClean:_Matlab_Code_for_Analyzing_FACS_Data.

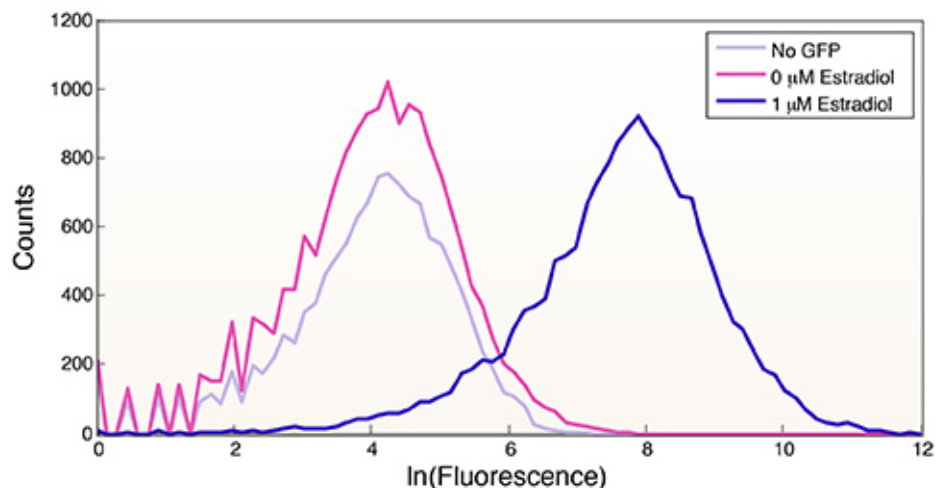
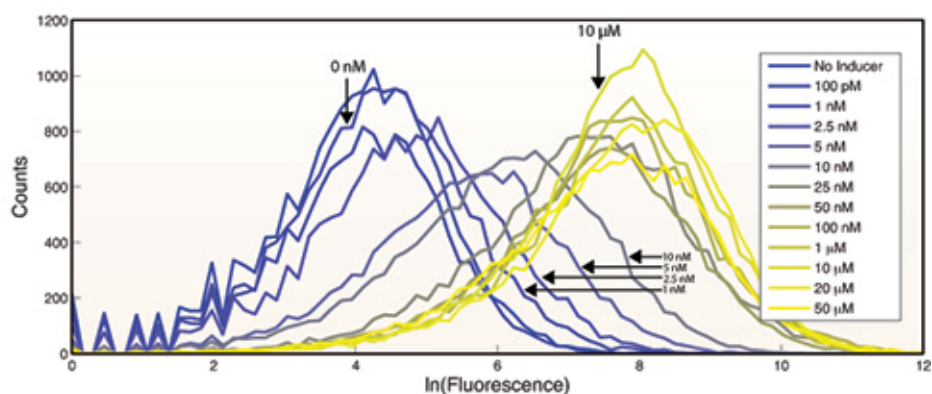


Figure 7. Induction of GFP by Z₃EV in response to 0 μ M β -estradiol and 1 μ M β -estradiol following 18 hr of induction. Fluorescence was also measured from cells lacking GFP to illustrate the tight regulation of the Z₃EV system. This figure was generated using the code in **Figure 6**.

A



B

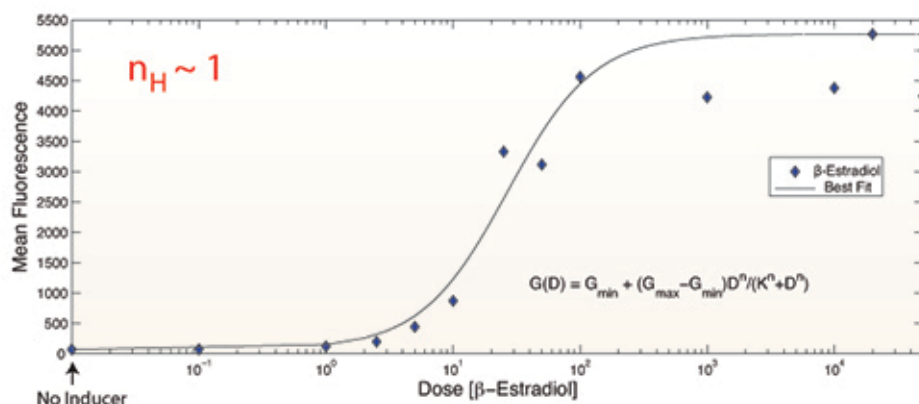


Figure 8. The relationship between inducer concentration and expression output is graded with a Hill coefficient of ~ 1 . **(A)** Distributions of GFP expression as a function of β -estradiol concentration. **(B)** The mean fluorescence of the distributions from **(A)**. The data were fit to the function $G(D) = G_{\min} + (G_{\max} - G_{\min})D^n / (K^n + D^n)$ where D is the β -estradiol dose, G_{\min} and G_{\max} are the minimum and maximum GFP values, respectively, n is the Hill coefficient, and K is the β -estradiol dose that yields $\frac{1}{2} (G_{\max} + G_{\min})$.

Oligonucleotide Sequence	Name
5'-TTGAAACCA AACTCGCCTCT-3'	DBD Check Forward
5'-tccagagac ttcagggtgct-3'	DBD Check Reverse

Table 1. Primers for confirming correct fusion of DBD of interest to the Estrogen Receptor. The forward primer is homologous the ACT1 promoter and the reverse primer is homologous to the estrogen receptor. For Typical DBDs (~300 bp), the PCR product is <1.5 kb.

Oligonucleotide Sequence	Name
5'-ggccgc...DBD binding site... t-3'	Top oligo
5'-ctaga...DNA binding site reverse...gc-3'	Bottom oligo
5'-gccgcGTGGGCGTGCCTGGGCGGG CGTGGGCGTGCCTGGGCGGGCGTG GGCGTGCCTGGGCGT-3'	Top oligo + 6x Zif268 Binding Sites
5'-ctagaCGCCACGCACGCCACGCC CGCCACGCACGCCACGCCCGCC CACGCACGCCACGc-3'	Bottom oligo + 6x Zif268 Binding Sites

Table 2. Structure of primers for creating dsDNA containing binding sites of interest. To create the Z₃EV-responsive promoter, the oligonucleotides Top oligo + 6x Zif268 Binding Sites and Bottom oligo + 6x Zif268 Binding were used. Sequences for creating the correct DNA overhangs are in lowercase. The Zif268 consensus binding site, GCGTGGGCG, is underlined in the top oligo.

Reagent	Amount
T4 Polynucleotide Kinase Buffer	5 µl
T4 Polynucleotide Kinase (@10,000 units/ml)	1 µl
Top oligo (100 µM)	1.5 µl
Bottom oligo (100 µM)	1.5 µl
Water	41 µl

Table 3. Phosphorylate and anneal primers in a thermocycler using 50 µl of reaction described above. There are two thermocycler steps. Step 1: 37 °C 30 min (Phosphorylate), and step 2: 95 °C 30 sec (step down 1 °C every 30 sec until at 4 °C; Anneal).

Reagent	Amount
XbaI	1 µl
NotI-HF	1 µl
Plasmid DNA	5 µl (1-2 µg)
10x Cut Smart Buffer	5 µl
Water	38 µl

Table 4. Restriction digest of plasmid pMN8 with XbaI and NotI.

Reagent	Amount
T4 Ligase Buffer	2 µl
T4 Ligase	0.2 µl
Backbone @ 10 ng/µl	1 µl
Binding Sequences@ 5x molar concentration/µl	1 µl
Water	15.8 µl

Table 5. Ligate binding sites into plasmid DNA.

Primer	Description
~40 bp upstream of ATG + CGCACTTAACCTTCGCATCTG-3'	Forward primer for amplifying KanMX-Promoter
5'- reverse complement(ATG + ~37bp) + TATAGTTTTTCTCCTTGACG-3'	Reverse primer for amplifying KanMX-Promoter
5'-caatttgcctgctcaagaaataaa ttaaatacaataaaCGCAC TTAACTTCGCATCTG-3'	Forward primer for making <i>GCN4</i> promoter swap.
5'-tggaattaaagcaaataaactgg ctgatattcgacatTATAGTT TTTTCTCCTTGACG-3'	Reverse primer for making <i>GCN4</i> promoter swap.

Table 6. PCR amplify KanMX-Promoter for making titratable allele.

Discussion

The hormone-based switches described here have myriad applications, from studying native cell biology to testing the ability of a DNA binding domain of interest to target a DNA sequence *in vivo*. The system has many desirable features, including a graded response to inducer, fast action, and tight regulation (*i.e.* no measurable leakiness, see **Figure 7**). However, there is still room for improvement and expansion.

Recent work in synthetic biology has emphasized multiplexing synthetic systems and expanding the repertoire of well-characterized, programmable DNA parts¹⁶. Independent, conditional expression of distinct target genes requires both multiple inducers and DNA binding domains that lack overlapping specificity. The availability of engineered and naturally occurring receptors provides a large set of parts with which to develop new artificial transcription factors. Expanding the repertoire of mutually orthogonal switches has been greatly aided by directed evolution approaches^{17,18}. Current efforts to reprogram the ligand-binding specificity of our artificial transcription factors will be presented in the future.

Previously, the phenotypic consequences of gene deletion/overexpression have been extensively studied in yeast^{19,20}. However, it has not been straightforward to study the effect of expression on different phenotypes over a range of expression levels. A primary feature of the system presented herein is its graded nature (**Figure 8**). While often assumed, the relationship between gene expression and phenotypes of interest may not necessarily be monotonic. Artificial transcription factors such as Z₃EV and Z₄EV will make the task of assaying the phenotypic responses to changes in gene expression straightforward.

Finally, the protocols discussed in this manuscript are for engineering and characterizing artificial transcription factors that respond to β -estradiol; however, an important alternative to chemically responsive domains is the use of light-responsive domains (such as the PhyB/Pif3, LOV, and BLUF), whose activities are reversible without having to perturb the culture growth medium²¹⁻²³. Light-based systems facilitate spatiotemporal control of gene expression, protein-protein interactions, ion transport, and enzymatic activity, which have already proven powerful²¹⁻²⁶.

In conclusion, we have presented methods for the rapid construction of inducible, artificial transcription factors in yeast. This protocol provides a template for their construction in conjunction with cognate GFP reporters, as well as methods for analyzing the reporter data using flow cytometry and assaying the effect of ATF activation on growth.

Disclosures

Mclsaac, Noyes, and Botstein (with others) have submitted part of this system as a Patent Disclosure.

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