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Synthetic RNA switches for yeast metabolic engineering

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Summary

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.

Keywords

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]. The switch couples an input domain, an RNA aptamer, to an output domain, a ribozyme [4]. 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

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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 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. 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. 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. 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. 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, 1x 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 10x 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 1M Tris-HCl, pH 7.5. Filter sterilize and freeze at -20 °C.
4. Buffer E: 10 mM Tris-HCl, pH 7.5, 270 mM sucrose, 2 mM MgCl₂. 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 MgCl₂. 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 Mn²⁺ 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: 1x phosphate-buffered saline (PBS), 1% (w/v) bovine serum albumin (BSA). Combine 100 mL 10x PBS (1.37 M NaCl, 27 mM KCl, 100 mM Na₂HPO₄, 18 mM KH₂PO₄) with 900 mL ultrapure water. Dissolve 10 g BSA (Fraction V) in 1L 1x 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 μ L reaction for each mutagenic level (see Note ³). Extract the resulting PCR products from an agarose gel, 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 μ L 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 μ g of plasmid DNA per transformation.

¹Ultimately, 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.

²Reducing 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.

³It 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.

⁴Gel 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.

⁵For 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.

⁶With 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.

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 benchtop. Grow the yeast culture to OD 1.3, then add 500 µL of Tris-DTT to aid in cell permeabilization [8]. Incubate, shaking, at 30 °C for an additional 15 minutes. Centrifuge at 2500g and 4 °C for 3 minutes, decant supernatant, and gently resuspend in 25 mL of ice-cold Buffer E. Centrifuge again at 2500 g and 4 °C for 3 minutes, decant supernatant, wash with 1 mL ice-cold Buffer E, transfer to a 1.5 mL tube, and centrifuge once more at 2500 g and 4 °C for 3 minutes. 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 1000 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 hr. Centrifuge at 5000g and 4 °C for 5 minutes, 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 two hours, 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.

⁷Using 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.

⁸The cell pellet may be >100 µL in volume, so the additional volume of Buffer E may be substantially lower than 300 µL.

⁹If 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).

¹⁰Screening a single plate on the Beckman Quanta flow cytometer described above takes ~90 minutes. Spacing the plates by 2 hours 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 twelve plates, limiting daily throughput to 6 plates. Using a different plate shaker may allow for higher or lower daily throughput.

3. Day 2: every two hours, inoculate a new growth plate as before, for a total of another six plates. Similarly, every two hours, dilute the oldest growth plate 100x into a new deep-well “assay” plate. For the assay plate, aliquot 400 μ L of SD media containing the desired substrate concentration (e.g. 1 mM caffeine). Transfer 4 μ L 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 two hours, 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 μ L of cells into 300 μ L 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). Rank the samples, and identify the top hits (Figure 1A). Centrifuge the stored assay plates at 4000 $\times$ g and 4 °C for 10 minutes. 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 two hours, dilute the oldest remaining growth plate into a new assay plate and assay the oldest assay plate (see Note ¹³).
6. Day 5: every two hours, measure the oldest assay plate.
7. Collect the top hits from each day of screening (Figure 1B). 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).

¹¹Diluting the cells 4x reduces the clogging rate of the flow cytometer.

¹²This 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.

¹³The process may be continued for as long as desired, inoculating 6 deep-well plates each day. This protocol describes a typical screen, using 18 deep-well plates and screening ~1600 colonies.

¹⁴Yeast 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.

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 hours, dilute 10x into 250 mL fresh SD media. After another 24 hours, dilute 30x into 250 mL fresh SD media. After a further 24 hours, dilute 100x into fresh SD media containing your enzymatic substrate (see Note ¹⁶). Grow for a final 24 hours, 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 6000 g and 4 °C for 5 minutes. Resuspend in 6 mL of PBS-BSA and filter through a cell strainer. Add DAPI to the cell suspension at 1:1000, 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 Figure 2A). Screen the library, preferably oversampling the expected library diversity by at least 3-fold. 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 hours. 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 (Figure 2B). Oversample the expected library diversity by at least 3x. Collect the sorted cells as before.
5. Third Sort – Positive: Dilute the sorted cells into 25 mL of SD media. Grow 24 hours, then dilute 100x into 25 mL fresh SD media containing your substrate (e.g. 1

¹⁵If 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.

¹⁶Given 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, crossfeeding 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, the minimize the effects of crossfeeding.

mM caffeine). Grow for a final 24 hours, then sort as in steps 2 and 3. Set a more stringent sorting gate, collecting only the top ~0.5% (Figure 2C). Collect ~2000 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 Section 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.

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Figure 1. 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. ~1600 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.

Figure 2. 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, collect the ~0.5% of cells with the highest relative GFP fluorescence.