Chapter 17

Design, Engineering, and Characterization of Prokaryotic Ligand-Binding Transcriptional Activators as Biosensors in Yeast

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Abstract

In cell factory development, screening procedures, often relying on low-throughput analytical methods, are lagging far behind diversity generation methods. This renders the identification and selection of the best cell factory designs tiresome and costly, conclusively hindering the manufacturing process. In the yeast *Saccharomyces cerevisiae*, implementation of allosterically regulated transcription factors from prokaryotes as metabolite biosensors has proven a valuable strategy to alleviate this screening bottleneck. Here, we present a protocol to select and incorporate prokaryotic transcriptional activators as metabolite biosensors in *S. cerevisiae*. As an example, we outline the engineering and characterization of the LysR-type transcriptional regulator (LTTR) family member BenM from *Acetinobacter* sp. ADP1 for monitoring accumulation of *cis,cis*-muconic acid, a bioplast precursor, in yeast by means of flow cytometry.

Key words Biosensor, Transcription factor, Cell factory, Synthetic biology, Screening, Yeast

1 Introduction

In the last two decades, metabolic engineering has proven itself as a key technology for the manufacturing of valuable molecules from renewable feedstocks [1–3]. The constant development of synthetic biology tools for cloning, library construction, and genome engineering, coupled with the sharp decrease in DNA synthesis costs, has greatly aided the process of building more efficient microbial cell factories [1–3]. Adversely, when aiming to engineer a high-performing cell factory, most heterologous compounds do not give a clear phenotype, rendering the screening procedure of a population of cell factory designs through conventional spectrophotometry-based analytics methods slow and often costly.

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The fact that screening and selection methods lag far behind the techniques that allow the generation of large libraries ultimately hampers the identification of cell factory designs with optimal titers, rates, and yields.

Genetically encoded biosensors convey a huge potential to overcome this screening bottleneck. In general, biosensors are able to sense input, like extra- or intracellular metabolite perturbations, and subsequently actuate an adequate output akin to logic gates in electrical circuits. Natural biosensors include molecular gating components like RNA aptamers and allosterically regulated transcription factors, which can regulate transcription of target genes in the presence of an adequate input [1–6]. In the case of ligand-binding allosterically regulated transcription factors, placing a reporter gene or non-native selection gene under the control of the ligand-binding transcription factor offers a sensitive and specific synthetic system that can couple input (intracellular ligand concentration) to a high-throughput screenable output (e.g. fluorescence or antibiotic resistance) [1–7].

Prokaryotes harbor an enormous reservoir of ligand-binding transcriptional regulators, which can be applied as biosensors [8]. These transcriptional regulators can be grouped into transcriptional repressors and transcriptional activators (Fig. 1). Transcriptional repressors have abundantly been applied as biosensors both in prokaryotes and eukaryotes [1–7]. Perhaps the most famous example is the TetR system. Specifically, when tetracyclin (tc) is absent, the transcription factor TetR represses the expression of a membrane protein (TetA) through binding to an operator in the *tetA* promoter (TetO), thereby preventing transcription. In contrast, the presence of tc induces a conformational change of TetR rendering it unable to bind TetO and thereby relieving the repression of *tetA* expression, ultimately forcing tc out of the cell [9]. Because of the efficiency of TetR to sense sub-inhibitory tc concentrations ($K_a = 10^{10}\ M^{-1}$) and its high specificity for TetO, the prokaryote TetR-TetO system has been engineered and transplanted into eukaryote hosts for monitoring and regulation purposes [10–12]. Other examples of prokaryotic repressors that have been successfully transplanted as biosensors into *Saccharomyces cerevisiae* include FapR, for the detection of malonyl-CoA, and XylR, detecting xylose [13–15].

Until recently, transcriptional activators had not been reported as metabolite biosensors in a eukaryote, limiting the reservoir of transcriptional regulators that can be tapped from for biosensor development. The different mode of action for activators compared to repressors, as well as the inherent need for more extensive engineering of the biosensor in order obtain a relevant input to output relationship (Fig. 1), are possible explanations for why prokaryotic transcriptional activators had not been transplanted as biosensors into eukaryotes yet. Indeed, in our recent study, both

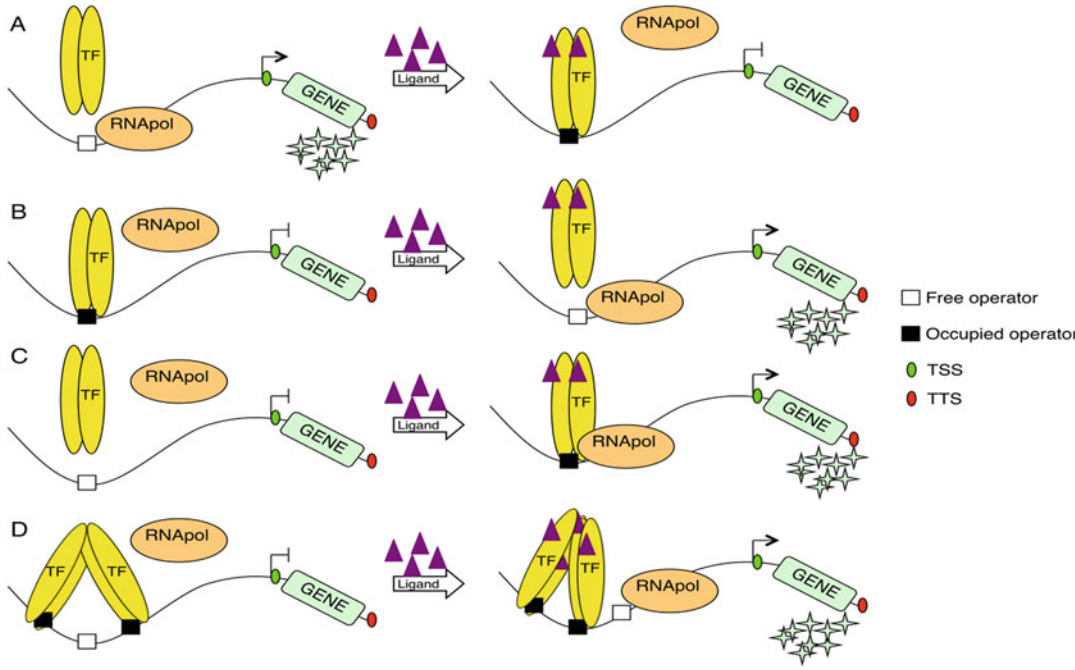

Fig. 1 Mechanisms for gene expression regulation. (**a**) Repressor-based repression: in the presence of the ligand, the transcription factor (TF) binds to the operator site within the promoter region and physically hinders the RNA polymerase activity; (**b**) Activator-based regulation: in the presence of the ligand, the TF acts as a recruiter for RNA polymerase when bound to the operator site; (**c**) Repressor-based activation: the repressor is constitutively bound to the operator site and the presence of the ligand triggers a conformational change in the TF impairing the binding, consequently relieving the repression; (**d**) BenM-based gene regulation in *Acinetobacter* sp. ADP1: CCM, a degradation compound from aromatic acid catabolism, triggers a conformational change in BenM that results in a shift of the sites being bound, facilitating access of RNA polymerase and activate transcription of the downstream operon (*see* **Note 1** for more details)

tuning of the expression level of transcriptional activators and extensive engineering of reporter promoters turned out to be important for establishing small-molecule biosensors based on prokaryotic transcriptional activators [16].

In this protocol we present the (1) selection criteria, (2) design, (3) engineering, and (4) application of a biosensor for *cis,cis*-muconic acid (CCM) in the budding yeast *S. cerevisiae* as we previously reported [16]. CCM is an important bioplastics precursor, the production of which from renewable carbon sources has been engineered in yeast [17]. In the bacterium *Acinetobacter* sp. ADP1, BenM is a LysR-type transcriptional regulator (LTTR) that upon binding of CCM undergoes a conformational change resulting in transcriptional activation of genes involved with catabolism of aromatic compounds (Fig. 1) [18]. BenM is a well-studied gene and protein; with annotated gene sequence, crystal structure, and operator sequence [19]. In our original study, we optimized various parameters to establish BenM as a CCM biosensor in yeast,

and proved the validity of the biosensor design for other biosensors based on LTTR-type of regulators. In this protocol we will focus on the engineering of the optimal CCM biosensor design and how to characterize its performance.

2 Materials

2.1 Strains, Media and Reagents

1. Bacterial strain: *Escherichia coli* DH5α is used as a host for cloning and plasmid propagation.
2. Yeast strain: *S. cerevisiae* CEN.PK113-5A (MAT*a*, *trp1 his3Δ1 leu2-3/112 MAL2-8^c SUC2*) is used as the basic strain in which the biosensor-reporter will be built in.
3. Media: For *E. coli*; Luria-Bertani (LB) medium with ampicillin (10 g tryptone, 5 g yeast extract, 10 g NaCl, deionized water up to 1 L, autoclave, add 100 μg/mL ampicillin). For *S. cerevisiae*; Synthetic Complete (SC) medium (6.7 g yeast nitrogen base without amino acids, appropriate amount of drop-out medium supplement (Sigma-Aldrich), deionized water up to approximately 880 mL, adjust pH to 5.6, deionized water up to 900 mL, (20 g agar in case of plates), autoclave, 100 mL 20% (w/v) glucose); mineral medium with tryptophan (per L: 7.5 g $(NH_4)_2SO_4$, 14.4 g KH_2PO_4, 0.5 g $MgSO_4{\cdot}7H_2O$, 20 g glucose, 2 mL trace metals solution, 1 mL vitamin solution, 4 mL tryptophan solution (5 g/L)); and yeast extract peptone dextrose (YPD) complete medium (10 g BactoYeast extract, 20 g BactoPeptone, 20 g Dextrose, (20 g agar in case of plates), deionized water up to approximately 1000 mL, autoclave); pre-mineral medium (75 mL ammonium solution $(NH_4)_2SO_4$ (100 g/L), 120 mL phosphate solution KH_2PO_4 (120 g/L), 10 mL magnesium solution $MgSO_4$, $7H_2O$ (50 g/L), 100 mL 20% (w/v) glucose, 2 mL trace metals, 1 mL vitamins, 4 mL 250× tryptophan stock solution (5 g/L) deionized water up to 900 mL, mix using stirring rod, filter sterile, store at 4 °C).

2.2 Molecular Biology

1. DNA polymerases: High-fidelity Phusion U Hot Start DNA Polymerase (Thermo Fisher Scientific, Inc.), 2xOneTaq® Master Mix DNA Polymerase (New England Biolabs).
2. Synthetic genes: Commercially synthesized (Integrated DNA Technologies, Inc.). Genes are codon-optimized for expression in yeast using manufacturer's software (Table 1).
3. Oligonucleotides: Commercially synthesized (Integrated DNA Technologies, Inc.) (Tables 2, 3, 4).

Table 1
Synthetic DNA fragments

BenM coding sequence codon-optimized for *S. cerevisiae*:

```
1 ATGGAATTGA GACACTTGAG ATACTTCGTT GCCGTTGTTG AAGAACAATC TTTTACAAAG 61
  GCTGCCGACA AGTTGTGTAT TGCTCAACCA CCATTATCCA GACAAATCCA AAACTTGGAA
  121 GAAGAATTGG GTATCCAATT ATTGGAAAGA GGTTCCAGAC CAGTTAAGAC
  TACTCCAGAA 181 GGTCATTTCT TTTACCAATA CGCCATCAAG TTGTTGTCCA
  ACGTTGATCA AATGGTCAGT 241 ATGACCAAGA GAATTGCCTC TGTTGAAAAG
  ACCATTAGAA TCGGTTTTGT TGGTTCCTTG 301 TTGTTCGGTT TGTTGCCAAG
  AATTATCCAC TTGTACAGAC AAGCTCATCC AAACTTGAGA 361 ATCGAATTAT
  ACGAAATGGG TACTAAGGCT CAAACCGAAG CTTTGAAAGA AGGTAGAATT 421
  GACGCTGGTT TTGGTAGATT GAAGATTTCT GATCCAGCCA TCAAGAGAAC CTTGTTGAGA
  481 AACGAAAGAT TGATGGTTGC TGTTCATGCT TCCCATCCAT TGAATCAAAT
  GAAGGATAAG 541 GGTGTTCACT TGAACGATTT GATCGACGAA AAGATCTTGT
  TGTACCCATC TTCTCCAAAG 601 CCAAACTTCT CTACTCATGT TATGAACATC
  TTCTCTGACC ATGGTTTGGA ACCTACCAAG 661 ATTAACGAAG TTAGAGAAGT
  CCAATTGGCC TTGGGTTTGG TTGCTGCTGG TGAAGGTATT 721 TCATTGGTTC
  CAGCTTCTAC CCAATCCATT CAATTATTCA ACTTGTCCTA CGTCCCATTA 781
  TTAGATCCAG ATGCTATTAC CCCAATCTAC ATTGCTGTTA GAAACATGGA AGAATCCACC
  841 TACATCTACT CATTATACGA AACCATCAGA CAAATCTACG CCTACGAAGG
  TTTTACTGAA 901 CCACCAAATT GGTAA
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Sequence of 209bp_CYC1p_*BenO*_T1: the minimal *CYC1* promoter with *BenO* (underlined) inserted 6 bp upstream of TATA-1β (indicated in bold):

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1 CCAGGCAACT TTAGTGCTGA CACATAATAC TCCATAGGTA TTTTATTATA CAAATAATGT 61
  GTTTGAACTT ATTAAAACAT TCTTTTAAGG TATAAACAAC AGGCATATAT ATATGTGTGC
  121 GACGACACAT GATCATATGG CATGCATGTG CTCTGTATGT ATATAAAACT
  CTTGTTTTCT 181 TCTTTTCTCT AAATATTCTT TCCTTATACA TTAGGACCTT
  TGCAGCATAA ATTACTATAC 241 TTCTATAGAC ACACAAACAC AAATACACAC
  ACTAAATTAA TA
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4. Plasmids: The EasyClone plasmids used in this protocol are from Jensen et al. [20]. All constructed plasmids are sequence-verified by Sanger sequencing.
5. Gel purification: Amplified genes, promoters and digested vectors are gel-purified using NucleoSpin® Gel and PCR Clean-up kit (Machery-Nagel).
6. Restriction enzymes: FastDigest® SfaAI and FastDigest® NotI with the corresponding FastDigest® buffer; Nb.BsmI and corresponding buffer 3.1 (New England Biolabs).
7. USER cloning: USER™ enzyme (New England Biolabs), 5× Phusion HF Buffer (Life Technologies).

2.3 Reagents

1. 0.02 M NaOH is used for yeast cell lysis.
2. 1% Agarose is used for routine analysis of nucleic acids by gel electrophoresis.

Table 2
Primers for USER cloning of genes and promoters

Description primer	Sequence 5′-3′
Forward primer for USER cloning of REV1p	**CGTGCGAU**TTCTTAGGCACAACAATATTTATAAAAGAAG
Reverse primer for USER cloning of REV1p	**ATGACAGA**UCGCTGGATATGCCTAGAAATGC
Forward primer for USER cloning of BenM (Kozak, start codon)	**ATCTGTCAU**AAAACAATGGAATTGAGACAC
Reverse primer for USER cloning of BenM	**CACGCGAU**TTACCAATTTGGTGGTTCAG
Forward primer for USER cloning of 209bp_CYC1p_*BenO*_T1	**CGTGCGAU**CCAGGCAACTTTAGTGCTGACAC
Reverse primer for USER cloning of 209bp_CYC1p_*BenO*_T1	**ATGACAGAU**TATTAATTTAGTGTGTGTATTTGTGTTTGTG
Forward primer for USER cloning of yeGFP (Kozak, start codon)	**ATCTGTCAU**AAAACAATGTCTAAAGGTG
Reverse primer for USER cloning of yeGFP	**CACGCGAU**TTATTTGTACAATTCATCCA

Uracil-containing overhangs are indicated in bold

Table 3
Universal primers binding common EasyClone backbone vectors are used to confirm the correct assembling of the expression cassette into the integrative vector backbone

Primer ID	Sequence 5′-3′	Description
224	GAAATTCGCTTATTTAGAAGTGTC	Universal forward primer
225	CTCCTTCCTTTTCGGTTAGAG	Universal reverse primer

3. Phosphate buffered saline (PBS) is used for cell dilution prior to flow cytometry.
4. *Cis,cis*-muconic acid (Sigma-Aldrich).
5. Trace metals solution (per L: 4.5 g $CaCl_2{\cdot}2H_2O$, 4.5 g $ZnSO_4{\cdot}7H_2O$, 3 g $FeSO_4{\cdot}7H_2O$, 1 g H_3BO_3, 1 g $MnCl_2{\cdot}4H_2O$, 0.4 g $Na_2MoO_4{\cdot}2H_2O$, 0.3 g $CoCl_2{\cdot}6H_2O$, 0.1 g $CuSO_4{\cdot}5H_2O$, 0.1 g KI, 15 g EDTA. Add the salts (without EDTA) one by one to 900 mL deionized water and dissolve them, while keeping the pH at 6. Then, gently heat the solution and add EDTA. Finally, adjust pH to 4, bring final volume to 1 L, autoclave and store at 4 °C).

Table 4
Primers 2220 and 2221 are universal primers annealing to any EasyClone vector of choice. The other primers correspond to an insertion into a particular genomic integration site. Either one or both primer pairs can be used for verification of the particular correct integration

Site	Primer ID	Sequence 5′-3′	Description	Fragment size (bp)
X-3	2220	CCTGCAGGACTAGTGCTGAG	X-3 DOWN	667
	904	CCGTGCAATACCAAAATCG		
	2221	GTTGACACTTCTAAATAAGCGAATTTC	X-3 UP	1059
	903	TGACGAATCGTTAGGCACAG		
XII-4	2220	CCTGCAGGACTAGTGCTGAG	XII-4 DOWN	667
	898	CGTGAAATCTCTTTGCGGTAG		
	2221	GTTGACACTTCTAAATAAGCGAATTTC	XII-4 UP	828
	897	GAACTGACGTCGAAGGCTCT		

6. Vitamin solution (per L: 50 mg biotin, 200 mg p-aminobenzoic acid, 1 g nicotinic acid, 1 g Ca-pantothenate, 1 g pyridoxine-HCl, 1 g thiamine-HCl, 25 g myo-inositol. Dissolve biotin in 20 mL 0.1 M NaOH, and then add 900 mL deionized water. Adjust pH to 6.5 and add the remaining vitamins. Readjust pH to 6.5 just before and after adding m-inositol. Adjust the final volume to 1 L. Filter-sterilize and store at 4 °C).
7. Yeast transformation mix: 50% w/v Polyethylene glycol solution MW 3350 (PEG3350), 1 M Lithium acetate pH 7.5 (LiAc), 2 mg/mL Deoxyribonucleic acid (DNA) single stranded from salmon testes (ssDNA) dissolved in sterile TE (10 mM Tris–HCl, 1 mM Na_2EDTA pH 8.0) boil for 5 min and then keep on ice (Sigma-Aldrich).

2.4 Flow Cytometry

1. Culturing: Regular 96-well plates (volume: 360 μL per well) are used for pre-culturing, polypropylene deep well plates (volume: 1 mL per well) are used for the subculturing in induction (+ CCM) or control (−CCM) medium prior to flow cytometry analysis.
2. Instrument: Becton Dickinson LSR FORTESSA with a blue 488 nm laser.
3. Software: BD FACSDIVA™ is used by the BD LSR FORTESSA machine for data acquisition; FlowJo is used for data analysis.

3 Methods

In the following section the step-by-step design, engineering, and characterization of BenM as a CCM biosensor in *S. cerevisiae* is described (*see* **Note 2**). The procedure starts with identification of

the candidate transcription factor and its corresponding operator sequence and ligand from literature. Based on this information, the protocol describes how to generate a yeast strain that incorporates a sensor-reporter system that basically consists of two constructs: (1) the gene encoding the TF driven by a yeast promoter, and (2) the yeast-enhanced GFP (yeGFP) reporter gene driven by an engineered yeast promoter with the operator sequence for the ligand-binding TF. For the former, it is generally advisable to compare different expression levels (i.e. promoters with various strengths), whereas for the latter, the design of the reporter promoter, in particular the number and positioning of the operator sequence, is important [9, 21]. In the case of transcriptional activator-based biosensors, an ideal scenario is a low basal activity of the reporter (output) in the absence of the ligand (input), combined with a strong dose-response output with increasing concentrations of the target ligand. Indeed, finding this optimal input–output configuration required the generation of a library of strains, in which each reporter promoter variant was combined with various expression levels of the TF. The resulting library covered a wide range of modulated responses to the same inducer enabling a more reliable and sensitive detection of the ligand permitting ad hoc high-throughput screening of strains [16]. For simplicity, this protocol only outlines the design and engineering of the optimal sensor-reporter design as identified in our study [16]. Next, this protocol also describes how to characterize the performance of the biosensor using flow cytometry.

3.1 Selection of Candidate Biosensor

The first step to construct a transcription factor-based biosensor is the identification of a transcription factor, which can bind, or in other indirect ways sense, the chemical of interest.

Consider whether the following criteria are fulfilled:

1. Gene sequence of the ligand-binding TF is known.
2. Operator sequence of the TF is known.
3. The ligand is commercially available, is not toxic to yeast at relevant concentrations, and can be taken up by yeast at a tolerable pH (3.5–7.5).
4. Optional: Consideration of the need for expression of additional genes in yeast that would allow for the TF to function (e.g. cofactors) or for the ligand to be taken up (e.g. transporters) (*see* **Note 3**).

Predictably the starting point to identify these elements is literature mining for either publications of transcription factor discovery, operator sequence, molecule of interest, motifs' similarity or distinctive characteristics of the system under study. In addition, public databases are available for browsing the genome of many species enabling a direct approach when looking for operator sites (e.g. http://www.pseudomonas.com/).

In the case of BenM, both gene sequence and operator sequence are known, CCM is commercially available and its production has been engineered in yeast before [17–19].

3.2 Preparation of Gene and Promoter Fragments for USER Cloning

The next step is the design and ordering of synthetic DNA constructs for both the gene encoding the transcription factor and the reporter promoter, as well as primers that can amplify these parts, which will result in fragments for cloning into the appropriate vectors. In order to robustly characterize biosensor designs, it is advisable to stably integrate both the sensor and the reporter construct into defined genomic loci. In this protocol USER cloning and the EasyClone system are used for creating integrative vectors and inserting the resulting constructs into the genome using homologous recombination [20, 22].

3.2.1 Design of Synthetic DNA Constructs

In order to engineer a genetically encoded biosensor based on a transcription factor, two synthetic DNA constructs have to be synthesized (Table 1):

1. Open reading frame BenM (sensor): copy and paste gene sequence (*see* **Note 4**) and use the codon optimization for *S. cerevisiae* option in the web tool on the IDT website (http://eu.idtdna.com/CodonOpt).
2. Reporter promoter: In the optimal CCM biosensor design identified in our study, the BenM operator sequence is integrated 6 bp upstream of TATA-1β in the truncated 209 bp CYC1 minimal promoter [16]. We will refer to this promoter as 209bp_CYC1p_*BenO*_T1.

In addition to these two synthetic constructs, two other parts, i.e. the promoter driving expression of the transcription factor, and the reporter gene coding for yeGFP (*see* **Note 5**), can be obtained from amplification of genomic DNA or commercially available resources (e.g. Addgene plasmid ID 40235), respectively. We will illustrate the example for constructing the optimal CCM biosensor using the weak *REV1* promoter (REV1p) to control the expression of BenM.

3.2.2 Primer Design

Primers with uracil overhangs are used for the amplification of genes and promoters. As illustrated in the article describing the EasyClone system [20], the overhangs vary depending on the chosen combination of promoter-gene and insertion position in the backbone [20]. In this example genes and promoters are always inserted into position 2 (*see* Fig. 2).

3.2.3 PCRs

All the genes and promoters are amplified with primers containing uracil, which requires the use of a DNA polymerase that can read through uracil without hindering its proofreading activity (Fig. 2a).

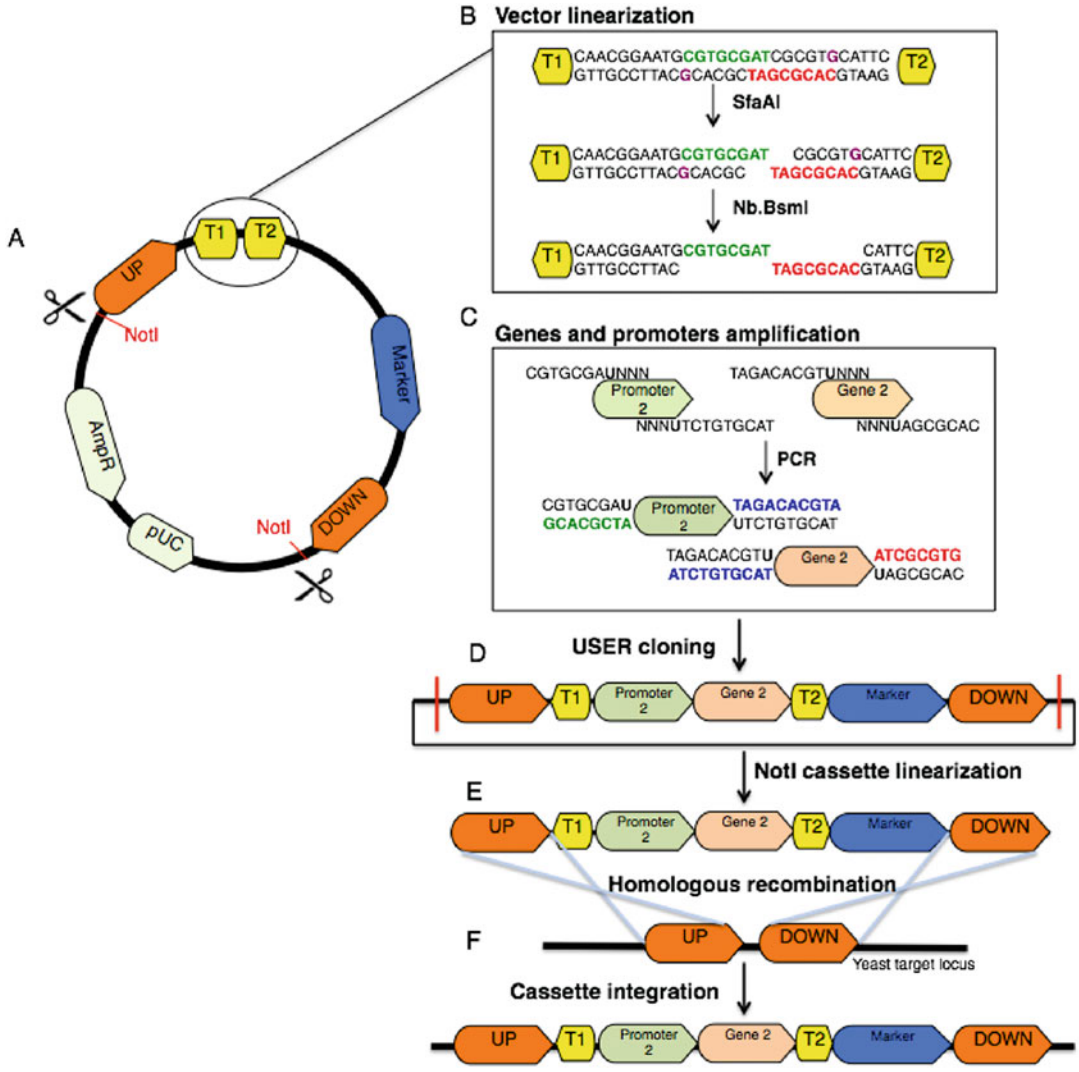

Fig. 2 Cloning genes and promoters into EasyClone vectors: (**a**) The basic vector backbone includes genetic element for vector replication—pUC—and selection—AmpR—in bacteria; NotI restriction sites flanking yeast homologous regions—UP and DOWN; an auxotrophic selectable marker gene; two yeast terminators—ADH1 and CYC1—T1 and T2 separated. (**b**, **c**) A two-step digestion of the vector generates long overhangs complementary to the USER tails of the amplified gene and promoter fragments. (**d**) The resulting vector carrying the expression cassette is propagated and purified from *E. coli*. (**e**) The purified plasmid is linearized by NotI digestion. (**f**) The linearized construct from the backbone is then transformed into yeast, where the homology regions (UP and DOWN) trigger homologous recombination of the construct into the yeast genome

The primers used for the optimal BenM biosensor design are listed in Table 2.

1. Prepare the PCRs as follows:

   Add to 50 μL nuclease-free water.

   10 μL 5× Phusion HF Buffer.

2 μL forward primer (10 μM).

2 μL reverse primer (10 μM).

1 μL dNTP mix (10 mM each).

X μL template DNA (typically 1–100 ng of plasmid or synthetic DNA).

1 μL Phusion U Hot Start DNA Polymerase.

2. Run the following PCR program:
98 °C for 30 s.

30 cycles of

98 °C for 10 s.

58 °C for 30 s (or another suitable annealing temperature).

72 °C for 30 s per 1 kb of the PCR product.

72 °C for 5 min.

10 °C hold.

3.2.4 Analysis and Purification of PCR Results

PCR products are analyzed by 1% agarose gel electrophoresis and purified from the gel using the NucleoSpin® Gel and PCR Clean-up from Macherey Nagel, eluting with 50 μL of elution solution. Amplified genes and promoters are stored at −20 °C.

3.3 Vectors Preparation

We use yeast-integrative plasmids from the EasyClone system as vector backbones (*see* **Note 6**). The biosensor and reporter will ultimately each be cloned into a different integration vector. In this example vector pCfB257 (targeting EasyClone integration site X-3) and pCfB262 (targeting site XII-4) will be prepared.

EasyClone vectors are composed of: pUC plasmid origin of replication and the ampicillin-resistance gene (AmpR), which allow for replication and selection in *E. coli*; NotI USER restriction sites flanking 500 bp homologous regions—named UP and DOWN—for homologous recombination into a target yeast genomic locus; an auxotrophic selectable marker gene (*Kl.LEU2* for pCfB257 and *Sp.HIS5* for pCfB262) to select yeast transformants; and, two yeast terminators ADH1 and CYC1 separated by an SfaAI restriction site. USER-cloned constructs of the biosensor and the reporter are designed to be inserted between the double terminators (Fig. 2c).

3.3.1 Composition of Expression Cassettes

To construct the CCM biosensor in yeast, we originally tested several promoters for controlling the expression of BenM and the yeGFP reporter gene. In the following we illustrate the example for constructing the optimal CCM biosensor using REV1p to control the expression of BenM, and 209bp_CYC1p_*BenO*_T1 to control the yeGFP reporter gene [16].

3.3.2 Vector Linearization

The backbone linearization is a two-step digestion to create first a double-strand cut and then a single-strand cut to generate long overhangs compatible with USER overhangs (Fig. 2b).

1. Prepare the reaction as follows:

   Add to 50 μL nuclease-free water.

   x μL of EasyClone vector (5 μg).

   5 μL of FastDigest® buffer.

   5 μL of FastDigest SfaAI® restriction enzyme.

2. Incubate for at least 2 h at 37 °C (*see* **Note 7**).
3. Purify the plasmid from solution, using NucleoSpin® Gel and PCR Clean-up kit from Macherey Nagel, eluting with 50 μL elution buffer.
4. Determine the DNA concentration.
5. Prepare the reactions as follows:

   Eluent (all).

   1 μL of Nb.BsmI/μg of digested vector.

   x μL of Buffer 3.1 (making up a 1:10 of the total volume).

6. Incubate for 1 h at 65 °C.
7. Purify the digested and nicked vector from the gel, using NucleoSpin® Gel and PCR Clean-up from Macherey Nagel. Elute with 50 μL of elution buffer (*see* **Note 8**).
8. Determine DNA concentration.
9. Store the USER-ready vectors at −20 °C.

3.4 Cloning of Genes and Promoters into Yeast Integration Vectors

The next step is USER cloning of the genes and promoters, generated in Subheading 3.2, into USER-ready integration vectors, generated in Subheading 3.3. Two integration vectors will be created. The first vector will contain BenM under the control of REV1p and will be targeted to EasyClone integration site X-3, whereas the second vector will contain the yeGFP reporter gene driven by 209bp_CYC1p_*BenO*_T1, and will be targeted to integration site XII-4.

3.4.1 USER Cloning (Fig. 2d)

1. Prepare the USER reaction as follows:

   Add to 6 μL nuclease-free water.

   1 μL of SfaAI/Nb.BsmI-treated vector (pCfB257 or pCfB262 (*see* **Note 9**)).

   1 μL of promoter fragment for position Promoter 2 (REV1p or reporter promoter).

   1 μL of gene fragment for position Gene 2 (BenM or yeGFP).

   1.2 μL 5× Phusion HF buffer.

   0.5 μL USER™ enzyme.

2. Incubate the mixture in PCR machine at the following conditions:

   37 °C for 25 min.

   25 °C for 10 min.

   20 °C for 10 min.

   15 °C for 10 min.

   10 °C pause.

3.4.2 Plasmid Amplification

The reaction mix is transformed into DH5α competent *E. coli* cells (kept at −80 °C)

1. Cool the USER reaction tubes on ice and add 50 μL of competent cells.
2. After 10 min on ice, perform heat shock at 42 °C for 45 s and place the tubes on ice for 1–2 min.
3. Plate the cells on LB plates with ampicillin and incubate at 37 °C overnight.

3.4.3 Vector Verification and Purification

The correct cloning of gene and promoter fragments into the EasyClone vector is established by PCR on bacteria colonies employing standard primers (*see* **Note 10**) that amplify the entire expression cassette (Table 3).

The colony PCR is performed as following:

1. Mix the following in a PCR tube:

   5 μL 2xOneTaq Master Mix polymerase.

   1 μL forward verification primer (10 μM) ID224.

   1 μL reverse verification primer (10 μM) ID225.

   3 μL nuclease-free water.

2. Add a small amount of *E. coli* colony biomass (it is enough to touch the colony with a pipette tip) to the PCR tube.
3. Run the following PCR program:

   94 °C for 3 min.

   35 cycles of

   94 °C for 20 s.

   50 °C for 30 s (or another suitable annealing temperature).

   68 °C for 1 min per 1 kb of the PCR product.

   68 °C for 5 min.

   10 °C pause.

4. Analyze the PCR reactions on 1% agarose gel.

5. The colony corresponding to the correct gel band size (*see* **Note 11**) is inoculated into 3 mL LB medium with ampicillin and cultivated at 37 °C overnight.
6. The plasmid is purified from the culture using NucleoSpin® kit from Macherey-Nagel.
7. The constructed vector is sequence validated using standard Sanger sequencing and DNA alignment tools.

3.5 Genomic Integration of Expression Constructs

Genomic integration ensures stable expression and reduced cell-to-cell variation in fluorescence output. Therefore, it is advised to integrate both the sensor and reporter constructs into defined loci (*see* **Note 12**). In this example the sensor construct is integrated into EasyClone site X-3, whereas the reporter construct is integrated into site XII-4.

3.5.1 Expression Vector Linearization

NotI restriction sites flanking UP and DOWN regions in the vector permit the release of the expression cassette needed for homologous recombination in yeast (Fig. 2e).

1. The reaction is set up as follows:
   x μL of expression vector (5 μg).
   2 μL of FastDigest® buffer.
   X μL of FastDigest NotI® (use 0.2 μL per 1 μg DNA).
   Add to 20 μL nuclease-free water.
2. Incubate the reaction at 37 °C for 1 h (*see* **Note 13**).
3. If the fragment is not purified from agarose, deactivate the enzyme by incubating the reaction at 65 °C for 15 min.
4. The digested vector can be stored at −20 °C for future use.

3.5.2 Transformation of Expression Vector into Yeast (Fig. 2f)

1. Streak CEN.PK113-5A on YPD agar and incubate at 30 °C for 1–2 days.
2. Inoculate 3–5 mL liquid YPD with CEN.PK113-5A and incubate at 30 °C O/N.
3. In the morning: Measure the OD_{600} of the O/N culture. To a culture flask containing 25–50 mL YPD (or media of choice) (*see* **Note 14**) add cells to give a starting density of approximately 5×10^6 cells/mL. Incubate the flask on a shaker at 30 °C and 200 rpm for two population doublings (typically 4–5 h).
4. When the culture has reached a density of at least 2×10^7 cells/mL harvest the cells by centrifugation, 3000 rpm (1630 × *g* in a 16.2 cm radius rotor) for 5 min.
5. Remove the supernatant and resuspend the cells in 25 mL sterile H_2O, centrifuge 3000 rpm (1630 × *g*) for 5 min and discard the supernatant.

6. Resuspend the cells in 1 mL sterile H_2O and transfer to an Eppendorf tube.
7. Centrifuge 3000 rpm (1630 × *g*) for 30 s and discard the supernatant.
8. Resuspend cells in a total volume of 1 mL H_2O.
9. For each transformation, including a water control, transfer the volume of cell suspension corresponding to 1×10^8 cells (typically 100 μL) to an Eppendorf tube.
10. Centrifuge at 3000 rpm (1630 × *g*) for 30 s and discard supernatant.
11. Make a transformation mix for the planned number of transformations plus one extra and keep it on ice. For each transformation prepare as follows:

    240 μL of PEG3350.

    36 μL of LiAc 1 M.

    10 μL of ssDNA (10 mg/mL).

    X μL DNA to transform.

    74–x μL of MilliQ water.

12. First add X μL of linearized vector corresponding to 300–700 ng (typically 1–10 μL) directly to the cells. Then make a master mix of PEG, LiAc, and ssDNA and add 360-X μL for each transformation. Resuspend the cells by vortex mixing vigorously or pipetting up and down.
13. Incubate the tubes at 42 °C for 40 min.
14. Centrifuge at 3000 rpm (1630 × *g*) for 30 s and remove the transformation mix with a pipette.
15. For selection of amino acid markers: resuspend cells in 100 μL H_2O, and plate on SC-his-leu plates (or markers of choice) and incubate at 30 °C.
16. Check the transformation plate after 2–3 days.
17. Pick a chosen number – we recommend 8 – of colonies and replicate them on selective media. Incubate for 1–2 days at 30 °C.

3.5.3 Genome Integration Verification

The growth of transformants on selective media is not definitive proof of integration in a specific locus. To verify correct genome integration of the linearized fragments, carry out yeast colony PCR as follows:

1. Take a small amount of colony material and resuspend it in 50 μL of 20 mM NaOH.
2. Incubate for 10 min at 100 °C.
3. Spin down the debris and keep the supernatant (DNA).

4. Set up the following genotyping PCR:
   6 μL water.
   2 μL 2xOneTaq Master Mix polymerase.
   1 μL primer 1 (10 μM).
   1 μL primer 2 (10 μM).
   2 μL of DNA.
5. Run the following PCR program:
   94 °C for 1 min.
   35 cycles of
   94 °C for 20 s.
   50 °C for 30 s (or another suitable annealing temperature).
   68 °C for 1 min/kb of the PCR product.
   68 °C for 7 min.
   10 °C pause.
6. Analyze the samples on 1% agarose gel (*see* Table 4).

3.6 Flow Cytometry

In this protocol, biosensor performance is determined using flow cytometry. In short, each strain is pre-cultured individually, and then subcultured both in control medium and in medium with added inducer followed by flow cytometry analysis. Both the level of background fluorescence and the fold induction (fluorescence intensity in the induced state divided by fluorescence intensity in the non-induced state) are important parameters to determine the performance of the biosensor design.

There are two reasons a flow cytometer is used for this purpose. First, the reporter promoter activity of the biosensor design illustrated in this protocol is low, and therefore characterization requires sensitive apparatus to detect the fluorescent signal. Second, flow cytometry allows for single-cell measurements of fluorescent intensity, which allows for insight in the distribution of the signal within the population (Fig. 3).

3.6.1 Induction Media Preparation

It is advisable to prepare pre-mineral medium as described in Subheading 2.1 (*see* **Note 15**). The reason for this is that some inducers, such as CCM, are unstable, and should only be added to the medium on the day the medium is needed.

For the preparation of 100 mL of the induction medium, dissolve 20 mg CCM in 90 mL of pre-mineral medium with a magnetic stirrer, adjust the pH to 4.5 (*see* **Note 16**) and finally adjust the final volume to 100 mL using deionized water, filter-sterilize and use the same day (*see* **Note 17**).

For 100 mL of control mineral medium, the recipe is the aforementioned but without the addition of CCM.

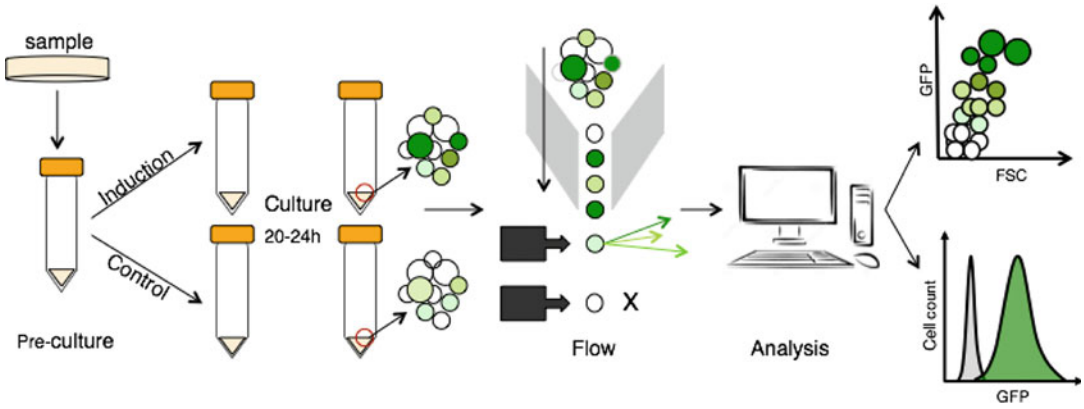

Fig. 3 Flow cytometry analysis of biosensor performance. The yeast strain harboring the sensor and reporter is inoculated for a 24 h pre-culture; subsequently the cells are subcultured into two fresh cultivations either in the absence or presence of the ligand and incubated for another 20–24 h. The cells are diluted in PBS and subjected to flow cytometry for single cell recording of fluorescence level. The output data are exported and more thoroughly analyzed with the use of external software

3.6.2 Culturing Strains and Induction of Biosensor

Ultimately, the performance of each strain is judged by fluorescence measurements of the culture grown in mineral medium with and without inducer. When analyzing a large number of strains, it is advisable to use 96-well plates both for the pre-culture as well as the subculturing step. In addition to the strains containing the biosensor design(s) of interest, it is recommended to analyze the following control strains:

1. WT CEN.PK strain: to assess auto-fluorescence.
2. No sensor, reporter-only control strain: to assess background expression of the reporter gene in the absence of the biosensor.

Day 1: Inoculate 3 single colonies of each strain into 3 different wells of a 96-well plate containing 150–500 μL SC-his-leu per well. Incubate at 250 rpm, 30 °C for 16–20 h.

Day 2: Subculture the strains 1:100 by transferring 5 μL of the overnight culture to both 500 μL control mineral medium and 500 μL induction mineral medium. Incubate at 250 rpm, 30 °C for 20–24 h.

Day 3: After 20–24 h growth, transfer 30 μL of each culture to 150 μL PBS in a 96-well plate right before flow cytometry analysis.

3.6.3 Flow Cytometry and Data Analysis

1. Analyze each culture by flow cytometry with a 488 nm laser for validation of single strains.
2. For each biological replicate of each strain, record 10,000 single-cell events by gating in the forward scatter (FSC) and side scatter (SSC) channels.
3. Export .FSC files.

4. Data can be analyzed using for example FlowJo software (TreeStar Inc.).
5. Calculate the mean fluorescence intensity (MFI) of each biological replicate.
6. Calculate the fold change induction by dividing the MFI of the induced (ON) state by the MFI of the control (OFF) state.
7. Calculate the average and standard deviation of each strain of the MFIs and fold-change induction based on the biological replicates ($n = 3$).

3.6.4 Determining the Operational Range and Specificity of the Biosensor

In order to apply a biosensor for screening cell factory performance, the operational range and specificity of the biosensor needs to be determined. In order to do this, the same steps as in Subheadings 3.6.1–3.6.3 are followed with the following modifications:

1. To determine the operational range: grow each strain in a series of different media that contain increments in the concentration of inducer, for examples 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4 mM CCM. Plot the average of the mean fluorescence intensity against the inducer concentration to obtain a response curve (Fig. 4a).
2. To determine the specificity: grow each strain in a series of media that have been supplemented with different inducers (*see* **Note 18**), such as pathway intermediates or chemicals that are structurally similar to the compound of interest. In the case of CCM, malonic acid, protocatecuic acid, fumaric acid, and succinic acid can be tested. Plot the average of the mean fluorescence intensity for both control medium, media with different inducers, as well as medium with CCM (Fig. 4b).

4 Notes

1. A minimal transcription unit (TU) in prokaryotes is composed of several genetic elements: a regulatory region termed a promoter, a transcription start site (TSS), open-reading frames (ORFs) encoding one or more genes, and a transcription termination sequence (TTS) termed a terminator. Importantly, the regulatory region contains the promoter where RNA polymerase and transcription factors (TFs) bind in order to modulate the activity of the promoter. Also, in many cases, prokaryotic transcription initiation requires proteins known as sigma factors (s) that enable proper promoter recognition by RNA polymerase. In general transcription regulation can have a negative effect on promoter activity when the TF, also known as a repressor, binds to the promoter and thereby physically

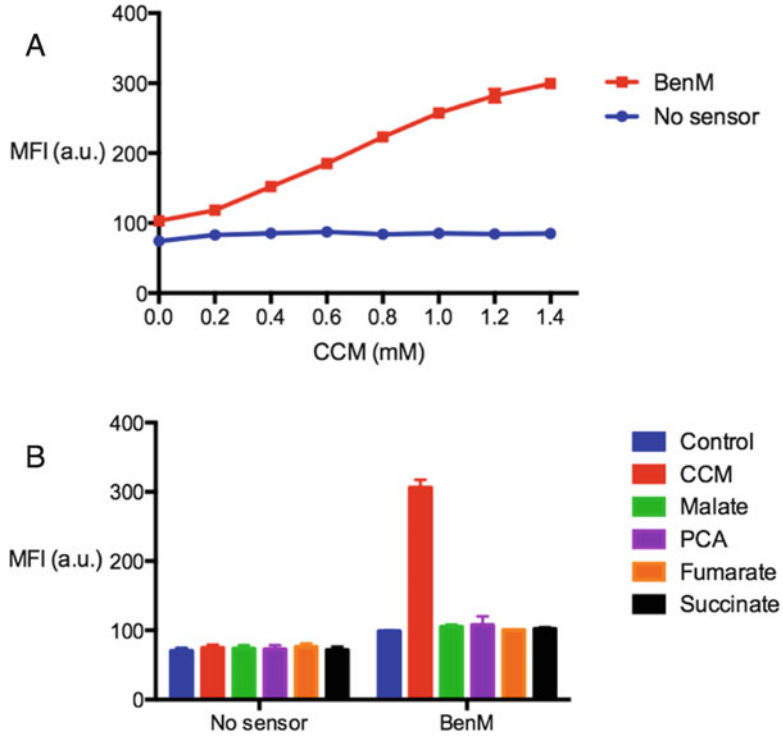

Fig. 4 Operational range and specificity of CCM biosensor. (**a**) Response function for a yeast harboring the optimal CCM biosensor design (BenM) as well as a control strain only expressing yeGFP from 209bp_CYC1p_-*BenO*_T1 (No sensor). The mean fluorescence intensity (MFI) was measured by flow cytometry 24 h after subculturing in the presence of different concentrations of CCM. (**b**) Specificity of CCM biosensor was determined by measuring yeGFP expression 24 h after subculturing in the presence of various dicarboxylic acids (1.4 mM) and in control medium

interferes with RNA polymerase binding to the promoter (Fig. 1a). In contrast, a positive effect on promoter activity can occur when the regulator, known as activator, bind to the promoter's upstream region to recruit RNA polymerase and initiate transcription (Fig. 1b), or when the repressor undergoes a conformational change disabling its DNA binding and thereby relieving repression (Fig. 1c). In the case of BenM, the TF is constitutively bound to DNA, and only in the presence of CCM the BenM tetramer changes conformation enabling a shift in its binding to the operators. This shift is believed to allow access to DNA binding of the RNA polymerase and thereby CCM-induced activation of expression [18] (Fig. 1d). In native hosts, TFs work jointly and a regulatory region can be occupied by several TFs. Moreover, different sites are able to recruit the same TF, and different TFs can recognize similar sites. Theoretically, the regulatory effect on expression depends on the TF concentration and TF–operator binding affinity: to function, strong sites work with a lower amount, likely for

fine-tuning regulation of important genes, while weak sites require high concentrations of TFs, such as global TFs that are known to be less specific. Furthermore, there are TFs with a dual regulatory role, functioning as activators and repressors at the same time. This is a common theme in sugar catabolism [23].

2. Our design has been tested and validated for other transcriptional activators of the LTTR superfamily as well. Here we just present the protocol for the transplantation of BenM.
3. For the malonic acid biosensor described in our research paper, we first integrated the *SpMAE1* gene, coding for a dicarboxylic acid plasma membrane transporter, to allow for uptake of malonic acid.
4. In case the bacterial sequence has the alternative start codon GTG, change this to ATG.
5. Other fluorescent proteins can be used as well. We also have good experience with sfGFP.
6. Any given integrative plasmid backbone can be used.
7. It is advised to incubate overnight for efficient digestion.
8. The quality of the digest can be tested by gel electrophoresis analysis.
9. Use vector to insert molar ratio 1:3.
10. Standard verification primers are suitable for the amplification of fragments no longer than 5 kb.
11. The sizes are 2025 bp for the construct REV1p-BenM; 1109 bp for 209bp_CYC1p_*BenO*_T1-yeGFP.
12. Integration of the reporter is in our experience most crucial. We have seen comparable results when the sensor is expressed from a centromeric plasmid.
13. Optional step to confirm linearization on the gel and if desired purify the correct fragment from the gel using NucleoSpin® Gel and PCR Clean-up from Macherey Nagel. NotI-digested fragment sizes are 6099 bp for the REV1pr-BenM expression cassette and 4102 bp for the 209bp_CYC1p_*BenO*_T1-yeGFP. Be aware of the other backbone fragment of 2.8 kb.
14. Adjust depending on the number of transformations needed. 5 mL culture is typically needed per transformation.
15. Add amino acids depending on the auxotrophies of your strain. Since our strains are auxotrophic for tryptophan, we add tryptophan here.
16. Only protonated acids will passively diffuse across the yeast cell membrane. In order to characterize the CCM biosensor we used medium with pH 4.5 in order to leave most of acid in the

protonated state (CCM pKa = 3.87). The pH of the medium should be tuned with the pK_a of your inducer in case uptake of the chemical relies on passive diffusion.

17. It is essential to add and dissolve CCM in mineral medium on the same day the medium is used. We noticed decreased induction of the sensor-reporter when using medium that has been standing for more than a few days. Other inducers might be more stable, though.
18. Use the same molarity of these inducers as the most relevant molarity that was used for the compound of interest (typically the highest molarity).

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