Chapter 7

Synthetic RNA Switches for Yeast Metabolic Engineering: Screening Recombinant Enzyme Libraries

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Abstract

Directed evolution is a powerful technique for increasing the activity of poorly active enzymes, for example when an enzyme is engineered to accept a new substrate or function in a new environment. Since enzyme activity greatly depends on the enzyme environment, screening should be performed under the same conditions as the ultimate application of the enzyme. When an enzyme will be used in live cells, RNA biosensors offer a powerful and flexible method of linking the desired phenotype, production of a small molecule of interest, to an easily measured phenotype, such as fluorescence. Here, we describe methods for screening enzyme libraries using an RNA biosensor, showing examples from the evolution of a P450 monooxygenase.

Key words RNA switch, Directed evolution, P450, High-throughput enzyme screening

1 Introduction

Metabolic engineers seek to modify microbial biosynthetic pathways with the ultimate goal of producing chemicals, such as antibiotics or pharmaceuticals, in an affordable and environmentally friendly fashion. While researchers have made great strides in developing rational methods for constructing and optimizing metabolic pathways, evolutionary methods can often provide a powerful alternative. In any evolutionary optimization, the challenge is to quickly separate out improved variants from the much larger pool of variants with neutral or deleterious changes. As a result, the assay throughput affects both the potential library size and the cost in time and materials required for screening. Unfortunately, many existing high-throughput assays function in vitro [1], while applications often require that an enzyme or pathway function in vivo. As a result, there exists a pressing need for high-throughput screening platforms that function in living cells [2].

In this chapter, we describe a method for screening enzyme libraries in *Saccharomyces cerevisiae* using a synthetic RNA switch [3].

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The switch couples an input domain, an RNA aptamer, to an output domain, a ribozyme [4] (Fig. 1a). The details of aptamer selection [5] and switch construction [6] are left to separate protocols. The switch we used in this example binds with high affinity to theophylline, a clinical bronchodilator, but does not bind to caffeine at physiologically relevant concentrations. The ability to distinguish between these closely related compounds allows us to detect the enzymatic conversion of caffeine into theophylline. We used as our starting enzyme a mutant of CYP102A1 (i.e., a P450 monooxygenase from *Bacillus megaterium*) that showed low, but detectable, activity as a caffeine demethylase. Using this method, we were able to iteratively screen mutant libraries of our model P450 monooxygenase in vivo, ultimately increasing the activity of the final variant by more than 30-fold.

In this protocol, we use error-prone PCR as an example of a library generation step (Fig. 1c, d). The RNA switch is then used to link enzymatic activity and GFP fluorescence, such that cells containing highly active enzymes also express high levels of GFP (Fig. 1e). As a result, enzyme libraries can be rapidly screened in vivo, using fluorescence measurements to quickly identify the variants with improved activity. We describe two methods for fluorescence screening. The first, screening in 96-well plates using flow cytometry, is very sensitive but is limited to library sizes of several thousand members (Fig. 1f). As a result, this method is most useful when evolving a poorly active enzyme or pathway. The second method, screening by fluorescence-activated cell sorting (FACS), is less sensitive but can easily screen libraries of several million members (Fig. 1g). Once the parental enzymatic activity is sufficiently high, screening by FACS can be extremely powerful.

2 Materials

2.1 Library Generation

1. SD (synthetic dropout) media: 5 g/L ammonium sulfate, 1.7 g/L yeast nitrogen base, 1× amino acid supplement, 2 % glucose. Combine 2.5 g ammonium sulfate and 0.85 g yeast nitrogen base without amino acids or ammonium sulfate in a final volume of 400 mL ultrapure water. Autoclave, then add 50 mL of filter-sterilized 10× amino acid dropout stocks (Clontech, Mountain View, CA) and 50 mL of autoclaved 20 % (w/v) glucose.
2. Yeast extract-peptone-dextrose (YPD) media: 10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose. Combine 5 g yeast extract and 10 g peptone in a final volume of 450 mL ultrapure water. Autoclave. Add 50 mL of autoclaved 20 % (w/v) glucose.
3. Tris-DTT: 2.5 M dithiothreitol, 1 M Tris–HCl, pH 7.5. Dissolve 0.39 g dithiothreitol in 1 mL of 1 M Tris–HCl, pH 7.5. Filter-sterilize and freeze at −20 °C.

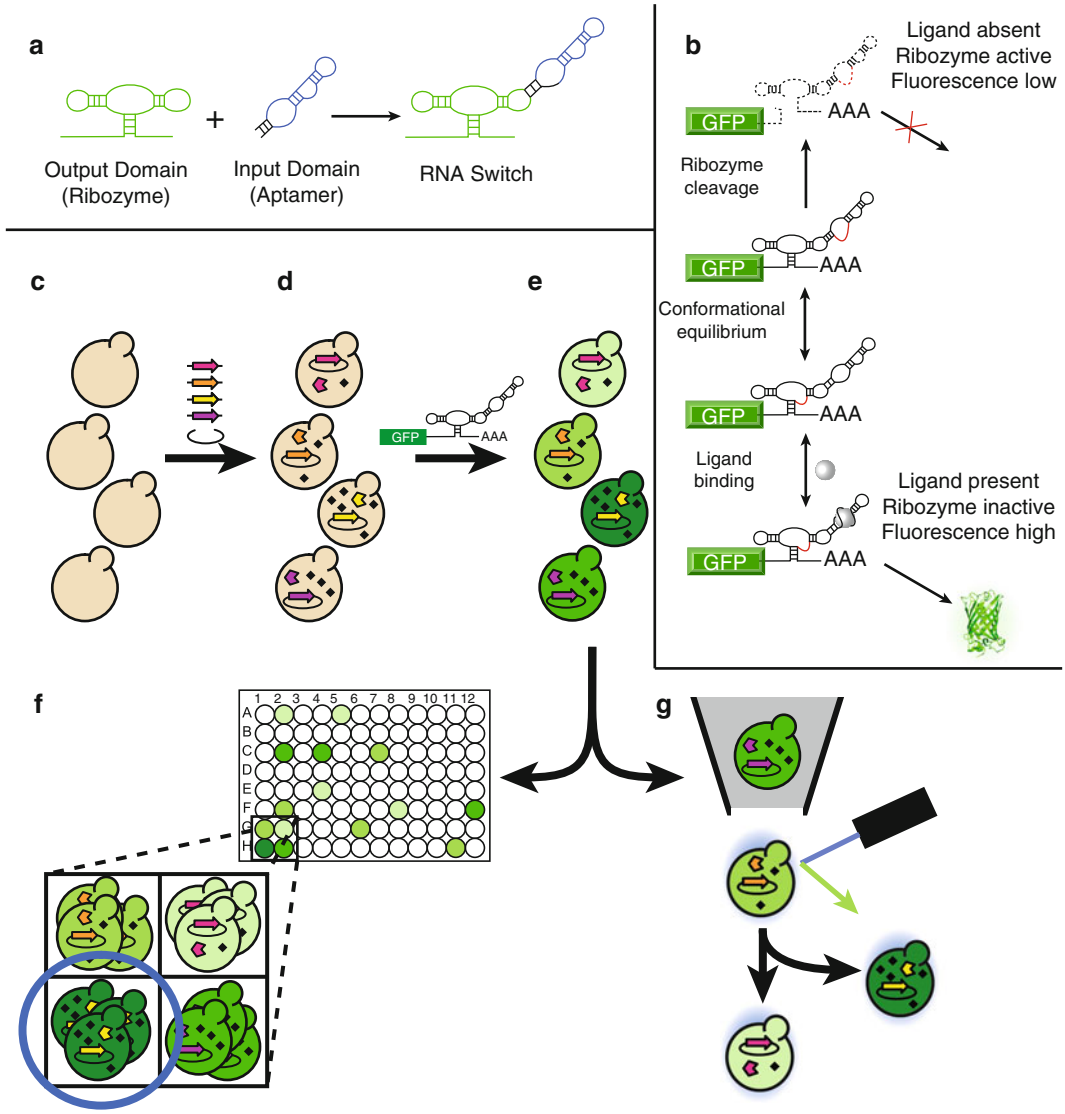

Fig. 1 Screening overview. (**a**) A synthetic RNA switch combines an input domain (an aptamer) with an output domain (a ribozyme) in such a way that only one of the two domains can properly fold at any given time. (**b**) When the RNA switch is placed in the 3′ UTR of a screenable gene such as GFP, it exists in a conformational equilibrium between the states in which the ribozyme is properly folded or the aptamer is properly folded. If the ribozyme folds, it will cleave, removing the poly-A tail from the associated mRNA, leading to degradation of the mRNA and low expression. Ligand binding stabilizes the form in which the aptamer is folded and the ribozyme is not, leading to decreased cleavage and increased expression. (**c**, **d**) An enzyme library is constructed in yeast using gap repair. Some enzymes are more active, making more product (*black diamonds*). (**e**) When combined with GFP under the control of the synthetic RNA switch, cells with highly active enzymes also make more GFP. (**f**) Screening can be performed in clonal culture in 96-well plates or (**g**) in single cells by FACS. Adapted, with permission, from Metabolic Engineering, 14(4). Michener JK and Smolke CD. High-throughput enzyme engineering in *Saccharomyces cerevisiae* using a synthetic RNA switch. pp. 306–316. 2012

4. Buffer E: 10 mM Tris–HCl, pH 7.5, 270 mM sucrose, 2 mM $MgCl_2$. Dissolve 92.4 g sucrose in a final volume of 990 mL ultrapure water. Add 10 mL of 1 M Tris–HCl, pH 7.5 and 1 mL of 2 M $MgCl_2$. Filter-sterilize.
5. Electroporator: (e.g., GenePulser Xcell, Bio-Rad, Hercules, CA).
6. Mutazyme II DNA polymerase (Agilent, Santa Clara, CA). This polymerase is engineered to reduce the bias in the types of mutations it introduces. A similar error-prone polymerase or a PCR reaction performed with Mn^{2+} will also suffice.
7. Gel extraction kit (e.g., Qiagen, Valencia, CA).
8. 2 mm gap electroporation cuvettes.

2.2 Plate-Based Library Screening Materials

1. Deep 96-well plates (e.g., #62406-496, BD, Franklin Lakes, NJ). Wrap in aluminum foil and autoclave.
2. Toothpicks: autoclave.
3. Enzyme substrate: 25 mM caffeine for the described example (i.e., mutants of CYP102A1). Dissolve 485 mg of caffeine in 100 mL of ultrapure water. Filter-sterilize. Choose the appropriate substrate and the appropriate concentration(s) according to the enzyme activity to be tested.
4. Flow cytometer with plate loader (e.g., Cell Lab Quanta MPL, Beckman Coulter, Brea, CA).
5. Plate shaker (e.g., LT-X, Kuhner, Basel, Switzerland).
6. AeraSeal film (e.g., Excel Scientific, Victorville, CA).
7. Yeast miniprep system (e.g., Yeast Miniprep II, Zymo Research, Irvine, CA).

2.3 FACS-Based Library Screening Materials

1. Resuspension buffer: 1× phosphate-buffered saline (PBS), 1 % (w/v) bovine serum albumin (BSA). Combine 100 mL 10× PBS (1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 18 mM KH_2PO_4) with 900 mL ultrapure water. Dissolve 10 g BSA (Fraction V) in 1 L 1× PBS and filter-sterilize.
2. DAPI: 5 g/L 4′,6-diamidino-2-phenylindole (DAPI) in ultrapure water. Filter-sterilize and store at −20 °C.
3. Cell strainer: 40 μm pore (Becton Dickinson, Franklin Lakes, NJ).
4. FACS: Aria II (Becton Dickinson, Franklin Lakes, NJ).

3 Methods

3.1 Screening Strain Construction

1. Identify or construct an RNA aptamer that binds to the small molecule of interest [5] and use that aptamer to build a synthetic RNA switch [6]. The switch must be able to distinguish

between functionally relevant concentrations of the desired small molecule (*see* **Note 1**). Use standard molecular biology techniques to clone the RNA switch downstream of the output fluorophore, such as GFP.

2. To reduce the effects of expression noise on the biosensor sensitivity [3], express the GFP-switch combination from a constitutive promoter such as TEF1 and integrate the resulting construct into the genome of the screening strain (*see* **Note 2**). If screening by FACS, integrate a second expression construct in which the GFP-switch combination is replaced by an unregulated mCherry coding sequence. The mCherry fluorescence will later be used to normalize for extrinsic noise in fluorescent protein expression [7].

3.2 Library Generation

1. Begin with a prep of pure plasmid DNA encoding your enzyme of interest (e.g., a caffeine demethylase) in a yeast expression vector. Design 50 bp primers that flank the region you wish to mutate, preferably just outside a pair of unique restriction sites.
2. Day 1: Amplify the enzyme region of interest in an error-prone PCR using the Mutazyme II kit, using a single 20 μL reaction for each mutagenic level (*see* **Note 3**). Extract the resulting PCR products from an agarose gel, and then treat the extracted DNA with DpnI (*see* **Note 4**). Finally, use the mutagenic PCR reactions as templates in a new set of high-fidelity PCRs, running two separate 100 μL PCRs for each transformation (*see* **Note 5**). Cut the plasmid overnight with the two unique restriction enzymes and, optionally, a third enzyme that cleaves the center of the insert (*see* **Note 6**). Digest 1 μg of plasmid DNA per transformation. Inoculate a 5 mL overnight culture of YPD with the destination yeast strain and grow at 30 °C (*see* **Note 7**).
3. Day 2: Dilute destination strain into 50 mL of fresh YPD at OD 0.01 and grow at 30 °C. Meanwhile, mix the PCR products and digested vector. For replicate transformations of the same library, all of the DNA may be mixed in a single tube. Phenol/chloroform extract and ethanol precipitate each DNA mixture. Dry the precipitated DNA on the bench top. Grow the yeast culture to OD 1.3, and then add 500 μL of Tris-DTT to aid in cell permeabilization [8]. Incubate, shaking, at 30 °C for an additional 15 min. Centrifuge at 2,500 × *g* and 4 °C for 3 min, decant supernatant, and gently resuspend in 25 mL of ice-cold Buffer E. Centrifuge again at 2,500 × *g* and 4 °C for 3 min, decant supernatant, wash with 1 mL ice-cold Buffer E, transfer to a 1.5 mL tube, and centrifuge once more at 2,500 × *g* and 4 °C for 3 min. Resuspend the cells in ice-cold Buffer E to a final volume of 300 μL (*see* **Note 8**).

4. Use the cell suspension to resuspend the precipitated DNA mixture (insert + vector), using 55 μL of cell suspension per transformation. Transfer 50 μL of the cell + DNA mixture into a 4 °C electroporation cuvette and electroporate at 540 V, 25 μF, and 1,000 Ω. Add 1 mL of 30 °C YPD to the cuvette and transfer to a 15 mL tube. Wash the cuvette with another 1 mL of 30 °C YPD and transfer to the same tube. Incubate the tube, shaking, at 30 °C for 1 h. Centrifuge at 5,000 × *g* and 4 °C for 5 min, and then resuspend the cells in 10 mL of appropriate SD media.

3.3 Plate-Based Library Screening

1. Plate dilutions of the transformation mix(es) on appropriate SD plates and incubate at 30 °C for 2 days. Meanwhile, store the excess transformation mix at 4 °C. Count colonies on the resulting plates to determine the complete library size (*see* **Note 9**). Replate the transformation mix at an appropriate dilution to yield ~200 colonies per plate, using as many plates as necessary to achieve the desired number of colonies.
2. Day 1: every 2 h, for a total of six plates, set up a new 96-well "growth" plate of colonies (*see* **Note 10**). Aliquot 400 μL of SD media into each well of a sterile 96-well plate. Using sterile toothpicks, pick colonies from the agar plate and inoculate each well with a unique colony. Reserve the corner wells for positive controls (cells containing the parental enzyme). Cover with AeraSeal film and incubate in the plate shaker, at 30 °C and 480 rpm, overnight.
3. Day 2: every 2 h, inoculate a new growth plate as before, for a total of another six plates. Similarly, every 2 h, dilute the oldest growth plate 100× into a new deep-well "assay" plate. For the assay plate, aliquot 400 μL of SD media containing the desired substrate concentration (e.g., 1 mM caffeine). Transfer 4 μL of cell suspension from each well of the growth plate to the corresponding well of the assay plate. Cover with new AeraSeal film and incubate the assay plate as before. Store the sealed growth plate at 4 °C.
4. Day 3: every 2 h, inoculate a new growth plate and dilute the oldest remaining growth plate into a new assay plate. Remove the oldest assay plate and dilute 100 μL of cells into 300 μL of ultrapure water (*see* **Note 11**). Store the remaining cell suspensions at 4 °C. Analyze the diluted cells on a flow cytometer equipped with a multi-plate loader. Excite cells at 488 nm and measure GFP fluorescence at 525 nm. Gate by cell volume and side scatter to isolate the population of live cells. Then normalize the fluorescence by cell volume and use the geometric mean as a measure of the sample fluorescent intensity. For each plate, compare the fluorescent intensities of the samples to the average intensity of the four positive controls (at the corners).

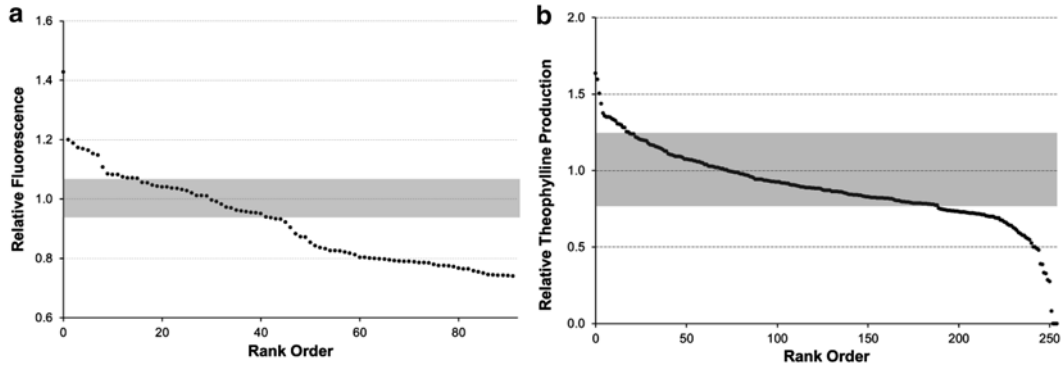

Fig. 2 Representative screening data. (**a**) Representative data screening a single 96-well plate by flow cytometry. Many cultures have mean fluorescence levels similar to the parent (the *grey box* shows one standard deviation around the parental fluorescence). A small fraction of the cultures have significantly higher fluorescence, and these are the cultures that should be screened for enzymatic productivity by HPLC. (**b**) Representative HPLC data for an entire batch of screening. ~1,600 cultures were assayed by flow cytometry, and the top ~250 by HPLC. Nearly every clone was active, compared to ~50 % activity in the unscreened population. Many clones showed activity similar to the parent (the *grey box* shows two standard deviations around the parental mean). However, several clones were significantly more active than the parent, and these clones were carried forward to the rescreening process

Rank the samples, and identify the top hits (Fig. 2a). Centrifuge the stored assay plates at 4,000 × *g* and 4 °C for 10 min. Remove the supernatant of the top hits and analyze it by HPLC using an appropriate protocol for the metabolite of interest (*see* **Note 12**). The assay plates can then be washed and autoclaved for reuse.

5. Day 4: every 2 h, dilute the oldest remaining growth plate into a new assay plate and assay the oldest assay plate (*see* **Note 13**).
6. Day 5: every 2 h, measure the oldest assay plate.
7. Collect the top hits from each day of screening (Fig. 2b). Return to the stored growth plates and inoculate a new growth plate of the top hits, again reserving the corners for controls. Grow overnight, then dilute into an assay plate as before. Regrow overnight, then assay by flow cytometry and HPLC as before.
8. Prep plasmid DNA from consistent hits using a yeast miniprep kit. Transform the plasmid DNA into *E. coli*, grow up the resulting colonies, and sequence. Also, retransform the purified plasmid back into *S. cerevisiae* to reconfirm activity (*see* **Note 14**).

3.4 FACS-Based Library Screening

1. Plate dilutions of the transformation mix on appropriate SD plates to determine the library size (*see* **Note 15**). A typical library size is 4×10^6–10^7. Meanwhile, dilute the transformation mix into 250 mL of appropriate SD media. Grow, shaking, at 30 °C. After 24 h, dilute 10× into 250 mL fresh SD media.

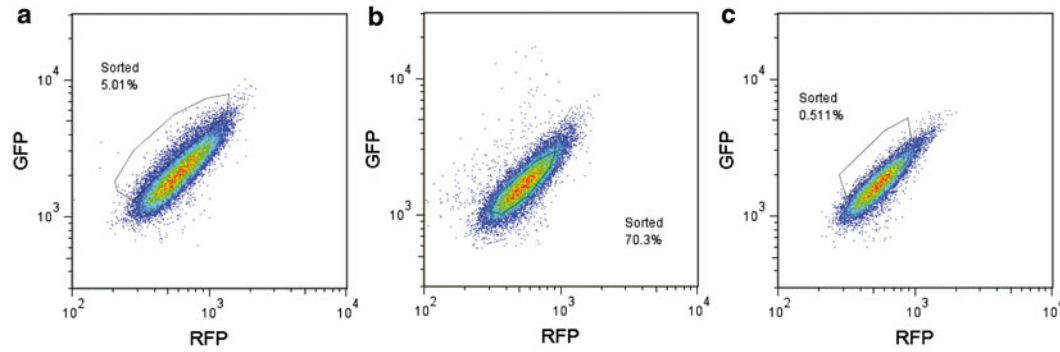

Fig. 3 Representative sorting results. (**a**) For the first positive sort, grow the cells in the presence of the enzymatic substrate and collect the ~5 % of cells with high GFP fluorescence relative to their RFP fluorescence. (**b**) In the second negative sort, grow the cells in the absence of the substrate and collect the ~70 % of cells with background levels of GFP fluorescence. This step removes cells with constitutive high GFP expression. (**c**) In the final positive sort, grow the cells in the presence of the enzymatic substrate and collect the ~0.5 % of cells with the highest relative GFP fluorescence

After another 24 h, dilute 30× into 250 mL fresh SD media. After a further 24 h, dilute 100× into fresh SD media containing your enzymatic substrate (*see* **Note 16**). Grow for a final 24 h, and then sort by FACS.

2. First sort—positive: The specific details of the sorting will depend on the instrument used. For the machine detailed above, spin down 10 mL of the sorting culture at 6,000 × *g* and 4 °C for 5 min. Resuspend in 6 mL of PBS-BSA and filter through a cell strainer. Add DAPI to the cell suspension at 1:1,000, to stain dead cells during sorting. Split the cell suspension into two 5 mL polystyrene tubes. Add 1 mL of SD media to a 5 mL polypropylene collection tube.
3. Excite GFP at 488 nm and measure at 525 nm. Excite mCherry at 532 nm and measure at 610 nm. Excite DAPI at 355 nm and measure at 450 nm. Gate your cells by forward and side scatter to remove debris. Then gate for DAPI-(-) cells to avoid dead cells. Sort 100,000 cells from the library to analyze the fluorescence distribution in two dimensions (GFP and RFP). Set a sorting gate to collect the appropriate percentage of cells with a significantly higher GFP/RFP ratio (for an initial sort, the top 5 % is a typical sorting window, *see* Fig. 3a). Screen the library, preferably oversampling the expected library diversity by at least threefold. Collect the sorted cells into the polypropylene collection tube.
4. Second sort—negative: Dilute the cells into 50 mL of fresh SD media, and inoculate a negative control (dual-color screening strain with an empty expression plasmid) into 5 mL of SD media. Grow both cultures for 24 h. Prepare the library for FACS as before. Spin down the entire negative control culture

and resuspend in 1 mL of PBS-BSA. Pass through the cell strainer, add DAPI, and transfer to a 5 mL polystyrene tube. When sorting, first analyze the negative control culture. Set your sorting gate based on the GFP/RFP profile of the negative control culture, collecting the region of highest density (containing ~80 % of the population). Then sort the library using that same gate, typically collecting ~70 % of the population (Fig. 3b). Oversample the expected library diversity by at least 3×. Collect the sorted cells as before.

5. Third Sort—Positive: Dilute the sorted cells into 25 mL of SD media. Grow 24 h, then dilute 100× into 25 mL fresh SD media containing your substrate (e.g., 1 mM caffeine). Grow for a final 24 h, and then sort as in **steps 2** and **3**. Set a more stringent sorting gate, collecting only the top ~0.5 % (Fig. 3c). Collect ~2,000 cells. Plate these cells on agar plates, at a plating density of ~300 cells per plate. Assay the individual colonies in 96-well plates as described above in Subheading 3.3. For any consistently improved clones, prep the plasmid DNA, transform into *E. coli* to sequence and transform into the screening strain to verify the improved activity.

4 Notes

1. Ultimately, the RNA switch will be used to identify rare enzyme or pathway variants that increase product titers. The most important design parameter for a switch, therefore, is the ratio of fluorescence produced by the improved variant relative to the parent. The higher this ratio, the easier it is to separate improved variants from those containing neutral or deleterious changes; screening by FACS, for example, requires a higher ratio than screening by flow cytometry. Several factors can affect this ratio: first, the change in product titer will depend on the optimization strategy, and smaller improvements in titer will produce smaller ratios. Second, the binding affinity of the switch should be matched as closely as possible to the concentration of the small molecule being sensed. If these values diverge significantly, even a large change in product titer may produce only a small change in fluorescence. Third, the dynamic range of the switch directly affects the fluorescence ratio. If the resolution of the switch is insufficient for the desired screening application, one of these factors will need to be improved. Finally, if the RNA switch will be used to iteratively screen for improved enzymes or pathways, then the switch transfer function must either have a graded response, as is true of the switch used in this method [4], or a tunable threshold [9]. A switch with a threshold response at a fixed

input concentration can only be used to discriminate between a small range of product concentrations and is unlikely to be useful for screening a progressive series of enzymes or pathways.

2. Reducing expression noise is more important when screening by flow cytometry (where expression noise affects the precision with which the population mean is determined) than when screening by FACS (where the second fluorophore helps compensate for the variability in plasmid copy number). However, FACS screening with the biosensor on a low-copy plasmid can lead to identification of variants with mutations in the plasmid centromere [10]. These mutations produce highly variable expression levels and, correspondingly, a phenotype that is difficult to remove when screening mixed populations. We are aware of no disadvantage to integrating the biosensor into the host genome.
3. It may be helpful to produce multiple libraries (~4) with different mutation rates from a single template. Initial screening can identify the library with the best mutation rate and thereby avoid screening an unproductive library.
4. Gel extraction may not be necessary if your PCR products have a single band. DpnI digests only methylated DNA and will, therefore, digest the template DNA without affecting the unmethylated PCR products. DpnI should be added after the gel extraction, since the amount of template DNA necessary for the Mutazyme kit may obscure your PCR product on the gel.
5. For plate-based screening, a single transformation per library is usually sufficient to yield more clones than can reasonably be screened. For FACS-based screening, 5 transformations per library produce a corresponding increase in the library size.
6. With triple-digested vector and extracted/DpnI treated insert, either control transformation (vector only or insert only) gave $<10^2$ transformants compared to $>10^5$ for the desired gap repair library.
7. Using SD media gives noticeably smaller library sizes. If it is necessary to grow the destination yeast in dropout media, one alternative is to grow the overnight culture in dropout media but then dilute into YPD for the final growth period.
8. The cell pellet may be >100 μL in volume, so the additional volume of Buffer E may be substantially lower than 300 μL.
9. If screening multiple libraries with different mutation rates, pick and assay a single 96-well plate from each library as described in **steps 2** and **3**. Measure the product concentration of each culture and plot the distribution of enzymatic activities in each library. Use this plot to choose the best library for screening (typically with ~50 % of the enzymes active).

10. Screening a single plate on the Beckman Quanta flow cytometer described above takes ~90 min. Spacing the plates by 2 h ensures that the incubation times are consistent and allows flexible timing in case of clogs or other disturbances. If a different flow cytometer is used, the plate timing should be adjusted accordingly. Additionally, the Kuhner plate shaker described above holds 12 plates, limiting daily throughput to six plates. Using a different plate shaker may allow for higher or lower daily throughput.
11. Diluting the cells 4× reduces the clogging rate of the flow cytometer.
12. This HPLC protocol is specific to compounds (such as theophylline in *S. cerevisiae*) where the extracellular metabolite concentration in a pure culture is informative as to the total enzyme activity. In other situations, such as when the product is preferentially retained within the cell, the cell pellet may need to be lysed and assayed using appropriate analytical techniques.
13. The process may be continued for as long as desired, inoculating six deep-well plates each day. This protocol describes a typical screen, using 18 deep-well plates and screening ~1,600 colonies.
14. Yeast cultures can occasionally harbor multiple high-copy plasmids. If retransformed cells show different levels of activity compared to the original culture, try sequencing more plasmids from the *E. coli* transformants to determine whether multiple plasmids may be present.
15. If testing multiple libraries for FACS-based screening, use a fraction (~5 μL) of each extracted, DpnI-treated mutagenic PCR product as the template for a single 100 μL non-mutagenic PCR. Transform that product into the single-color screening strain and use 96-well plates to measure the enzymatic activities of 46–92 clones per library. The desired mutation rate for a FACS-based library is higher than for a plate-based library, typically in the range where ~20 % of enzymes retain activity. Having identified the desired library, use the remainder of the mutagenic PCR product to set up 8 × 100 μL non-mutagenic PCRs and proceed as described above.
16. Given this dilution schedule, the cells will reach saturating densities before sorting. In some situations, the optimal sorting density may be significantly lower. The small molecule used in this example, theophylline, is preferentially retained in the cell in which it was synthesized. As a result, cross-feeding between producers and non-producers is insignificant. A more freely diffusible product would require a lower culture density to minimize the bulk product concentration and, as a result, minimize the effects of cross-feeding.

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