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Directed Enzyme Evolution

***Screening and Selection
Methods***

Edited by

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Genetic Complementation Protocols

Jessica L. Sneeden and Lawrence A. Loeb

1. Introduction

Genetic selection provides a powerful tool for the study of cellular processes. It is particularly useful in analyzing protein sequence constraints when used in conjunction with directed molecular evolution. Our lab has used this approach to analyze the function of enzymes involved in DNA metabolism, to study the mutability of protein domains, and to generate mutant proteins possessing properties different from those selected by natural evolution (*1–4*). To illustrate the concept, this chapter discusses genetic complementation of an *E. coli* strain defective in expression of the small subunit of ribonucleotide reductase (NrdB). Wild-type NrdB, in *trans*, is used to complement the hydroxyurea hypersensitivity of the defective strain. Cloning of the wild-type gene, expression, and complementation methods are discussed. The principles used for complementation with ribonucleotide reductase should be applicable to other enzymes for which a complementation system can be established.

Genetic complementation in bacteria is a powerful method with which to examine the biological function of a gene product. The concept is illustrated in **Fig. 1**. Briefly, a bacterial strain lacking or deficient in gene A is compared to a wild-type strain. Sometimes conditions can be found under which survival rates are similar or indistinguishable (permissive conditions). However, under conditions which restrict growth of strains failing to express gene A, only strains expressing gene A (in *cis* or *trans*) continue to multiply at rates similar to those under permissive conditions. This approach has been used for decades in a variety of systems, to obtain useful genetic information about protein function, inactivating mutations, and protein-protein relationships. With the advent of new molecular techniques and genome sequencing efforts, it is possible to disable or inactivate a specific gene and complement the inactivating mutation in *trans*, to obtain information about its physiological role.

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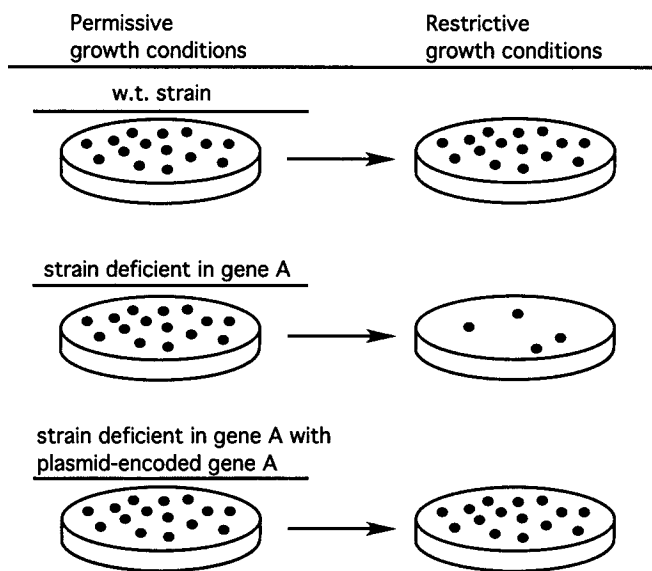


Fig 1. Schematic drawing of bacterial genetic complementation, where complementation is measured in a colony-forming assay.

In addition to its use in obtaining information about wild-type gene function, it is also possible to use complementation systems to select for mutant proteins with properties not selected in nature. One example is the conversion of a DNA polymerase into an enzyme capable of polymerizing ribonucleotides (*1*); another is the development of mutant enzymes highly resistant to anticancer agents that can be useful in the application of cancer gene therapy (*2–4*).

The key advantage of positive genetic selection is that one can grow cells under restrictive conditions that select for only those gene products that compensate for the deficiency. One can analyze large combinatorial libraries consisting of as many as 10^7 mutant genes for their ability to display a desired phenotype. The major limitation to the number of mutants that can be studied is the transformation efficiency of *E. coli* (10^6 – 10^8). This is sharply contrasted with screening methods, which rely on individual, not population, mutant analysis. Even with the advent of automated screening technologies, the throughput of this type of selection is much lower than that obtained by positive genetic selection. A critical feature of genetic selection is the window of selection, or the phenotypic difference between the wild-type strain vs the strain carrying the deficiency. When complementing the deficiency in *trans*, a difference of $>10^3$ is preferable, but a lower differential may be acceptable.

Prokaryotic selection systems offer a number of advantages over selection in eukaryotes. Transformation efficiencies, hence the ability to screen larger num-

bers of mutants, are much higher in prokaryotes; prokaryotic genomes are less complex, yet it is frequently possible to complement deficiencies using mammalian gene products; and the cell division times of prokaryotes are much shorter than for eukaryotes. Nevertheless, we have screened large libraries using genetic complementation of yeast (5), and it should be feasible to use mammalian cells in culture for analysis of libraries containing 10^4 – 10^5 mutant genes.

This chapter will focus on the cloning and expression of *Escherichia coli* NrdB to illustrate complementation methods. NrdB encodes the *E. coli* small subunit of ribonucleotide reductase. It catalyzes the removal of the 2'-hydroxyl of ribonucleoside diphosphates, generating deoxyribonucleoside diphosphate precursors for use in DNA synthesis. This gene has been extensively studied (6–9) and its sequence is known (10). NrdB is cloned from *E. coli* genomic DNA, and placed into a suitable expression vector. It is then transformed into a strain of *E. coli*, KK446 (7), which is deficient in NrdB; complementation is measured by the ability of NrdB in *trans* to complement the hydroxyurea hypersensitivity of KK446.

2. Materials

1. Plasmids TOPO-TA (Invitrogen) and pBR322.
2. *E. coli* genomic DNA, from strain carrying wild-type NrdB.
3. Primers flanking the gene of interest.
4. PCR components: *Taq* polymerase; dNTPs; *Taq* buffer, 1X concentration: 10 mM Tris-HCl, pH 9.0 at 25°C, 50 mM KCl, 0.1% Triton X-100.
5. *E. coli* strain with appropriate gene defect, here KK446 (6) which encodes a wild-type NrdB that is presumably defective in wild-type expression levels. Obtained from *E. coli* Genetic Stock Center at Yale (see Website: <http://cgsc.biology.yale.edu/>).
6. Restriction enzymes and buffers.
7. Agarose gel electrophoresis equipment.
8. Luria-Bertani (LB) medium.
9. Hydroxyurea.

3. Methods

The methods described outline construction of the plasmid containing the gene of interest (NrdB) and procedures to establish and test for complementation in *E. coli*.

3.1. Cloning of NrdB

The methods described in **Subheading 3.1.** outline the cloning and expression of NrdB, which can be generalized for use in cloning a variety of genes. The methods include 1) the design of PCR primers and PCR amplification of the gene, 2) cloning into Topo-TA vector, 3) verification by restriction mapping and sequence analysis, and 4) subcloning into pBR322 vector.

3.1.1. PCR of *NrdB*

Since the sequence of *NrdB* is known, it is possible to design primers for PCR amplification of the gene directly from *E. coli* genomic DNA (see **Note 1**). Ideally, the primers should flank the gene directly upstream and downstream of the coding sequence. Cloning vectors often contain a multiple cloning site (MCS) that is located within the coding frame of *LacZ*, allowing for blue/white screening. Therefore, design of primers should include a stop codon, followed by a Shine-Dalgarno sequence for ribosomal entry approx 8 nucleotides upstream of the initiator methionine (see **Fig. 2**). Because subcloning is often necessary, it is useful to include in the primer unique restriction sites on both ends of the gene, flanking the 5' stop codon and Shine-Dalgarno sequence upstream of the coding region (**Fig. 2A**).

PCR is carried out by standard molecular techniques. Briefly, add 10–50 ng *E. coli* genomic DNA, 10 mM Tris-HCl, pH 9.0 at 25°C, 50 mM KCl, 0.1% Triton X-100, 250 μ M (total) dNTP mix (dGTP, dCTP, dATP, dTTP), 1 mM $MgCl_2$, 20 pmoles each primer, and 2.5 U *Taq* DNA polymerase in a total volume of 50 μ L H_2O (see **Note 2**). Amplification is for 30 cycles of PCR. The length of the product should be determined by electrophoresis on an agarose gel. Ideally the product should contain a single band of the desired length (**Fig. 2B**) (see **Note 3**).

3.1.2. Cloning into TOPO-TA Vector (see Note 4)

After the desired product has been verified by agarose gel analysis, it is cloned into the TOPO-TA vector. The TOPO vectors have been developed by Invitrogen to contain covalently attached topoisomerases on each end of a linearized vector (**Fig. 2C**). This obviates the need for ligation cloning and gives a reasonably high insertion rate (Invitrogen).

1. Mix 5 μ L of unpurified PCR product (see **Note 5**) with 1 μ L TOPO vector and 1 μ L of 1X salt buffer (provided by Invitrogen).
2. Incubate 5 min at room temperature.
3. Transform into XL-1 (or your favorite strain) using standard methods (**II**).
4. Plate onto LB agar containing appropriate antibiotic selection.
5. Select single colonies and grow overnight in LB medium.
6. Isolate plasmid DNA by standard methods (**II**).
7. Check for incorporation of product of desired length by restriction analysis (**II**).
8. Verify construct by sequence analysis (**II**).

At this step, it is desirable to verify expression of *NrdB* in the TOPO vector, which is capable of expression under the *lac* promoter. However, expression of *NrdB* in a high-copy vector is toxic, as may be other genes. In the case of *NrdB*, it can be subcloned into a medium-copy vector (pBR322) to alleviate this problem (see **Note 6**).

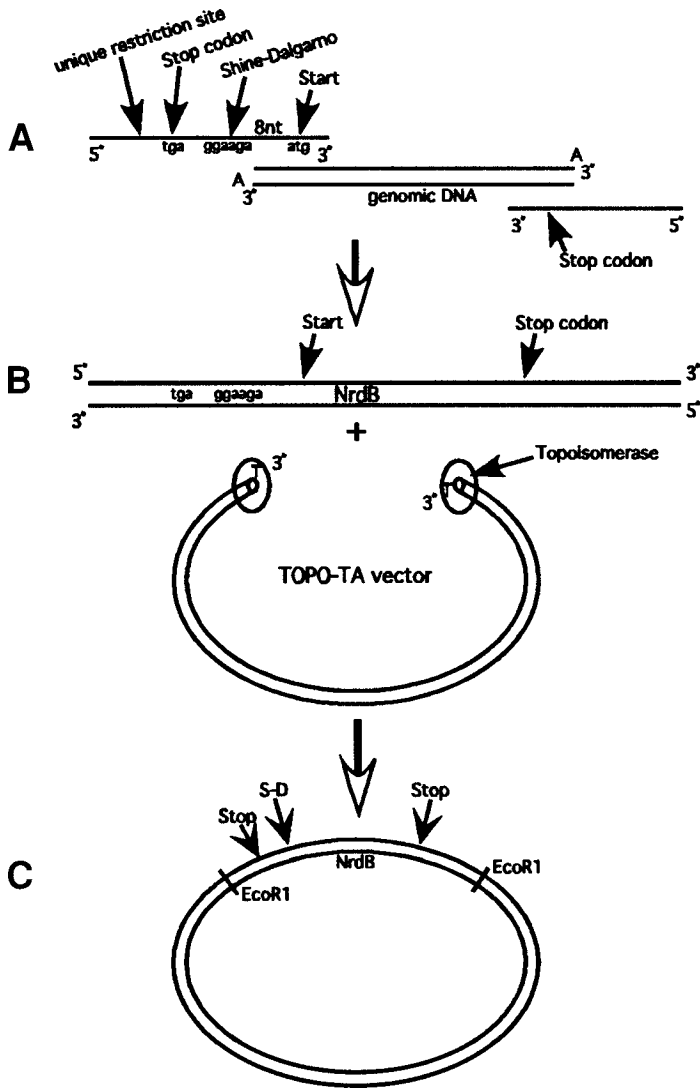


Fig 2. Schematic representation of (A) primer design for PCR cloning of genes from genomic DNA, (B) PCR product obtained added to Topo-TA vector, and (C) Topo-TA vector with NrdB, after transformation.

3.1.3. Subcloning into pBR322

Digest TOPO plasmid containing NrdB using restriction enzymes that cleave at flanking EcoRI sites. Clone into pBR322 using standard molecular biological methods (11).

3.2. Expression and Complementation

3.2.1. Expression of *NrdB*

When verifying expression of a protein where an antibody is available, Western blots are preferable (**II**). Since no commercial antibody is available for *E. coli* *NrdB*, verification of expression can be confirmed via complementation of an *E. coli* strain that is deficient in *NrdB* expression and displays hypersensitivity to hydroxyurea (see **Note 7**). A similar functional complementation may be required for verification of other genes.

3.2.2. Complementation

Complementation of sensitivity of *E. coli* strain KK446 to hydroxyurea is accomplished by expression of *NrdB*. This strain was described in 1976 by Fuchs and Karlstrom and the defect mapped to 48 min, the region encoding *NrdB*, the small subunit of ribonucleotide reductase (**7**). Hydroxyurea is a radical scavenger that removes the stable tyrosyl radical on the small subunit of ribonucleotide reductase, inactivating the enzyme. The defect was not further characterized, but was complemented by the authors with wild-type *NrdB* (**7**).

The ability of *NrdB* to complement hydroxyurea hypersensitivity of KK446 can be tested as follows:

1. Transform plasmids containing *NrdB* into KK446 cells via electroporation (**10**).
2. As a control, separately transform plasmid only into KK446 cells.
3. Isolate plasmids based on carbenicillin resistance, and verify the construct by restriction digestion analysis.
4. Inoculate KK446 only, KK446 bearing plasmid only, KK446 bearing plasmid encoding *NrdB*, and XL-1 blue cells (or other strain with wild-type *NrdB* expression) into LB medium and grow overnight at 37°C.
5. Dilute each culture 1:100 into fresh LB medium and grow to 0.6 OD.
6. Plate onto 0, 0.25, 0.5, and 1.0 mg/mL hydroxyurea-containing LB plates and grow overnight at 37°C.
7. Count colonies and determine differences in sensitivity to hydroxyurea.

Complementation is scored as a function of the colony-forming efficiency of plasmids with and without *NrdB*, as compared to KK446 without plasmid and XL-1 blue cells without plasmid (see **Note 8**). It is often not possible to obtain an isogenic strain which differs only by the one gene defect. Estimates using different cell strains may be used in this case.

4. Notes

1. This protocol is limited to cloning of genes with known sequence. It is important to note that often multiple sequences of a given gene exist in sequence databases and they are not always identical. Check different submitted sequences against each other, to avoid mistakes in primer design.

2. This procedure uses *Taq* DNA polymerase which creates an 3' overhanging adenine. It is also feasible to use polymerases which do not possess this function, and then to blunt-end clone the PCR product into a vector. However, this will decrease transformation efficiency.
3. *NrdB* is approx 1200 bp, which is relatively easy to PCR clone. For genes longer than 2.5 kb, optimization of PCR may be necessary to obtain a single gene product. It may also be necessary to gel purify the band of interest in the event that a single band is not obtained.
4. This method uses the TOPO-TA expression vector from Invitrogen, although other TA vectors exist.
5. Unpurified PCR product gives a higher transformation rate than purified product, likely because of the favorable salt concentration in the PCR mix. If the desired product has been gel purified, a higher transformation rate can be obtained by adding the product to a 50 μ L tube containing the standard PCR reaction mix.
6. Although *NrdB* has been extensively studied, it is not reported to be toxic at high expression levels. It is important to remember when establishing a complementation system that stability of the construct must be verified. When working with a potentially toxic gene, high expression levels should be avoided. In addition, the *lac* promoter is widely used in common expression vectors, but is leaky and cannot be fully suppressed. For our purposes, expression in a medium-copy vector under the *lac* promoter was sufficient to alleviate toxicity. It may be necessary in some cases to express in low-copy vector under a more tightly controllable promoter.
7. It is important to note that expression verified by complementation of a phenotype, even in a strain where the gene defect is known, while compelling evidence, is not absolute proof of expression of an active protein. Western blots are preferred where an antibody is available.
8. A critical feature of complementation, especially when used to select for mutant proteins, is the difference in phenotype between cells with and without the complementing gene. In general at least 1000-fold difference is preferable, although results may be obtained with somewhat smaller phenotypic differences.

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Use of Pol I-Deficient *E. coli* for Functional Complementation of DNA Polymerase

Manel Camps and Lawrence A. Loeb

1. Introduction

The *E. coli* JS200 strain carries a temperature-sensitive allele of DNA polymerase I that renders this strain conditional lethal. Growth under restrictive conditions is restored by small amounts of DNA polymerase activity. Even mutants with greatly reduced (1–10% of wild-type) catalytic activity or distantly-related polymerases of bacterial, eukaryotic, or viral origin effectively complement JS200 cells. The versatility of this complementation system makes it advantageous for selection of active polymerase mutants, for screening of polymerase inhibitors, or for screening of mutants with altered properties. Here we describe complementation of JS200 cells with the wild-type *E. coli* DNA polymerase I to illustrate such functional polymerase complementation.

Polymerases catalyze the template-directed incorporation of nucleotides or deoxynucleotides into a growing primer terminus. DNA polymerases and reverse transcriptases share a common structure and mechanism of catalysis in spite of low sequence conservation (*1*). As central players in replication, repair, and recombination, DNA polymerases have been intensely studied since the early days of molecular biology. Errors in nucleotide incorporation have been recognized as significant sources of mutations, contributing to the generation of genetic diversity, of which HIV reverse transcriptase is a dramatic example. Polymerase errors may also contribute to the genetic instability that characterizes certain disorders, such as cancer and trinucleotide expansion diseases. Finally, polymerases are finding an ever-growing number of applications in sequencing, amplification, mutagenesis, and cDNA library construction.

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E. coli DNA polymerase I is encoded by the *polA* gene. It has two relatively independent functional units: a polymerase (with a 3'→5' exonuclease proof-reading domain), and a separate 5'→3' exonuclease subunit. In vitro, the coordinated action of these two subunits results in efficient nick translation. In vivo, pol I is involved in lagging-strand synthesis during chromosomal replication and in DNA excision repair. Pol I mediates the processing of Okazaki fragments by extending from the 3' end of the RNA primer and by excising the RNA primer from the 5' end of the downstream fragment. Removal of all residues of the RNA primer is essential for joining of Okazaki fragments (2). Similarly, the coordinated action of polymerase and 5'→3' exonuclease activities on an RNA primer initiates ColE1 plasmid replication (3). On the DNA repair front, pol I catalyzes fill-in reactions in base and nucleotide excision repair. In the latter, pol I also contributes to releasing the oligonucleotide fragment and UvrC protein from the postincision complex (4,5). Pol I expression is constitutive, with an estimated 400 molecules/cell. It seems, however, that only a fraction of these molecules are engaged in lagging strand synthesis catalysis under normal circumstances, which would leave a substantial cellular complement available for DNA excision repair.

Pol I is not essential for growth in minimal medium, although pol I-deleted strains show slower growth rates. In rich medium, pol I is essential, presumably because cells are unable to complete lagging-strand synthesis before the next round of replication (6). Expression of either of the polymerase I subunits restores growth in rich media (6), implying that other enzymes are able to substitute for pol I in lagging-strand synthesis. In agreement with pol I's partial redundancy in vivo, *pol A* shows epistasis with a number of genes involved in DNA repair and recombination, including *rnhA* (7), *polC* (8,9), *uvrD* (10), *recA* (11–13), and *recB* (11).

PolA12 encodes a misfolding form of pol I that is defective in the coordination between the polymerase and 5'-exonuclease activities (14). *PolA12* also exhibits reduced temperature stability, and in vivo, its polymerase and 5'-exonuclease activities decrease 4-fold at 42°C (14). In combination with *recA*- and *recB*-inactivating mutations, *polA12* is lethal in rich medium (11). Surprisingly, RecA-mediated constitutive expression of the SOS response also renders *polA12* cell growth sensitive to high temperature (13). The *polA12 recA718* temperature-sensitive strain (JS200 strain) probably falls into this category (9). *RecA718* is a sensitized allele of *recA* (15) that is likely activated as a result of slow Okazaki fragment joining under conditions that are restrictive for *polA12*. The combination of a 5'→3' exonuclease- inactivating mutation and constitutive SOS expression is viable under restrictive conditions (13), however, and expression of polymerase activity alone (without 5'→3' exonuclease) relieves *polA12 recA718* conditional lethality (9). These two observations point to poly-

merase as the rate-limiting activity in pol I-deficient, SOS-induced cell conditional lethality. Complementing polymerase activity can be provided even by distantly-related polymerases of bacterial, eukaryotic, or viral origin, although polymerase overexpression may be required for complementation in some cases (9,17). Examples of complementing polymerases include *E. coli* pol III α subunit (9), *Thermus aquaticus* (Taq) polymerase (16), rat pol β (17), and HIV and MLV reverse transcriptases (18). JS200 complementation by some of these polymerases occurs even after partial inactivation by mutagenesis (19–22) (the threshold being 10% of wild-type activity for Taq and pol I, based on colony formation). With its great versatility, the *polA12 recA718* complementation system in *E. coli* has been used for selection of active mutants of *Thermus aquaticus* (Taq), and *E. coli* pol I (19,21,22), pol β (20), and HIV reverse transcriptase (23). These mutants were further screened for altered properties. A *TrpE65* ochre mutation was used as a secondary screen for pol β mutators (24). Finally, expression of low-fidelity pol I mutants in this system achieved in vivo mutagenesis with some specificity for a ColE1 plasmid (25).

In the following chapter we present a protocol for functional complementation of *polA12 recA718* cells by *E. coli* DNA polymerase I. This protocol can be easily adapted for complementation by other DNA polymerases, for mutator screening and for in vivo mutagenesis.

2. Materials

1. JS200 (*recA718 polA12 (ts) uvrA155 trpE65 lon-11 sulA*) competent cells *see* Notes 1–3).
2. pHSG576 empty vector control (*see* Note 4) and pECpol I construct containing the *E. coli* pol I gene (or another polymerase) under the tac promoter (*see* Note 5) in water solution (from mini, midi, or maxiprep).
3. LB (Luria-Bertani) medium.
4. Tetracycline solution: 12.5 mg/mL stock in 50% ethanol, light-sensitive, keep at -20°C .
5. Chloramphenicol solution: 30 mg/mL stock in 100% ethanol, keep at -20°C .
6. Isopropyl- β -D-1-thiogalactopyranoside (IPTG) solution: 100 mM stock in water, sterile-filtered, keep at -20°C .
7. 15-mL plastic, 1.5-mL eppendorf tubes, and racks to hold them.
8. Biorad Gene pulserTM electroporator and 0.2-cm electroporation cuvetts.
9. Sterile toothpicks.
10. LB tetracycline (12 $\mu\text{g/mL}$) and LB tetracycline (12 $\mu\text{g/mL}$) chloramphenicol (30 $\mu\text{g/mL}$) plates.
11. Petri dish turntable, 10 μL inoculation loop, and ethanol for flaming.
12. Bunsen burner.
13. 30 and 37°C incubators.
14. 30°C shakers.

3. Methods

1. Combine 40 μL (5×10^9 cells) competent cells with 1 μL of pHSG576 or pECpoll construct in electroporation cuvetts.
2. Electroporate the cells (at 400 Ω , 2.20 V, and 2.5 μFD).
3. Resuspend in 1 mL LB (*see Note 6*) immediately after electroporation and transfer to a 15-mL plastic tube.
4. Place in a shaker at 30°C for 1 h (*see Note 7*).
5. Plate a 1:0 and a 1:10³ dilution of cells (to ensure single colony formation) on LB tetracycline chloramphenicol plates (*see Note 6*).
6. Incubate at 30°C for 24 h (*see Note 7*).
7. Pick at least two single colonies from each electroporation into 5 mL LB with tetracycline (12 $\mu\text{g/mL}$) and chloramphenicol (30 $\mu\text{g/mL}$) (*see Note 8*).
8. Grow overnight in a 30°C incubator (without shaking). The next morning vortex briefly and shake at 30°C until the culture reaches mid-exponential phase (1–2 h) (*see Note 7*).
9. Test for temperature sensitivity in rich medium: Inoculate a spiral of increasing dilution in two LB agar plates with tetracycline and chloramphenicol (*see Note 8*). One of the plates needs to be pre-warmed at 37°C and the other plate pre-warmed at 30°C (*see Note 9*). This is done placing the loop of the inoculation rod ($\sim 2 \times 10^6$ cells) in the center of a plate and moving the loop toward the periphery as the plate spins. Incubate 1 plate at 37°C (*see Note 10*) and the duplicate plate at 30°C for 24–30 h (*see Notes 11 and 12*). Some growth in the center of the plate (where there is a high cell density) is expected, but there should be no growth in low cell density areas (*see Fig. 1, Note 13*).

4. Notes

1. JS200 cells were originally designated SC18-12 (9) and are tetracycline-resistant.
2. The *uvrA155* genotype means JS200 cells are deficient in nucleotide excision repair. This might contribute to the relative deficiency in polymerase (compared to 5'→3' exonuclease) activity in these cells, as 5'→3' exonuclease activity has a prominent role in nucleotide excision repair (26).
3. Competent cells can be prepared as follows: single JS200 colonies growing on LB plates with appropriate antibiotic selection (in this case, 12.5 $\mu\text{g/mL}$ tetracycline) are picked into a flask containing 50 mL of LB plus antibiotic and grown at 30°C overnight without shaking (*see Notes 6 and 7*). The next morning, cells are shaken for 1 h at 30°C. All 50 mL of bacterial culture are transferred to a flask containing 450 mL LB with antibiotic, and left in the 30°C shaker for 3–4 h (to an OD₆₀₀ of 0.5–1). Cells are chilled on ice for 20 min, pelleted in a Sorval® RC 5B plus centrifuge (10 min at 6000 rpm 4°C), and washed twice in 10% glycerol. The last spin is performed in bottles with conical bottom for easy removal of the supernatant in a Sorval® RC 3B centrifuge (10 min at 4000 rpm 4°C). The pellet is resuspended in ~ 2 mL 10% glycerol, stored in 120 μL aliquots, and quick-frozen in dry ice.

4. pHSG576 is a low-copy plasmid encoding chloramphenicol resistance (27). This plasmid carries the pol I-independent pSC100 origin of replication (28). Providing the test polymerase in a pol I-independent vector is of relevance, as maintenance of a ColE1 plasmid in JS200 cells under restrictive conditions would compete for residual or redundant pol I activity and effectively increase the threshold for functional complementation. On the other hand, increasing the threshold for complementation might be desirable in some cases (for example to minimize the likelihood of reversion [see Note 6]).
5. pECpol I construction: the entire open reading frame of the pol I gene (*polA*) of *E. coli* DH5 α was amplified with primers 5'-ATATATATAAGCTTATGGTT CAGATCCCCCAAATCCACTTATC-3' (initiating methionine in bold) and 5'-ATATATAATGAATTCTTAGTGCGCCTGATCCCAGTTTTCGCCACT (stop codon in bold) and cloned into the *HindIII* *EcoRI* sites of the pHSG576 polylinker using *HindIII* *EcoRI* adapters (italics). This places the pol I gene under transcriptional control of the *tac* promoter.
6. Nutrient Broth has been used instead in the work reported in the literature (17–19,21). In our hands, growth in LB appears to be similar in the rates of loss of temperature-sensitivity or in the strength of the conditional lethal phenotype.
7. Pol I-deficient strains in combination with alterations in RecA, RecB or UvrD are easily overgrown by suppressors or revertants under non-permissive conditions (10). This problem is less severe for *polA12 recA718* double mutants (9), but revertants/suppressors still occur at a detectable frequency (about 1 in 500 after overnight culture). To avoid overgrowth by these revertants, we maintain conditions as permissible as possible, growing the cultures at 30°C, and keeping the cell density to less than OD₆₀₀ = 1. The temperature sensitivity of these cells should be checked periodically (see step 9 in Subheading 3.). Most of the cells that lose temperature sensitivity appear to be suppressors rather than simple revertants and often exhibit a milder but not wild-type phenotype (Tsai, C.-H., personal communication and our own observations). In the *polA12 uvrE502* background one apparent revertant was found to be an intragenic suppressor (10).
8. Overexpression of the polymerase can be induced at this point by adding 1 mM IPTG to the medium. IPTG induction of transcription was required for complementation in the case of pol III α subunit and pol β (9,17).
9. Pre-warming of the plates is critical. The temperature-sensitive phenotype of JS200 cells (see Fig. 1) and that of other *polA12 recA*, *polA12 recB*, or *polA12 uvrD* derivatives is only apparent in isolated cells. These cells lose viability quickly (2–4 h) after switching to the restrictive temperature, at least in liquid culture (11,13). In consequence, for tests or selections that depend on conditional lethality it is essential that the plates achieve the restrictive temperature before the JS200 cells plated on them reach the local cell density that allows survival.
10. Initially 42°C was chosen as the restrictive temperature for functional complementation in JS200 cells (9,17,20,29). We have since switched to 37°C (16,19,22,23,25), as we still see strong conditional lethality at this temperature (see ref. 18 for a comparison).

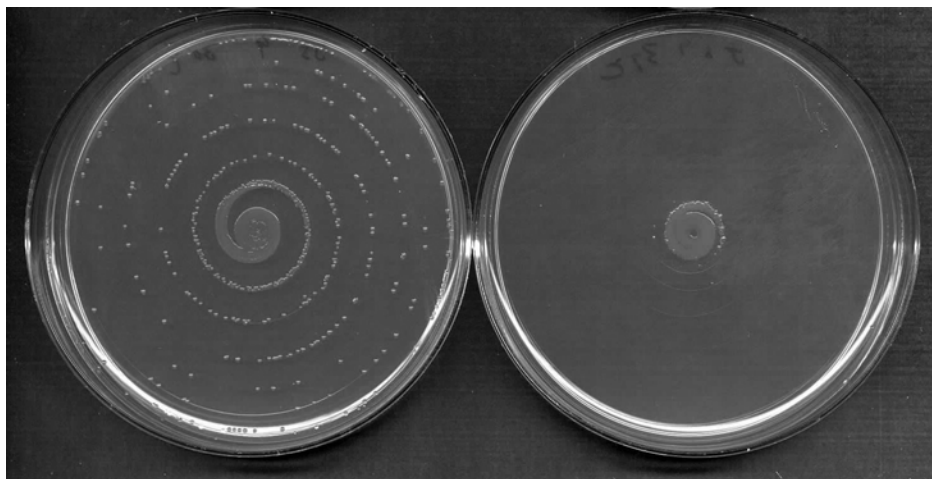


Fig. 1. Spiral assay for temperature sensitivity. *PolA12 rec718* cells were plated and grown as described in **Subheading 3., step 9**. On the left, growth at 30°C, on the right growth at 37°C (Modified from Ern Loh, unpublished).

11. In cases of partial functional complementation plates can be incubated for longer periods of time, up to 48 h, to detect growth at 37°C (**17–19**).
12. The plates should be placed upside-down in the incubator to prevent excessive evaporation from the agar.
13. Alternatively, the temperature-sensitivity assay can be done in a quantitative manner by plating approx 10^3 cells (in duplicate or triplicate) instead of inoculating them. Briefly, add 100 μ L of a dilution containing 10^4 cells/mL to 4 LB agar plates with tetracycline and chloramphenicol, 2 of them pre-warmed to 30°C, and the other 2 pre-warmed to 37°C. Spin the plate on the turntable while evenly spreading the bacterial dilution with a glass rod (previously flamed in ethanol). Place the duplicate plates in the 30°C and 37°C incubators, and incubate for 24–30 h. No more than 2 or 3 cells should grow at 37°C for every 1000 cells that grow at 30°C.

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Selection of Novel Eukaryotic DNA Polymerases by Mutagenesis and Genetic Complementation of Yeast

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1. Introduction

DNA-directed DNA polymerases have been broadly classified into seven families based on their sequence homology (1). It is surprising to learn that enzymes such as DNA polymerases, which carry out pivotal role during DNA replication, repair, and recombination, are poorly conserved amongst different families, but within a given family, all the members are highly conserved. These observations have profound implications and suggest that DNA polymerases have been plastic during evolution, but can tolerate multiple mutations (2). The mutability of DNA polymerases has been utilized extensively in our studies and has shed light on structure-function relationships of each domain. Any single amino acid residue or the entire domain can be randomly mutagenized and the active mutants can be selected by genetic complementation. Here we describe the complementation of *Saccharomyces cerevisiae* Pol3 (Pol δ) by utilizing a common technique in yeast genetics known as “plasmid shuffling,” where the wild-type copy of the Pol3 present in a Ura3 selective marker plasmid is exchanged or genetically complemented for in vitro mutated version(s) of Pol3 in the domain-of-interest. Since Pol3p is essential for viability of yeast, only those mutants that genetically complement the loss of wild-type Pol3p survive.

2. Materials

1. pYcplac 111 and pYcplac 33 (ATCC, Manassas, VA).
2. *Saccharomyces cerevisiae* genomic DNA (Invitrogen, Carlsbad, CA).
3. *E. coli* strains DH5 α and XL1-blue (Invitrogen and Stratagene, La Jolla, CA).

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4. Yeast strains (ATCC) (*see Note 1*).
5. Standard microbiological culture media (*E. coli*): Luria Bertani.
6. Standard microbiological culture media (*S. cerevisiae*): YPD.
7. *S. cerevisiae* selection medium: SC-amino acid drop out mixture.
8. Oligonucleotide primers.
9. Thermocycler.
10. Quik-change PCR mutagenesis kit (Stratagene).
11. Restriction enzymes, T4 DNA ligase.
12. Agarose gel electrophoresis apparatus.
13. DNA sequencing apparatus or available core facility.
14. Qiagen gel and plasmid purification kit (Qiagen, Valencia, CA).
13. Carbenicillin (Sigma, St. Louis, MO).
14. Canavanine (Sigma).
15. 5-Fluoro-orotic acid (5-FOA) (Qbiogene, Carlsbad, CA).
16. G418 (Invitrogen).
17. Frozen-EZ yeast transformation II kit (Zymo Research, Orange, CA).

3. Methods

The methodology presented here is applicable to the “essential” replicative DNA polymerase α , δ , and ϵ , whose complete loss of function is lethal to the viability of haploid yeast (*see Note 2*). Theoretically this methodology can also be utilized to study “non-essential” DNA polymerases, if mutant allele of the enzyme exhibits a selectable phenotype, for example, enhanced sensitivity to UV radiation or temperature sensitivity to growth that can be rescued by genetic complementation (3,4). Here we describe genetic complementation of the DNA polymerase δ “knock out” strain with any (Pol3p) library of interest.

3.1. Amplification of Yeast Pol3 Targeting Module

Standard recombinant DNA techniques were followed throughout this chapter (5). One of the most important parameters in this protocol is the choice of appropriate haploid yeast strain. Technically any wild-type yeast strain can be used and the minimum prerequisites are sensitivity to canavanine and auxotrophy for *Leu2*, *Ura3*, and/or *Trp1*, *His3*, *Lys2* markers. We used YGL27-3D (MATa, *leu2 his3 trp1 lys2 ura3* CAN1, *pol3::KanMX*) engineered by Simon and co workers (6) and Singh and co workers (7). The chromosomal copy of the Pol3 was replaced with KanMX cassette that provided resistance to the antibiotic G418 and the lethality was rescued by presence of wild-type Pol3 on an episomal plasmid with the *Ura3* selective marker (8,9).

3.1.1. Generation of Designer Polymerase “Knock Out” Strain

1. Transform the haploid yeast strain with the wild-type copy of the DNA polymerase gene-of-interest cloned into Ycplac33 vector that has *Ura3* selection marker (*see Notes 3–7* for information on molecular cloning, purification and

propagation of the Ycplac 33 vectors). Select for the transformants on SC-Ura plates by incubation at 30°C for 2–4 d (see **Note 8**). For routine yeast transformation, use the Frozen-EZ II kit (see **Note 9**).

- Entire ORF of any yeast gene can be easily deleted by utilizing PCR-based gene-disruption method (8). To delete the chromosomal copy of any DNA polymerase gene-of-interest, design chimeric primers in following manner. For the forward and reverse primers fuse 50 bases flanking the start and stop codon upstream of 20 bases which anneals to the KanMX cassette. The Pol3 primers are shown as an example, KanMX annealing sequence is in bold, start and stop codon are in bold italics.

Forward primer:

5' CTTGCTATTAAGCATTAATCTTTATACATATACGCACAGCA
ATGAGTGAAGTGGTTAGCTTGCCTCGTCC 3'

Reverse primer:

5' GCCTTTCTTAATCCTAATATGATGTGCCACCCTATCGTTTTTAC
CATTTGAATCGACAGCAGTATAGCG 3'

- Amplify the KanMX cassette in the plasmid pFA₆KanMX4 (obtained from Dr. Philippsen, **ref. 8**) using the above primers. Start with the following conditions before optimizing for the specific primers. Combine 10 ng of template, 200 μ M of dNTPs, 20–50 pmoles of primers, 2–3 mM MgCl₂, 5 units of *Taq* DNA polymerase, 1X PCR buffer and sterile ddH₂O to 50 μ L total volume, and amplify using the following conditions: initial denaturation at 94°C for 1 min, 94°C for 30 s, 60°C for 30 s, 72°C for 1.5 min, 30 cycles, final extension at 72°C for 7 min. Set up a negative control PCR reaction by including all the components except the DNA template (see **Note 10**).
- Resolve 10 μ L of the PCR reactions on a 1% agarose gel to assess yield. Successful amplification results in a sharp band that migrates at 1.5 kb as delineated by size markers in adjacent lanes. Set up 5–15 PCR reactions (depending on your yield), resolve the reactions on a quantitative 1% agarose gel, photo-document the gel and excise the 1.5 kb band from the gel using a new razor blade. Trim as much excess agarose from gel band as possible. Chop the excised agarose bands into 5–6 mm sized pieces and transfer them into a 15-mL centrifuge tube. Gene-targeting experiments require at least 1–2 μ g of DNA (from a preferably high concentration stock) and the PCR reactions can be scaled accordingly.
- Purify the DNA using Qiagen gel extraction kit (see **Note 6**). Quantitate the DNA yield using UV absorption spectrophotometer.
- Transform the yeast strain from **step 1** (Ura3 selected) with 1–2 μ g of the PCR product by scaling up the reaction 2–4-fold according to the Frozen-EZ II transformation kit. The gene-targeted integrands can be selected by either of two ways:
 - After incubation of the yeast at 30°C (**step 4** in the kit instructions), pellet the yeast, suspend them in 5 mL of YPD and culture for 4 h (two generations) at 30°C. Re-pellet yeast cells, suspend them in 0.5 mL of sterile water and plate them in 3–5 YPD+G418 plates (G418 200 μ g/mL). Incubate at

30°C for 2–4 d, reconfirm G418 resistance by streaking 10–20 colonies on a new YPD+G418 plate.

- b. Alternatively after **step 4**, plate all the cells on 5–7 YPD plates, incubate at 30°C for 24 h and replica plate onto YPD+G418 plates. Incubate for 2–4 d at 30°C. Reconfirm G418 resistance as above.
7. Inoculate 4–6 independent colonies into 5 mL YPD+G418 medium (200 µg/mL) and a single colony from wild-type strain into 5 mL YPD. Culture them overnight at 30°C by shaking at 250 rpm. Isolate genomic DNA using standard yeast molecular biology procedures.
8. Obtain the restriction map of ± 1 kb genomic DNA sequence flanking the gene of interest at <http://genome-www.stanford.edu/Saccharomyces>. Compare the restriction maps of the genomic DNA and the KanMX cassette and confirm the locus specific integration by Southern blot analysis and PCR.

3.2. Genetic Complementation of the “Designer Strain” with Library Allele of Interest

1. Transform the yeast strain generated according to **Subheading 3.1.1.** with the mutant library allele, positive and a negative control plasmid (*see Notes 11–13* for information on site-directed mutagenesis, if the Strategene’s Quik-Change kit is used for library construction). Use the Frozen EZ II transformation kit. Plate cells on SC-Leu, incubate at 30°C for 2–4 d.
2. Using a sharpie and a ruler, divide SC-Leu+5-FOA plate (5-FOA 1 g/L) into eight sectors, streak 4–8 colonies from the SC-Leu plate and incubate at 30°C for 2–6 d (*see Notes 14 and 15*). Using a sterile tooth pick, re-streak a small patch of 5-FOA-resistant colonies from at least three different sectors of each mutant on to a new SC-Leu+5-FOA plate and inoculate 5 mL SC-Leu media with the same toothpick and culture for 1–2 d at 30°C.
3. Pellet 4 mL of the culture, resuspend the pellet in 0.5 mL of sterile 15% v/v glycerol and store cells at –80°C. Next, use 0.5 mL of the cells for the plasmid rescue and store rest of culture (0.25–0.5 mL) at 4°C for further experiments.
4. Confirm the complementation by DNA sequencing and/or restriction analysis for the presence of the mutation in the plasmids isolated from yeast.

3.3. Selection of Novel Polymerases

The main objective behind our complementation experiment was to identify and characterize Pol3 enzymes that retained wild-type catalytic activity but were compromised in their fidelity. We used a forward mutation assay, specifically inactivation of CAN1 gene as the reporter to screen for the candidate mutants. Wild-type CAN1 codes for arginine permease, which transports arginine into the cell. Canavanine, an arginine analog, is cytotoxic to cells that have functional CAN1, and inactivation of CAN1 by spontaneous mutagenesis leads to canavanine resistance. Therefore rates of spontaneous mutation with different mutant alleles (polymerase-of-interest) can be readily assessed. The

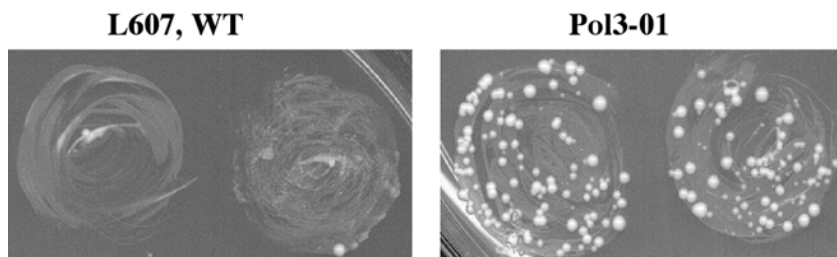


Fig. 1. Two single colonies of each strain were patched on SC-Leu-Arg+canavanine plates. Number of resistant colonies can be approximately correlated with rate of spontaneous mutagenesis in that strain. Pol3-01 is a strong mutator polymerase that is deficient in exonuclease proofreading.

spontaneous mutation in the *CAN1* locus acquired by the wild-type strain and an isogenic strain with exonuclease-deficient DNA polymerase δ is shown in **Fig. 1** as an example of the canavanine patch assay (*see Note 16*).

3.3.1. Canavanine Patch Assay

1. Using a soft edge toothpick or inoculation loop, randomly pick 3–5 colonies of equal size of each mutant, the wild-type and patch them on SC-Leu-Arg+canavanine plates (canavanine 60 mg/L). Starting from the center, gradually move outward in a circular motion until the diameter of the patch is about 1.5–2 cm. Incubate the plate at 30°C for 2–3 d.
2. Count the number of canavanine-resistant colonies in the wild-type strain and compare with the mutants.

4. Notes

Standard techniques in manipulating yeast (*S. cerevisiae*) have been assumed in this chapter, and the reader with no previous experience working with yeast is urged to refer to the commonly used molecular biology protocol book (**14**).

1. Any wild-type haploid strain can be used and minimum requirements are the presence of *Leu2*, *Ura3*, and/or *Trp1*, *His3* markers, which make them auxotrophic for leucine, uracil, tryptophan, and histidine biosynthesis, respectively. Usually, well-characterized strains like W303, BY4741 are preferable as data can be more meaningfully compared with the literature.
2. DNA polymerases α , δ , ϵ , and ϕ are essential for viability of haploid yeast and as an alternative to **Note 1**, a yeast strain that harbors a temperature-sensitive mutation in the DNA polymerase gene-of-interest can also be used. It is preferable to use a strain whose viability is compromised at non-permissive temperatures. For example, the yeast strain S111 *pol1-17*; *trp1-289 tyr1 ura3-1 ura3-2 ade2-101 gal2 can1 pol1-17* has been used for mutagenesis and selection of novel DNA polymerase α alleles by complementation and selection of the library at 37°C (**4**).

3. Expression of DNA polymerase genes in all eukaryotes including *S. cerevisiae* is cell-cycle regulated. The transcriptional elements that control the expression of these genes during G₁/S phase are usually present within approximately 700 bp upstream of the start site. Therefore it is imperative to search the literature for any information on the promoter region of the gene-of-interest, as this information is required to design the PCR primers for cloning into the appropriate vectors. The genetic complementation assay described in this chapter utilizes the native promoter element of Pol3 as both Ycplac III and Ycplac 33 vector have no yeast promoters upstream of their multiple cloning site (MCS). We recommend utilization of the native promoter as pleiotropic effects due to constitutive overexpression of DNA polymerases may cause aberrant growth defects. If the information on the promoter region is not documented in the literature, genomic DNA sequence starting from about 150–700 bp upstream of the start site can be reasonably assumed to encompass all the cell-cycle specific elements. If the expression vectors are constructed (and also complements the chromosomal “knock out” strain) without clear knowledge of the promoter region, it is also prudent to compare the growth rates, expression levels by Western blotting and examine the mutant cells on the wild-type strain by microscope.
4. If the multiple cloning site of Ycplac III and Ycplac 33 vectors are incompatible with the genomic DNA being cloned, consider cloning the DNA using either “linkers” or “adapters” or devise an alternative strategy by referring to the section titled “Generating new cleavage sites” in the technical appendix of New England BioLab’s product catalog. Other low-copy yeast vectors that carry Leu2 and Ura3 markers can also be considered.
5. We have found empirically in our lab that it is not necessary to use multiple spin columns for purification of DNA embedded in the agarose gel matrix according to the kit instructions. We have reliably purified up to 5 µg of DNA using one spin column; this enables DNA from several lanes to be pooled and purified in two-to-four columns. From the agarose gel, estimate the DNA yield (use quantitative DNA size standards), excise the bands and pool them into 15-mL centrifuge tubes, weigh the mass of the agarose and scale up the amount of buffer G (provided with the kit). We routinely elute DNA in 10 mM Tris-HCl, pH 7.5 buffer heated to 65°C.
6. Qiagen gel extraction can also be conveniently used to purify DNA after restriction digestion. Heat inactivate the restriction enzyme, weigh the mass of the liquid, and proceed with the purification according to the instructions. If necessary, several identical reactions can also be pooled together before purification.
7. Full-length Pol3 is unstable when propagated in *E. coli* and cultured at 37°C. Hence the *E. coli* transformed with full-length Pol3 was cultured at 30°C (7). The stability of the gene product of interest may have to be empirically determined.
8. The colonies that grow (transformants) on the SC-Ura plate can also be confirmed by restreaking them on a new SC-Ura plate. Inoculate 2–3 independent colonies into 5 mL SC-Ura medium, culture overnight till saturation at 30°C. Remove 0.5 mL of cells and confirm the presence of the plasmid by “plasmid rescue” and make glycerol stock of rest of the cells for long term storage.

9. We have transformed yeast by two different procedures:
 - a. The LioAc/PEG is normally used for transformation when high efficiency is required (15).
 - b. But for routine transformation and very small libraries, we use Frozen-EZ II kit (Zymo research, Orange, CA). The instructions are easy to follow and results are reliable.
10. If the amplification of the KanMX cassette is inefficient with the custom primers, optimize the PCR cycling conditions by lowering the annealing temperature to 54°C and incrementally raising the temperature by 2°C. Alternatively, the entire PCR cycling conditions can be divided into two stages. In the first stage, the reactions can be cycled for 4–6 cycles at lower annealing temperature (55°C) and for next 20–25 cycles the annealing temperature can be raised to 60°C.
11. The site-directed mutagenesis procedure described here is identical to the Stratagene's Quik-Change kit, but the entire procedure can be performed without purchasing the kit. Only custom oligonucleotides, DPN I, and Turbo *Pfu* DNA polymerase are required.
12. For site-directed mutagenesis the most critical parameter is the genotype of the *E. coli* strain that is used for propagation of the DNA template (YcPlac 111-gene-of-interest) used in the PCR reaction. Only Dam⁺ *E. coli* strains should be used.
13. We have had >90% success in identification of the mutant clone after site-directed mutagenesis. If wild-type sequence is identified, try screening 3–5 colonies instead of one.
14. In absence of any selection pressure, yeast cells randomly lose plasmids. Therefore the loss of Ycplac33-Pol 3(wt) can be selected by growing yeast cells on 5-FOA. It is usually easy to find cells that have lost the Ycplac33 plasmid among 4–8 colonies that are being streaked. It is also important to realize that 5-FOA resistance does not always guarantee loss of the plasmid unless confirmed by plasmid rescue. Yeast cells can also acquire mutations on the Ura3 marker gene thus inactivating them and gaining resistance to 5-FOA.
15. Those mutants that failed to grow on the 5-FOA plate by 2–4 d were left at 30°C for another week; we observed many discrete colonies for each of the mutants. The survivors were treated as suppressors and were not characterized further.
16. The assay described is purely qualitative and more thorough quantitative analysis of the mutation rates can be obtained from fluctuation assays (16,17).

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Autogene Selections

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1. Introduction

The evolution of proteins is more difficult than the evolution of nucleic acids both in principle and in practice. While nucleic acid sequence space has a dimensionality of 4^n , where n is the size of the nucleic acid pool (i.e., G, C, A, and T), protein sequence space has a dimensionality of 20^n . Similarly, while nucleic acids can frequently be directly selected for function from a random sequence population, the corresponding methods for the directed evolution of proteins are generally not as robust, in part because of the larger sequence spaces that must be explored, and in part because protein selection requires a translation step that in turn often requires cellular transformation, an inherently inefficient procedure that limits library size. In addition, the requirement for expression of the protein library in a host places limits on the numbers and types of selections that can be performed. Selecting individual colonies on plates is not well-suited to truly high-throughput methods and generally limits library sizes to on the order of 10^5 . Moreover, the complexity of cellular metabolism provides an almost limitless source of potential artifacts to confound the selection of a given phenotype. For example, attempts to evolve an antibiotic resistance element can be thwarted by the evolution of chromosomal resistance elements or by the evolution of plasmid copy number or promoter strength rather than protein efficiency (1,2). While there are frequently work-arounds for many of the artifacts that might be encountered, they nonetheless ultimately limit the phenotypes that can be selected.

In an attempt to make protein selection more like nucleic acid selection, we have explored methods that more closely couple information essential to survival and amplification. In a nucleic acid selection, the information in a selected sequence can be immediately amplified (that is, the selected sequence itself is

amplified). In a protein selection, the information in a selected sequence is frequently amplified as part of a larger genetic unit, whether it be a phage or a cell.

The ability of a protein to amplify its own gene or sequence should potentially provide a short-cut to more traditional protein selection methods. To this end, we wondered if it might be possible to develop a directed evolution technique based on so-called ‘autogene’ technologies (3–5). For example, when the RNA polymerase from bacteriophage T7 is cloned behind its own promoter sequence, it will self-amplify, generating large amounts of protein. Variants of a polymerase that ‘self-amplified’ more efficiently under any given set of conditions (higher temperatures, in the presence of unnatural amino acids, with a different promoter sequence) should accumulate. In polymerase autogene selections, the desired mutants are enriched by *in vivo* enzyme activity rather than host growth advantage or *in vitro* protein-substrate binding.

While we have embodied this method for RNA polymerases, similar autogene selection schemes can be envisaged for transcription factors, ligases, and other enzymes commonly involved in molecular biology manipulations. In addition, the cellular barrier between individual autogenes and their products need not be absolute: Ghadessy et al. (6) have described a similar scheme wherein *Taq* polymerase variants are embedded in water-in-oil emulsions, and upon thermal cycling the cell disintegrates, yet the polymerases and their genes remain in contact, allowing the critical self-amplification required of an autogene format.

1.1. The T7 RNA Polymerase Autogene

A T7 RNA polymerase autogene is a construct where the gene for T7 RNA polymerase is cloned downstream of its own cognate promoter. Expression systems based on T7 RNA polymerase are very useful because the enzyme is both highly active (T7 RNA polymerase is five times more efficient than *E. coli* RNA polymerase in elongating transcripts) and highly specific for its own promoter. Hence, T7 RNA polymerase can be used to overexpress particular proteins without expressing host cell genes or interfering with host cell polymerases, and a T7 RNA polymerase autogene can potentially be used as part of a protein overexpression system. The first T7 RNA polymerase autogene was created by Dubendorff et al. (7,8). This autogene was first cloned in a derivative of plasmid pBR322 in *E. coli*. However, autogene expression is potentially so powerful that either the polymerase construct or the target genes may confer a selective disadvantage on cells and may fail to be maintained over time (*see Note 1*). In order to keep basal T7 RNA polymerase activity sufficiently low, two different strategies were used: first, transcription initiation was blocked by cloning a *lac* operator in front of the polymerase gene, and second, polymerase activity was inhibited by co-expressing phage T7 lysozyme, which binds to and inactivates the polymerase.

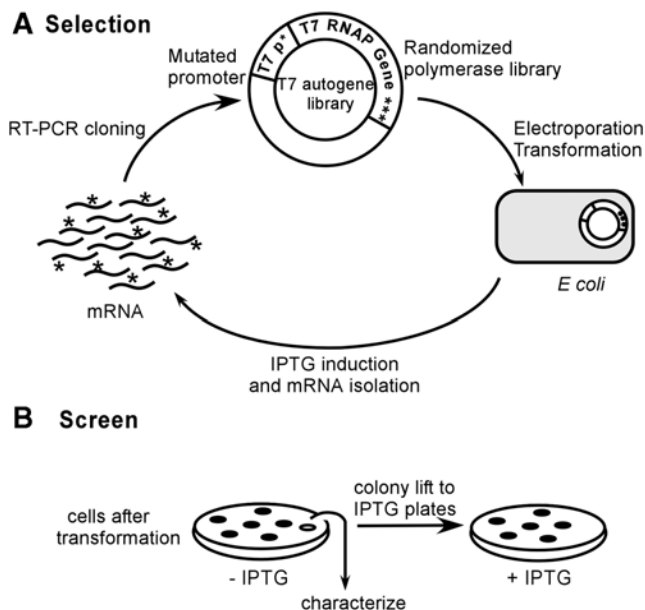


Fig. 1. Autogene selection scheme (see **Subheading 3.3.**). (A) An autogene library containing the polymerase pool and promoter mutations as described in **Subheading 3.2.** is transformed into cells and induced with IPTG. Active autogenes overexpress T7 RNA polymerases and the mRNAs encoding the polymerases. The total mRNA is extracted, and the gene for T7 RNA polymerase is reverse-transcribed and PCR-amplified. The gene fragments containing sequence variations (shown as *) are re-cloned and re-transformed. Several rounds of selection and amplification lead to the accumulation of polymerase variants with altered promoter specificities. (B) Screen for active variants (see **Subheading 3.3.10.**). The autogene library is initially plated on LB agar plates without IPTG. Colonies are lifted via nitrocellulose filters to a new plate containing IPTG and protein expression is induced. Colonies that have active autogenes cease to grow due to high polymerase expression levels. These colonies can be identified on the original plate, and subsequently picked and characterized by sequencing.

1.2. Autogene Selection

A combined *in vitro* / *in vivo* selection scheme was designed to promote the self-amplification of novel polymerase variants (9) (see **Fig. 1**). For example, when the polymerase was cloned adjacent to mutant T7 RNA polymerase promoters, little T7 RNA polymerase expression was observed. Any polymerase variant in the autogene pool that could recognize the mutant promoter should presumably re-establish the feedback loop and concomitantly lead not only to high protein expression levels, but also to high mRNA expression levels.

If a population of variants were to be transformed into cells, each cell should act as a discrete test tube, fostering the accumulation of the mRNA representing a given polymerase variant. At the conclusion of the self-amplification process, the variants could be thrown together, and polymerase mRNAs should be roughly represented in the mixed population according to the enzymatic success of the polymerases they encoded. In the case of a library cloned behind a mutant promoter, mRNA extracted from the population of cells should represent polymerase variants in rough proportion to their ability to utilize the mutant promoter. Re-cloning the successful sequences should over-represent successful polymerases relative to unsuccessful polymerases, and provide a means to carry out iterative rounds of selection and amplification. Multiple cycles of selection and amplification should ultimately lead to the accumulation of those polymerase variants that were most successful at facilitating their own expression.

1.3. Selections for Novel Promoter Specificities

As a proof of principle, we searched for polymerase variants that could utilize a promoter variant in which there was a G to C change at position -11. This mutation resembles the bacteriophage T3 promoter (10–12). A single asparagine to aspartate substitution at position 748 in T7 RNA polymerase was already known to facilitate the utilization of the T3-like promoter (13). A library of polymerase variants was constructed in which amino acid residues 746, 747, and 748 were completely randomized as described in **Subheading 3.2.2**. This library was then cloned behind the T3-like promoter and three rounds of selection and amplification (as described in **Fig. 1**) were carried out.

The progress of the selection was monitored in two ways. First, the autogene constructs were under the control of the lac repressor, and induction of the wild-type autogene by IPTG lead to cell death (*see Subheadings 1. and 3.3.10.*). Therefore, the fraction of colonies that were lost on replica plating to IPTG was hypothesized to be roughly proportional to the accumulation of active autogene variants. The proportion of IPTG-sensitive colonies was 20% after one round of selection, 88% after two rounds, and 96% after three rounds. Second, the number of PCR cycles that were required to amplify recovered mRNA molecules was assumed to correlate with the amount of mRNA that accumulated in bacteria during a given round of selection. It took 20 PCR cycles for Round 1 RT-PCR DNA to be visualized on an agarose gel, 14 cycles for Round 2, and 12 cycles for Round 3. The selection was therefore assumed to be essentially complete following round three.

Active polymerase variants were identified, cloned, and sequenced following each round of selection. It was found that the selection not only quickly re-established the wild-type amino acids at positions 746 (arginine) and 747 (leucine),

but also converged on the known N748D change, indicating that the autogene selection method is working as expected. Moreover, the convergence on the expected sequence indicated that the screening methods described above did in fact accurately reflect the extent of selection.

A second selection was also carried out to examine a wider range of promoter and polymerase combinations. For this experiment, a promoter library was constructed in which positions -8 through -11 in the promoter were completely randomized; each of these nucleotides had previously been shown to be extremely important for the specificity of interactions with T7 RNA polymerase (13-16). The promoter library was combined with the gene library in order to create approx 51,200 ($4^4 \times 20^3$) combinations of promoters and polymerases. The joint promoter:polymerase library was transformed into *E. coli* and variants were again selected as described in **Subheading 3.3**. To begin each new round, the polymerase variants were re-cloned behind the promoter library. However, after each round of selection only the polymerase mRNA could be recovered by reverse transcriptase-PCR (RT-PCR) for the next round, since the corresponding promoters were not part of the transcript. This is meant that at each round a given polymerase variant had to randomly re-find one or more promoters that it could productively utilize. However, this was not an overly daunting task, since there were only ca. 256 promoters. At the conclusion of the selection, successful combinations of promoters and polymerase variants were identified by screening for colonies that could not grow on isopropyl- β -D-thiogalactopyranoside (IPTG). This selection identified polymerase variants that could utilize a variety of T7 promoters; a summary of the selected polymerase variants and the promoters that they can utilize in vivo is shown in **Table 1**.

2. Materials

2.1. Kits and Reagents

1. Restriction endonucleases (New England Biolabs, Beverly, MA).
2. Topo TA cloning kit (Life Technologies, Carlsbad, CA).
3. T4 polynucleotide kinase (New England Biolabs).
4. QIAquick gel purification kit (Qiagen, Valencia, CA).
5. DNA mini, midi, and maxi prep kits (Qiagen).
6. QIAquick PCR purification kit (Qiagen).
7. T4 DNA ligase (Life Technologies).
8. *Taq* DNA polymerase (Promega, Madison, WI).
9. DNase I (Promega).
10. Shrimp alkaline phosphatase (USB, Cleveland, OH).
11. MasterPure RNA purification kit (Epicenter Technologies, Madison, WI).
12. AMV reverse transcriptase and 5X RTase buffer (USB).

Table 1
Summary of Selection for Polymerases
with Altered Promoter Specificities

<i>Polymerases</i>	<i>Positions in the polymerase gene*</i>			<i>Promoter Sequence</i> (−11 to −8)
	748	756	758	
Round 3				
R3 – 9	Asn	Arg	Cys	GACT
R3 – 32	Asn	Arg	Cys	GAC <u>G</u>
R3 – 26	Asn	Arg	Cys	<u>TGTA</u>
R3 – 17	Ala	Met	Ser	<u>GGTA</u>
R3 – 5	Phe	Gly	Ile	<u>GCTA</u>
R3 – 29	Thr	Lys	Gln	GACT
Round 4				
R4 – 14	Asn	Arg	Cys	GACT
R4 – 16	Asn	Arg	Cys	<u>GTTA</u>
R4 – 17	Asn	Arg	Cys	<u>GTCA</u>
ppC6	Asn	Arg	Cys	<u>GATA</u>
R4 – 11	Asn	Arg	Ser	GACT
*Wild-type	Asn	Arg	Gln	GACT

2.2. Cell Lines

1. DH5Δlac was a kind gift from Dr. Brian Sauer (Stowers Institute of Medical Research, Kansas City, MO).
2. INVαF' was purchased from Life Technologies.
3. NovaBlue and HMS174 were purchased from Novagen (Madison, WI).

2.3. Plasmids

1. pET28a+ and pLysS were purchased from Novagen.
2. pAR1219 was a kind gift of Dr. David Hoffman, University of Texas at Austin; this plasmid was originally developed by Studier (8).

3. Methods

The methods described below outline the construction of the wild-type T7 RNA polymerase autogene, the generation of autogene libraries, and selections using the autogene libraries.

3.1. Construction of the Wild-Type T7 Autogene Construct (pET/T7/T7)

The T7 autogene was made by cloning the T7 RNA polymerase gene into the plasmid pET28a. The T7 *lac* promoter in the plasmid pET28a contains a 25

base pair *lac* operator sequence immediately downstream from the 17 base-pair T7 RNA polymerase promoter. It also carries the natural promoter and coding sequence for the *lac* repressor (*lacI*). Binding of the *lac* repressor, to the *lac* operator effectively blocks transcription by T7 RNA polymerase. Addition of IPTG derepresses the T7 RNA polymerase promoter and induces the expression of the T7 RNA polymerase gene. The plasmid pET28a also contains the T7 terminator and a ribosomal binding site for translation of the cloned gene.

The steps in the construction of the wild-type T7 autogene are listed below:

1. Using plasmid pAR1219, (originally made by Studier [8]) as a template, a 2.7 kb fragment containing the T7 RNA polymerase gene was amplified using the primers ae32.1 and ae29.1. These primers were designed to have EcoRI and BsmBI restriction sites and had the following sequences:

ae32.1:

GGG CGT CTC GCA TGA ACA CGA TTA ACA TCG CT
(BsmBI site is underlined)

ae29.1:

GGG AAT TCT TAC GCG AAC GCG AAG TCC GA
(EcoRI site is underlined)

2. The PCR product was first directly cloned into the vector pCR2.1 (Topo TA cloning kit, Life Technologies) using the protocol suggested in the kit (*see Note 2*).
3. The resulting plasmid was then digested with BsmBI and EcoRI and the T7 RNA polymerase gene was cloned into the expression plasmid pET28a+ after digesting the latter with NcoI and EcoRI (Novagen). BsmBI cleaves downstream of its restriction site in primer ae32.1 and generates a sticky end compatible with NcoI.
4. The wild-type autogene thus obtained (pET/T7p/T7) was transformed into strain HMS174 pLysS (Novagen), the same cell line Studier et al. initially used to express the autogene (8). This strain contains plasmid pLysS which encodes T7 lysozyme, the natural inhibitor of T7 RNA polymerase. The plasmid pLysS also confers resistance to chloramphenicol and is compatible with pET28a.

3.2. Construction of Autogene Libraries

Autogene libraries were constructed by first generating vectors containing promoter mutations, and then ligating randomized T7 RNA polymerase genes into these vectors. The libraries were then transformed into DH5Δ*lac* pLysS cells for selection (*see Note 3*).

3.2.1. Libraries with Promoter Mutations (pET/T7p*/T7)

The promoter point mutation G(−11)C and the *lac* operator region were introduced adjacent to the T7 RNA polymerase promoter using the oligonucleotides ae66.1 and ae66.2. Annealing these oligonucleotides generated sticky ends that were suitable for ligation into the pET/T7/T7 autogene construct cleaved with BglII and XbaI.

ae66.1:

GAT CTC GAT CCC GCG AAA TTA ATA CCA CTC ACT ATA GGG
GAA TTG TGA GCG GAT AAC AAT TCC CCT (BglII site underlined)

ae66.2:

CTA GAG GGG AAT TGT TAT CCG CTC ACA ATT CCC CTA TAG
TGA GTG GTA TTA ATT TCG CGG GAT CGA (XbaI site underlined)

Similarly, oligonucleotides gcP1.66 and gcP2.66 were used to randomize positions -8 to -11 in the T7 RNA polymerase promoter.

gcP1.66:

GAT CTC GAT CCC GCG AAA TTA ATA CNN NNC ACT ATA GGG
GAA TTG TGA GCG GAT AAC AAT TCC CCT (BglII site underlined; N
indicates an equimolar mix of the four bases)

gcP2.66:

CTA GAG GGG AAT TGT TAT CCG CTC ACA ATT CCC CTA TAG
TGN NNN GTA TTA ATT TCG CGG GAT CGA (XbaI site underlined; N
indicates an equimolar mix of the four bases)

Oligonucleotides were synthesized in our lab on an ABI 394 DNA synthesizer (PE Biosystems Foster City, CA). For annealing, the oligonucleotides were mixed together, heated at 94°C for 1 min and allowed to cool to room temperature over 10 min. The annealed, double-stranded DNAs were phosphorylated with T4 DNA polynucleotide kinase prior to ligation (*see Subheading 3.2.1.1.*). Throughout the cloning procedures, oligonucleotides were visualized on a 4% agarose gel.

3.2.1.1. PHOSPHORYLATION OF OLIGONUCLEOTIDES

1. Mix 1 μ L 100 μ M double-stranded DNA, 1 μ L 10X T4 DNA kinase buffer (New England Biolabs) and 5 Units T4 DNA polynucleotide kinase (New England Biolabs). Add water to 10 μ L.
2. Incubate at 37°C for 1 h.
3. Inactivate the T4 DNA polynucleotide kinase by incubating at 65°C for 15 min.

3.2.1.2. CLONING TO CREATE THE LIBRARY WITH PROMOTER MUTATIONS (pET/T7P*/T7)

1. Cleave pET/T7/T7 autogene construct with BglII and XbaI, and recover the right size vector DNA fragment using the QIAquick gel extraction kit.
2. Quantitate the concentration of the vector and the phosphorylated oligonucleotide insert. For ligation, a vector to insert molar ratio of 1:3 was used (30 fmol vector ends to 90 fmol insert end).
3. Set up ligation reactions in 20 μ L volumes with 5X T4 DNA ligase buffer, and 1 unit of T4 DNA ligase. Perform ligations at 19°C for 6 h.
4. Deactivate the ligase by incubating the ligation mix at 70°C for 10 min.
5. Transform the ligated DNA into the electrocompetent cells prepared as described in the **Subheading 3.3.1.** to form the library with promoter mutations (pET/T7p*/T7).

3.2.2. Libraries with Random Regions in the Polymerase Gene (pET/T7p*/T7*)

In the selection for T3-like promoter specificity, T7 RNA polymerase was randomized at amino acid positions 746–748. Two pairs of primers (gcT7a.6 and gcT7lib1; gcT7a.9 and gc3'pET) and the wild-type (pET/T7p/T7) plasmid were used to generate two gene fragments, which were in turn assembled by overlap PCR (17) (*see Subheading 3.2.2.1.*). In the selection in which both the promoter and the polymerase were varied, T7 RNA polymerase was similarly randomized at amino acid positions 748, 756, and 758 using the primer gcT7lib2.80.

gcT7a.6: GGACCGATAA CGAAGTAGTT ACCGTGACCG

gcT7lib1.66:

CTG cAA cCG GAA CTG cCC GAG GAA CAT CAG NNN NNN NNN
CGT CTG AAT gGG CTT CTT GTA TTC CTG (residues 2236–2244 are randomized, silent mutations are in lower case)

gcT7lib2.80:

C GCT ATC cTT GTT GGT GTT gAT GGT AGG NNN TAA NNN GAA
CTG cCC GAG GAA CAT CAG NNN CAA GCG CGT CTG AAT AGG C
(residues 2241–2244, 2266–2268, and 2272–2274 are randomized, silent mutations are in lower case)

gcT7a.9:

CTG ATG TTC CTC GGg CAG TTC CGg TTg CAG (mismatches are in lower case)

gc3'pET: GCT CAG CGG TGG CAG CAG CCA ACT C

3.2.2.1. OVERLAP PCR

1. Set up a polymerase chain reaction with the pET/T7/T7 plasmid and primers gcT7a.6 and gcT7lib1 to yield the upstream, double-stranded fragment. Set up a PCR with primers gcT7a.9 and gc3'pET to yield the downstream, double-stranded fragment.
2. Purify both fragments using a QIAquick gel purification kit.
3. In the overlap PCR, for a 50 μ L total volume reaction, add upstream and downstream fragments in an equimolar ratio (less than 200 ng per fragment) and requisite amounts of PCR buffer, dNTPs and Taq polymerase. Carry out 5 thermal cycles to generate the initial, full-length template. Then add 0.5 μ L each of 20 μ M solutions of the gcT7a.6 and gc3'pET primers. Carry out 20 more thermal cycles.
4. Gel-purify the PCR amplification product using the QIAquick gel purification kit. Quantitate the amount of product generated by visualization in a 3% agarose gel.

3.2.2.2. CLONING THE RANDOMIZED POLYMERASE GENE INTO THE AUTOGENE VECTOR

1. Digest the purified overlap PCR products and autogene vectors containing mutated promoters (e.g., pET/T7p*/T7) (*see Subheading 3.3.7.*) with the restriction enzymes AflIII and EcoRI.

2. Gel purify the fragments containing randomized regions using the QIAquick gel purification kit.
3. Ligate the purified fragments into the appropriate vectors (*see Subheading 3.3.8.*).

3.3. Selection Procedure

The scheme for autogene selections is shown in **Fig. 1**.

1. Transform the autogene pool into DH5 Δ lac pLysS cells by electroporation (*see Subheadings 3.3.1. and 3.3.2.*)
2. Incubate the culture at 37°C for 7–10 h and induce T7 RNA polymerase expression by adding IPTG to a final concentration of 0.4 mM (*see Subheading 3.3.3.*).
3. After an hour of induction, extract RNA using the Masterpure RNA purification kit (Epicenter Technologies) following the protocol suggested in the kit.
4. Treat the purified RNA with DNaseI (*see Subheading 3.3.4.*) to remove trace DNA contamination and extract it with phenol-chloroform to remove the DNaseI.
5. Reverse-transcribe the extracted RNA (*see Subheading 3.3.5.*) using AMV-reverse transcriptase (USB) and the primer gc3'pET.
6. PCR-amplify the resulting cDNA using the primers gcT7a.6 and gc3'pET. The PCR mix should be treated with proteinase K to remove *Taq* DNA polymerase (*see Subheading 3.3.6.*).
7. Gel purify the PCR products using the QIAquick PCR purification kit and digest it with AflIII and EcoRI (*see Subheading 3.3.7.*).
8. Ligate the purified insert back into the original autogene vector (*see Subheading 3.3.8.*) to form a fresh autogene pool for subsequent rounds of selection.

Several cycles of selection and amplification lead to the enrichment of those polymerase variants that are most successful at recognizing the variant promoter and facilitating their own expression. Bacteria containing these polymerase variants can be further identified by screening for colonies that are unable to grow on IPTG plates (*see Subheading 3.3.10.*). The different steps in the selection process are described in detail below.

3.3.1. Preparing Competent Cells for Electroporation (modified from Dower's protocol [18])

1. Pick a single colony of DH5 Δ lac pLysS from a fresh plate. Grow in LB media containing appropriate antibiotics at 37°C for 8 h with vigorous shaking.
2. Dilute the initial culture 1:100 into a 1-L culture. Vigorously shake at 19°C to an OD₆₀₀ of 0.3–0.8.
3. Harvest the cells by chilling on ice for 5 min and centrifuging at 3250g for 10 min at 4°C.
4. Gently resuspend the cell pellet in 1 L cold, autoclaved, double-distilled water.
5. Centrifuge at 3250g for 10 min at 4°C.
6. Gently resuspend the cell pellet in 0.5 L cold, autoclaved, double-distilled water.
7. Centrifuge at 3250g for 10 min at 4°C.

8. Finally, gently resuspend the pellet in 2–20 mL of cold 10% glycerol (or cold, autoclaved, double-distilled water).

3.3.2. Transformation by Electroporation

1. Prepare fresh, electroporation competent cells for every transformation. Keep the cells on ice at all times.
2. Add ligated DNA to the competent cells on ice.
3. Transfer the concentrated, competent cells containing DNA to a 0.2-cm electroporation cuvet. Use at most 300 μ L cells per cuvet. Electroporate at 2.5 kv (we use the E. Pulser from Bio-Rad Laboratories, Hercules, CA). Add a 50-fold excess volume of SOC immediately following electroporation.
4. Shake vigorously at 37°C for one hour. Add a 100-fold excess volume of Luria-Bertani (LB) with antibiotics. Then shake at 37°C for no more than 10 h.

3.3.3. Induction and Total mRNA Purification Following Selection

1. At OD₆₀₀ about 0.5, induce with IPTG to a final concentration of 0.4 mM.
2. After one hour of induction with IPTG, isolate total mRNA using the Masterpure RNA purification kit (Epicenter Technologies) following the protocol suggested in the kit (*see Note 5*).

3.3.4. DNaseI Treatment and mRNA Purification

The isolated mRNA should be further treated with DNase to ensure the complete removal of contaminating DNA.

1. For 500 μ g of total RNA, add 25 Units of DNase I (Promega) with 50 μ L 10X RNase-free DNase I buffer (Promega). Add diethylpyrocarbonate (DEPC)-treated water to 500 μ L. Incubate at 37°C for 1 h.
2. Carry out a phenol-chloroform extraction to remove the DNase I.
3. Ethanol precipitate the purified RNA.

3.3.5. Reverse Transcription Using AMV Reverse Transcriptase

A trial reverse transcription reaction and PCR should be done prior to carrying out a large scale RT-PCR reaction in order to optimize the amount of RNA that will be used to create the pool for the next round of selection. Control reactions without RNA input and without reverse transcriptase should also be carried out to make sure that there is no residual DNA contamination.

1. Mix RNA (~1–5 μ g), and primer (2.5 μ M final concentration), and heat denature at 72°C for 3 min. Cool on ice for 5 min.
2. Add 5X RTase buffer (USB), 4 Units AMV Reverse Transcriptase, and dNTP's (0.4 mM final concentration) in a total volume of 20 μ L; incubate the mixture at 42°C for one hour.
3. For the trial PCR, 10 μ L of the RT reaction is used as input for a 50 μ L total volume PCR. The trial reaction is iteratively thermal cycled in order to optimize

the number of cycles required for large scale RT (typically it takes about 15–20 cycles for DNA from Round 1 to be visualized). At each interval (typically every 3–5 thermal cycles), 10 μ L aliquots are resolved and visualized on a 1% agarose gel. Once the optimal number of cycles for amplification has been determined, this will be applied to the remainder of the RT reaction.

3.3.6. PCR Reaction Purification to Remove Taq Polymerase

1. For every 93 μ L of amplified DNA (still in the PCR mix), add 1 μ L 1 M Tris-HCl, pH 7.8, 1 μ L of 0.5 M ethylenediaminetetraacetic acid (EDTA), 5 μ L 10% sodium dodecylsulfate (SDS) (final concentrations of 10 mM Tris, 5 mM EDTA, and 1% SDS).
2. Add 2.5 μ L proteinase K (20 mg/mL). Incubate at 37°C for 30 min to 1 h.
3. Heat at 68°C for 15 min to inactivate proteinase K.
4. Purify the amplified DNA using the QIAquick PCR purification kit (Qiagen).

3.3.7. Digestion of the DNA Insert and Vector Using Restriction Enzymes

3.3.7.1. FRAGMENT CONTAINING THE LIBRARY

The DNA obtained after RT-PCR is digested using the restriction enzymes AflIII and EcoRI. The digestion reactions are usually carried out at 37°C for 12–14 h unless otherwise recommended by the manufacturer. The fragment containing the random region is incised and gel purified using the QIAquick gel purification kit.

3.3.7.2. VECTOR PREPARATION

1. Digest the pET/T7p/T7 plasmid at 37°C overnight using the restriction enzymes AflIII and EcoRI to yield fragments of lengths 7.4 and 0.6 kb (from pET/T7p/T7).
2. Heat the digestion mixture to 65°C for 15 min to inactivate the restriction enzymes.
3. Dephosphorylate the vector fragment using shrimp alkaline phosphatase (SAP; 10 units for ~1 μ g of vector DNA) at 37°C for 30 min.
4. Inactivate the phosphatase at 65°C for 15 min. Purify the 7.4 kb fragment using the QIAquick gel purification kit for subsequent ligation reactions.

3.3.8. Ligation Reactions

During each round of selection, a test ligation was performed prior to the large scale ligation in order to determine the optimum insert-to-vector ratio for library construction.

1. The test ligation reaction volume should be 20 μ L with 5X T4 DNA ligase buffer, and 1 unit of T4 DNA ligase. Perform ligations at 19°C for 6 h. Set up parallel reactions with varying molar ratios of vector to insert. (typically, the insert to vector ratios were varied between 1:1 to 4:1).

2. Deactivate the ligase by incubating the ligation at 70°C for 10 min.
3. Transform the ligated DNA into electrocompetent cells prepared as described in **Subheading 3.3.1**.
4. Plate an aliquot of cells on selective medium and incubate at 37°C overnight in order to assay the library size.

The optimal insert:vector molar ratio was typically found to be between 1:1 and 3:1. Large scale ligation is then performed by scaling up the test ligation that gave the best transformation efficiency. Approximately 1 µg vector DNA is used in the large ligation reaction during each round of selection along with 10 units of T4 DNA ligase in a total volume of 200 µL.

3.3.9. Cleaning up Ligation Products for Efficient Transformation (19)

This procedure has been found to increase electroporation transformation efficiency. Therefore, following the ligation step at each round of selection:

1. Add 4X vol of water to 1X vol of ligation reaction.
2. Add 50X vol of butanol and mix thoroughly.
3. Centrifuge at 13,000g for 10 min at 4°C.
4. Remove the supernatant completely. Air dry for 5 min.
5. Suspend the DNA pellet in 0.5X vol of water.

3.3.10. Screen for Active Mutants

A colony lift technique was used to monitor the progress of the selection. Cells containing very active autogenes cease to grow when lifted to LB plates containing IPTG.

1. Roughly 1 h after electroporation and growth at 37°C, plate an aliquot of the cell culture containing the autogene pool onto LB plates containing appropriate antibiotics, and incubate at 37°C for 8–12 h.
2. Lift the colonies from these plates to plates containing IPTG using a butterfly nitrocellulose membrane (Midwest Scientific, Valley Park, MO).
3. Incubate both plates at 37°C for approx 8 h.
4. Compare the sizes of corresponding colonies in the plates containing IPTG and the ones without IPTG.
5. Pick colonies that did not grow well upon lifting to IPTG from the original plate and characterize them by sequencing. In each round at least 5000 or more individual variants can be examined.

4. Notes

1. The high level of T7 RNA polymerase expression from an active, wild-type T7 autogene has been found to be detrimental to the host cell growth (7–9). Therefore, it is critical to control the activities of autogenes inside cells. In the T7 RNA polymerase autogene construct, a lac operator was included between the T7 promoter and the T7 RNA polymerase gene. The lac operator is bound tightly by the

lac repressor (dissociation constant $K \sim 10^{-13} M^{-1}$). This binding efficiently represses transcription (7,20,21). In the presence of the gratuitous inducer IPTG, the affinity of lac repressor for the lac operator is reduced and transcription can proceed. Each subunit of the lac repressor is capable of binding one IPTG molecule with a dissociation constant $K \sim 10^{-6} M^{-1}$. However, transcriptional regulation by the lac operator alone proved to be inadequate to completely stabilize plasmids carrying the wild-type T7 RNA polymerase autogene. Therefore, an additional layer of inhibition of transcription was added. T7 lysozyme is a phage protein that naturally sequesters T7 RNA polymerase from transcription, thereby regulating the expression of phage proteins (22). Plasmids containing the wild-type T7 autogene could only be established in uninduced *E. coli* under both lac repression and lysozyme inhibition.

2. The PCR-amplified fragment containing the T7 RNA polymerase gene was initially digested with BsmBI and EcoRI and ligated into a pET28a vector. The ligation products were transformed into competent Novablue pLysS cells by heat shock. However, only a few colonies grew and plasmid digestion patterns indicated that the transformants contained vectors with no insert. After several more trials of digestion, purification and ligation, the T7 RNA polymerase gene still could not be successfully cloned into pET28a. In the ae32.1 primers, the BsmBI restriction site was only 3 base-pairs away from the end of the fragment; therefore the BsmBI endonuclease may have failed to cleave. As an alternative, PCR-amplified fragments containing the T7 RNA polymerase gene were first cloned into pCR2.1 using the Topo TA cloning kit. The ligation reactions were then transformed into competent Novablue pLysS cells.
3. Prior to the autogene selection, the toxicity of the wild-type autogene was monitored on plates and in liquid cultures with or without IPTG induction. The HMS174 pLysS cell line was first used to establish the plasmid pET/T7/T7, which contains an active autogene. Nonetheless, these cells still grew slower than cells that contained an inactive autogene (pET/T7p*/T7). Transformation with pET/T7/T7 also gave substantially fewer colony-forming units (CFUs) than transformation with pET/T7p*/T7. Therefore, a variety of cell lines were assayed to identify which strain seemed to be most tolerant of the autogene. Transformation efficiencies with pET/T7/T7 were determined for more than 10 different cell lines. In all instances, it was observed that if a cell line did not contain pLysS, pET/T7/T7 could not be established. Among the cell lines tested, DH5Δ*lac*(pLysS) cells gave the best transformation efficiencies and hence was chosen for selections.
4. In constructing the autogene pool for the selection where both the polymerase and promoter were randomized, two stop codons were first introduced at amino acid positions 747 and 748 in the wild-type RNA polymerase gene to form pET/T7/T7stop. This safeguard eliminated the possibility that wild-type RNA polymerases could be selected due to undigested vector background. Also, preventing the expression of active T7 RNA polymerase removed possible selection pressure and obviated skewing of the promoter library due to the toxicities of active

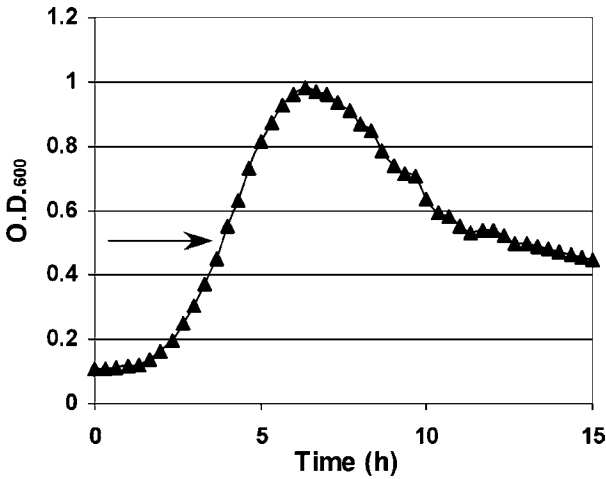


Fig. 2. Growth characteristics of cells containing a wild-type autogene (pET/T7/T7; see **Notes 1** and **5**). Cells containing the wild-type autogene (pET/T7p/T7) were grown at 37°C and induced with 0.4 mM IPTG at an OD₆₀₀ of 0.5 (indicated by the arrow). The OD₆₀₀ was monitored every 15 min for 15 h using an automated microbiology workstation, the Bioscreen C (Labsystems Oy, Finland).

autogene constructs. Oligonucleotides containing promoters randomized between the -8 and -11 positions were then cloned into pET/T7/T7stop to form an autogene construct with a promoter pool, pET/T7pp/T7stop. Unselected clones from this pool were sequenced, and the distribution of random sequence nucleotides was estimated to be 29% G, 21% A, 19% T, and 24% C.

5. The growth curve of DH5Δ*lac* pLysS cells containing an active autogene, pET/T7/T7, is shown in **Fig. 2**. At an OD₆₀₀ of 0.5, the culture was induced by adding IPTG to a concentration of 0.4 mM. It is apparent that cells containing an active autogene are viable for at least 2 h following induction with IPTG. Therefore, total RNA was isolated one hour after induction with IPTG during each round of selection. The timing of RNA harvesting could be varied in order to identify autogene variants that were quickly transcribed or translated, or were slowly turned over.
6. Large-scale RT and PCR can be performed by scaling up the test reactions, either in terms of volumes or the number of tubes. Typically 25–40 μg of RNA is introduced into the reverse transcriptase reaction at each round of selection.
7. Because of the deleterious nature of autogene expression on both transformation and cell growth, it is entirely possible that the most active autogenes were never selected from our population. We do not believe this was not a problem given the small library sizes that were used and the limited attempts to alter specificity that have so far been carried out, but toxicity problems could confound other, more ambitious selection experiments.

8. While these experiments describe the selection of T7 RNA polymerase autogenes, it is relatively easy to envision other selection experiments that similarly ask for a 'reflective' interaction between a gene and its gene product or cellular phenotype. For example, similar methods could be used to evolve transcription factors that enhanced their own synthesis. A tRNA synthetase gene placed in parallel with the T7 RNA polymerase gene could function as a selectable 'mini-operon' that could evolve to suppress stop codons within either gene. Another example is the evolution of a mutator allele to yield higher mutation frequencies, allowing faster adaptation to a variety of antibiotics.

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Selection for Soluble Proteins via Fusion with Chloramphenicol Acetyltransferase

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1. Introduction

The low solubility of a protein is one of the most frequent impediments for its structural and functional analysis and, on a more practical aspect, for its application as an industrial enzyme. The reason for low solubility can lie in low conformational stability (1), in a high number of surface-exposed hydrophobic amino acids (2) or in certain structural features, such as membrane binding regions (3). By changing the amino acid sequence of these proteins, their solubility can be significantly improved (4,5). Hence a general method that can efficiently identify (i.e., select) more soluble protein variants from a large repertoire is very useful in evolving such proteins.

A solubility selection can also be used to accelerate a general screening process. The limit in evolutionary approaches is rarely the creation of the repertoire but instead the analysis, i.e., the identification of the few interesting candidates. When evolving enzymes for certain activities, unwelcome mutations usually extensively dilute the repertoire. To most efficiently sieve through the many variants, it is advisable to apply a tiered screen. A good first tier should accommodate a high number of variants and should target a property that is sensitive to negative mutations. Both are provided by a selection for solubility.

Protein solubility and protein stability are often closely related. The PROSIDE approach (6) (see **Chapter 6**) is useful in selecting more stable proteins and can in general also be applied for the selection of more soluble variants. One limitation, though, is that it requires both termini to be on the surface of the protein and that it cannot be applied to enzymes that require the processing of proteins with pro- or prepro-domains to reach an active state. A possible approach to overcome this is to fuse the protein of choice to a reporter protein whose activity will

depend on the solubility and stability of the former. Waldo et al. (5,6) have shown that the activity of a reporter protein can correlate with the stability of different variants of one protein when these were fused with the green fluorescent protein (GFP). Maxwell et al. (4) used chloramphenicol acetyltransferase (CAT) as fusion partner for variants of HIV integrase and also found a very good correlation between the solubility of this protein and the ability of *E. coli* to grow on chloramphenicol (cam). Their approach has the advantage of allowing a true selection: negative variants are directly eliminated. Sieber et al. (7) used a fusion with CAT in order to select protein variants of cytochrome P450 that had lost their affinity to the membrane and were more soluble. Their approach was to use the selection as a first-tier filter before an enzymatic activity screen. It also helped to eliminate all variants that were not completely translated (shift in the reading frame). This identification of more soluble variants of cytochrome P450 enzymes from a library of chimeric P450 enzymes will be used here to illustrate the methods utilized for a successful application of a solubility selection based on a fusion with chloramphenicol acetyltransferase.

2. Materials

2.1. Solutions

1. Luria-Bertani (LB) broth with appropriate antibiotic as liquid and as agar.
2. Chloramphenicol stock solution of 4 mg/mL in ethanol.
3. Chloramphenicol stock solution of 40 mg/mL in ethanol.
4. Oligonucleotide primers, 10 pmol/ μ L stock in ddH₂O.
5. dNTP mixture, 10 mM each dATP, dCTP, dGTP, dTTP.

2.2. Enzymes

1. Restriction endonucleases with appropriate buffers.
2. T4 DNA ligase, 2000 U/ μ L (New England Biolabs, Beverly, MA).
3. *Pfu* DNA Polymerase 2.5 U/ μ L (Fermentas, Hanover, MD).
4. T4 DNA Polymerase (New England Biolabs).

2.3. Miscellaneous

1. Parental gene in expression vector.
2. Plasmid pACYC184 (New England Biolabs).
3. *Escherichia coli* competent cells with a minimum competency of 10⁸/ μ g DNA and cells with a minimum competency of 10⁷/ μ g DNA, e.g., XL1-Blue (Stratagene, La Jolla, CA).
4. 37°C incubator and shaker.
5. Equipment, buffer and chemicals to run and analyze agarose gels.
6. Spectrophotometer or microplate reader to measure bacterial growth via absorption at 600 nm.

7. QIAEXII Gel Extraction Kit (Qiagen; Valencia, CA).
8. QIAquick PCR Purification Kit (Qiagen).
9. Centri-Spin 10 columns (Princeton Separations; Adelphia, NJ).

3. Methods

The methods below outline 1) the fusion of the gene for chloramphenicol acetyltransferase (CAT-gene) to the gene of interest, 2) construction of control variants, 3) analysis of resistance towards chloramphenicol for adjustment of selection conditions and 4) the actual selection/screen of more soluble variants.

3.1. Construction of Gene Fusion

A fusion between the gene of the protein of interest and the CAT-gene, both connected through an oligonucleotide linker, is first constructed (*see Note 1*). When the gene is already available in an expression vector and when a convenient restriction site is available at the end of the gene, upstream of the stop codon, the CAT-gene together with a suitable linker sequence is amplified by PCR and ligated directly into the vector 3' of the gene of interest using this restriction site. When no restriction site is available the genes should be fused by splicing by overlap extension (GeneSOEing) (**8**). For our example given here, we have a restriction site for *MfeI* directly in the last codon of the gene (*see Fig. 1*). The peptide linker between the two proteins should be flexible, hydrophilic and not a first hand substrate for proteases (*see Note 2*). It is important to eliminate the start codon of the CAT-gene (*see Note 3*). The gene of the protein of interest can be terminated with an amber stop codon, which allows the chloramphenicol-selection to be performed in an amber-suppressor strain while the expression of the isolated protein can be done in a non-suppressing strain (*see Note 4*).

The resulting gene fusion is cloned into an expression vector and verified by sequencing (*see Note 5*).

3.1.1. Primer Design and Amplification of CAT-Gene

Design your primers the following way:

5' region of CAT-gene:

5'- GCGGACCAAT TGG CCT GGG TCC CCT GCT AGC GAGAAA
AAAATCACTGGATATACC -3'. The singly underlined sequence binds to the 5' end of the CAT-gene (T_m of 57°C). The double underlined sequence encodes a recognition site also present at the 3' end of the gene (i.e., *MfeI*). The codons in bold are encoding the linker sequence (Trp-Pro-Gly-Ser-Pro-Ala-Ser). The start codon of the CAT-gene is removed.

3' region of CAT-gene:

5'-CGACGATCTAGATTA CGCCCCGCCCTGCCACTC-3'. The singly underlined sequence binds to the 3' end of the CAT-gene (T_m of 73°C; *see*

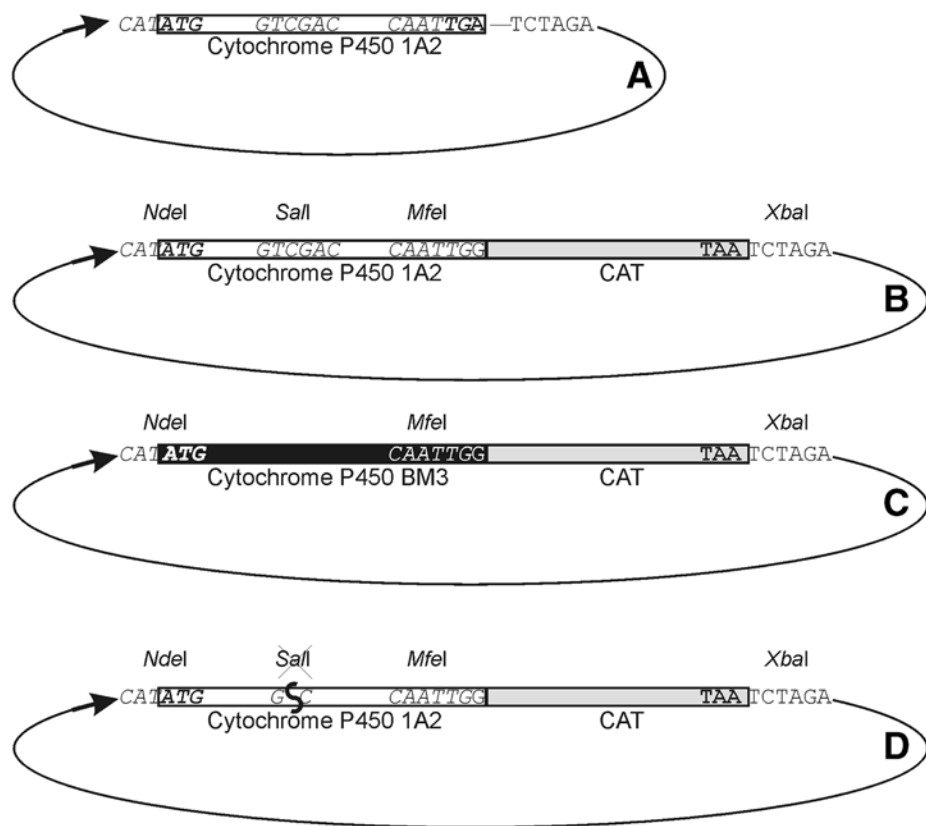


Fig. 1. Scheme of the different variants to be constructed from (A) the gene in the expression plasmid, (B) CAT-gene fusion, (C) positive control and (D) negative control. All are based on the selection performed with cytochrome P450 (7). The important restriction sites (recognition sequence in *italics*) and the start and stop codons (in **bold**) are shown. The arrow indicates a promoter region.

Note 6). The double underlined sequence encodes a different restriction site for directional cloning (i.e., *XbaI*).

1. Amplify the CAT-gene using the vector pACYC184 as template for four PCR reactions with each 50 μL (*see Note 7*) that contain 5 μL 10X Polymerase buffer with MgSO_4 , 0.2 μM dNTPs, 10 pmol of each primer, 10 ng template, and 1.25 U *Pfu* Polymerase. Keep the annealing temperature at 52°C and the extension time at 45 s (*see Note 8*).
2. Pool the reactions and purify the PCR product with the QIAquick PCR Purification kit.

3.1.2. Construction of Primary CAT-Fusion Vector

1. Digest the expression vector with the restriction enzyme that cuts at the 3' end of the gene and with a second enzyme that cuts further downstream (i.e., *MfeI* and *XbaI*), do a five-fold overdigestion using conditions as recommended by the enzyme supplier. Do the same digestion with the amplified CAT-gene.
2. Separate each DNA sample on a 1.0% tris-acetate EDTA (TAE) agarose gel using standard techniques.
3. Isolate the DNA from the gel using the QIAEXII gel extraction kit.
4. Determine the concentration of the purified DNA. If necessary concentrate to at least 50 ng/ μ L by debuffering the DNA with Centri-Spin10 spin columns, followed by freeze drying and dissolving in smaller amount of ddH₂O (see **Note 9**).
5. Ligate vector and CAT-gene insert by adding into 10 μ L final volume: 100 ng of vector and 250 ng of the CAT-gene, otherwise use conditions as recommended by the supplier of the ligase. Incubate for 3 h at room temperature.
6. Transform *E. coli* cells of a general cloning strain (e.g., XL1 Blue) that have a minimum competency of 10⁸/ μ g DNA.
7. Verify the correct sequence of the fusion by DNA sequencing.

3.2. Construction of Controls

As is the case for any selection, it is best to test the selection with negative as well as with positive controls. The controls should reflect your library. If you work with, on average, single exchange mutants, it would be good to have variants with different solubilities distinguished by single mutations. If, on the other hand, you produce chimeras between not-so-closely-related proteins, each parent should be tested in a fusion. When the library is very diverse and can contain many variants that are extensively divergent from the protein parents (e.g., have large deletions or shifts in reading frames), such variants should be simulated and tested in the context of the fusion to CAT.

The controls can be constructed the same way as your primary CAT-fusion vector (i.e., the CAT-gene is inserted behind the genes of the different controls in the same vector background). For this, please refer to **Subheading 3.1.** above. Alternatively, you can replace the gene of interest in your primary CAT-fusion vector by the genes of the controls. This is described below under **Subheading 3.2.1.** The protocols that we give here are derived for the application of the CAT-selection to find more soluble variants of cytochrome P450 enzymes (7).

3.2.1. Positive Control

As positive control, the gene of the parent (i.e., cytochrome P450 1A2 [9]) is replaced by a more soluble variant (i.e., the heme domain of cytochrome 450 from *Bacillus megaterium* [10]) (see **Note 10**). First, the gene for the positive control is PCR amplified and ligated into the plasmid containing the CAT-fusion, replacing the actual gene of interest (see **Fig. 1**).

1. Construct PCR primers for amplifying the gene of a more soluble variant. Include sequences of an N-terminal restriction site (e.g., *NdeI*) and the C-terminal restriction site (i.e., *MfeI*) within the primer sequence.
2. Amplify the gene by PCR as template for four PCR reactions with each 50 μL that contain 5 μL 10X Polymerase buffer with MgSO_4 , 0.2 μM dNTPs, 10 pmol of each primer, 10 ng template, and 1.25 U *Pfu* Polymerase. Set the annealing temperature 5°C below the melting of the primer binding region and the extension time to 1 min/1 kb to be amplified (see **Note 8**).
3. Digest the CAT-fusion vector with the restriction enzyme that cuts at the 5' end of the gene (e.g., *NdeI*) and with the enzyme used at the 3' end of the gene (i.e., *MfeI*), do a five-fold overdigestion using conditions as recommended by the supplier of the enzymes. Do the same digestion with the amplified control gene.
4. Continue as described in the protocol in **Subheading 3.1.2., step 2**.

3.2.2. Negative Controls

The library of P450 enzymes is expected to contain variants that are truncated by incomplete translation (i.e., have a shift in the reading frame). With the background of the original amino acid sequences such a variant was simulated and tested in the CAT selection as negative control (shift in reading frame). The vector with the parent is linearized with a restriction enzyme that cuts within the gene and that leaves an overhang of 1, 2, or 4 bases (i.e., *SalI*). The overhang is removed by making the DNA ends blunt and the vector is recircularized.

1. Locate one single restriction site in the CAT-fusion vector that cuts within the gene of interest (e.g. *SalI*, see **Fig. 1**).
2. Digest the CAT-fusion vector with this restriction enzyme, as recommended by the enzyme supplier.
3. Treat the sample with T4 Polymerase as recommended by the enzyme supplier to convert staggered ends to blunt ends.
4. Isolate the linearized DNA from a 1% agarose gel in TAE, measure amount of purified DNA.
5. Recircularize the DNA by ligating at a concentration of 10 ng/ μL or less in ligase buffer by incubating overnight at 16°C.
6. Transform *E. coli* cells of a general cloning strain (e.g., XL1 Blue) that have a minimum competency of $10^7/\mu\text{g}$ DNA.
7. Verify the control by preparing the plasmid from one transformant, restricting the plasmid with the enzyme used to create this control (i.e., *SalI*) and analysis by agarose gel electrophoresis. Verification by DNA sequencing should not be necessary.

3.3. Chloramphenicol (*cam*) Resistance Analysis and Adjustment of Selection Conditions

The chloramphenicol selection can either be performed in liquid media or on agar plates. Each method has its advantages. Liquid medium is best used for an

enrichment culture in which the most soluble variants will outgrow the less soluble variants. It is best applied when indeed only the most soluble variants are desired. Selection on agar plates is most suitable for a tiered screen. When the chloramphenicol concentration is correctly adjusted, much more soluble and slightly more soluble variants will all give rise to bacterial growth, but with differences in colony size. Slightly more soluble variants will not be lost. When colonies are picked for activity screening it is important to not only analyze the most soluble variants in order not to lose variants that are most active but only second best in solubility.

3.3.1. Liquid Selection

1. Transform the bacterial strain for protein expression (e.g., XL1-Blue) by the different fusion constructs (wild-type and controls).
2. Pick a colony each of the bacteria containing your fusion constructs.
3. Inoculate 5 mL LB and grow cells until an OD of about 1.
4. Dilute cells to an OD of 0.02 into a) growth medium that is optimal for protein expression (e.g., TB medium supplemented with trace elements (**II**), 1 mM thiamine and inducer) and b) growth medium that provides more strenuous growth conditions (e.g., minimal medium) (*see Note 12*). Add chloramphenicol at concentrations of 0, 5, 10, 20, 40, 80, 200, 400, and 1000 $\mu\text{g/mL}$.
5. Grow for 16 h at 37°C (or another temperature when required for protein expression). Measure the OD at 600 nm every 2–4 h.

3.3.2. Growth on Agar Plates

1. Transform the bacterial strain for protein expression (e.g., XL1-Blue) by the different fusion constructs (wild-type and controls).
2. Pick a colony each of the bacteria containing your fusion constructs.
3. Inoculate 5 mL LB and grow cells until an OD of about 1.
4. Dilute cells by a factor of 10^5 (to about 10^3 – 10^4 cells/mL) (*see Note 13*).
5. Plate 100 μL on agar plates with a) rich medium and b) poor medium (*see above for liquid selection*) and chloramphenicol at concentrations of 0, 5, 10, 20, 40, 80, 200, 400, and 1000 $\mu\text{g/mL}$ (*see Note 14*).
6. Incubate plates at 37°C (or another temperature when affecting protein expression) for several days and observe the appearance of colonies.
7. Readjust the concentrations of chloramphenicol and growth conditions in above experiments to find conditions where cells containing the negative control are not growing, those with the positive control are growing well, and those with the wild-type (parent) are just barely growing. Such conditions are optimal for selection (*see Note 15*).

Exemplary results as obtained for the analysis of the resistance of different variants of cytochrome P450 are given in **Table 1**. Based on these results, we decided to perform a tiered screen on agar plates containing 40 $\mu\text{g/mL}$ chloramphenicol.

Table 1
Resistance Towards Chloramphenicol Conferred
by Different CAT-Fusions When Expressed in XL1-Blue Cells
(The Resistance was Measured by the Growth Rate)

[Cam]	0 [†]	20 [†]	40 [†]	80 [†]	250 [†]	1000 [†]	40 [‡]	200 [‡]
Positive	100%	100%	100%	90%	75%	50%	+++	++
Wild Type	100%	80%	50%	≤10%	≤10%	≤10%	+	–
Negative	100%	40%	20%	≤10%	≤10%	≤10%	–	–

[Cam]—concentration of chloramphenicol in µg/mL; Positive—Gene of soluble protein (cytochrome P450 BM3) in CAT-fusion, Wild-Type—gene of protein to be solubilized (cytochrome P450 1A2) in CAT-fusion, Negative—Wild-Type with shift in reading frame within the gene.

[†]Cells were grown in TB medium with trace elements with different concentrations of chloramphenicol, the OD was measured after 12 h and compared to the OD of the cam-free control.

[‡]Cells were grown for several days at 30°C on TB agar with trace elements with different concentrations of chloramphenicol (shown are 40 or 200 µg/mL). Times that colonies appeared: +++ = 24 h or less, ++ = 36 h, + = 48 h, – no colonies after 60 h.

3.4. Pre-Selection and Selection

When optimal selection conditions are found the actual selection/screen can be approached. As described above, the decision whether to use liquid or solid growth medium depends on your problem. When performing a tiered screen, it is advisable to first test whether your enzyme is active when fused to CAT. If there is inhibition or an impediment, the pre-screened library has to be recloned (or at least re-transformed when amber suppression has been applied). When the activity can be detected within the fusion context, for practical reasons, it is advisable to do the screen without any recloning.

3.4.1. Chloramphenicol Selection in Liquid Medium

1. Transform *E. coli* cells with your library. Plate cells on LB agar containing the appropriate antibiotic.
2. Scrape cells from plate. Measure the OD. Use to inoculate selection medium with chloramphenicol at a concentration as found optimal in the resistance analysis (see **Subheading 3.3.**) (e.g., Terrific Broth (TB) medium, including 40 µg/mL chloramphenicol) with an OD of 0.05.
3. Grow cells until an OD of ca. 1–1.5 has been reached. Measure the time that it took the bacteria to reach OD 1 as an indicator for the state of the selection.
4. Store cells (for short period of time at 4°C, for longer periods as glycerine culture at –80°C) and use an aliquot to inoculate new medium as before.
5. Repeat cycle 2–6× or until the time to reach OD 1 does not change anymore.
6. From each cycle, plate a dilution of the cells on agar plate with chloramphenicol at a concentration that just inhibits growth of cells with parental variant and observe growth rate.

7. Reclone variants with good growth to eliminate the CAT-fusion.
8. Analyze free variants for solubility.

3.4.2. Chloramphenicol Selection in a Tiered Screen

1. Transform *E. coli* cells with your library. Plate cells on LB agar containing the appropriate antibiotic.
2. Scrape cells from plate. Measure the OD. Dilute cells to about 10^3 – 10^4 cells/mL (see **Note 13**).
3. Plate around 200–1000 cells (see **Note 16**) onto a normal-sized plate with selection agar with chloramphenicol at a concentration as found optimal in the resistance analysis (see **Subheading 3.3.**) (e.g., TB medium agar, including 40 μ g/mL chloramphenicol).
4. Grow colonies until they are big enough to be picked.
5. Pick colonies from chloramphenicol agar into microtiterplates for bacterial growth to perform enzyme assay (see **Note 17**).
6. Rescreen positive variants without recloning. Only variants that have passed the rescreen should be recloned to analyse the free proteins.

4. Notes

1. The chloramphenicol acetyltransferase can in theory be fused to the N- or the C-terminus of your protein. While it certainly depends on the protein you use (e.g., accessibility of the termini, N- or C-terminal processing), we recommend constructing a C-terminal fusion when no such bias is present. On the one hand, you have more flexibility with expressing the non-fused gene via amber non-suppression; on the other hand you are less susceptible to expression problems (e.g., mRNA instability). When using the chloramphenicol selection for eliminating out of reading frame mutants, you must use a C-terminal fusion.
2. We recommend using a sequence rich in glycine, serine, and proline. The former two leave the linker flexible and hydrophilic, while proline can confer resistance to proteases.
3. Even when the region upstream of the CAT-gene does not contain Shine-Delgano homolog sequences, we found expression of high CAT activity in scrambled variants that could only be eliminated after removal of the start codon, indicating that enough free CAT is produced to confound the selection.
4. Be careful when choosing your strain for protein expression. Most of the standard available *E. coli* K12 strains are amber-suppressor strains. When you already have good experience with the expression of your protein in a strain that is amber suppressing, do not switch to a different system. Instead, remove the CAT-gene before expressing the isolated protein.
5. For production of the library it is important that variants without insert are not pushing through, but will be eliminated directly. This is most easily achieved using a negative control as starting point for the cloning.
6. The melting temperatures of the primer binding regions are very different for the two primers suggested, which is normally not recommended for PCR. The prob-

lem lies in the high A/T content at the beginning and G/C content at the end of the CAT-gene. We obtained high yields of PCR products with these primers and an annealing temperature of 52°C.

7. In our experience, because of the better heat transfer, the yield of a PCR product is always better when PCR is performed in small volumes.
8. As general PCR protocol, we use 1 min at 95°C, 25–30 cycles of 30 s 95°C, 30 s at annealing temperature, extension time at 72°C; and 10 min at 72°C. The usual variables of annealing temperature and extension time depend on the primer and fragment to be amplified and are given specifically within the protocols.
9. It is important to have a high concentration of DNA for successful ligation. This procedure might not be fastest, but guarantees a high yield. The time spent is often saved in the search for correct clones among the transformants.
10. If there is no variant known that is more soluble, one is tempted to omit this control. However, we recommend trying hard to find a solution for this. For example, when slightly less soluble variants are available, those can be compared to the most soluble variant (i.e., wild-type), which then represents the positive control.
11. By the choice of the enzymes, the controls can be constructed that have a shift in the reading frame and a deletion combined. The protocol given here is only one example of how to construct good controls. Its utility depends on the availability of useful restriction sites.
12. It is important to try different growth conditions and it might be worth trying different bacterial strains as well. Conditions of high protein expression might not be best for the selection because even rather insoluble variants might still give rise to good growth. Depending on your system, it could well be that good differentiation can be achieved only when small amounts of protein are expressed.
13. For *E. coli*, an OD of 1 equals to approx 5×10^8 cells/mL.
14. An efficient way to produce agar plates with the different concentrations of chloramphenicol is to autoclave 900 mL of medium with agar, cool it down and keep it at 40°C. Using a pipet, remove 100 mL and pour 5 plates (no cam). Add 1 mL of 4 mg/mL cam, remove 100 mL to pour 5 plates (5 µg/mL). Add 0.875 mL of 4 mg/mL cam, remove 100 mL to pour 5 plates (10 µg/mL). Add 1.5 mL of 4 mg/mL cam, remove 100 mL to pour 5 plates (20 µg/mL). Add 250 µL of 40 mg/mL cam, remove 100 mL to pour 5 plates (40 µg/mL). Add 400 µL of 40 mg/mL cam, remove 100 mL to pour 5 plates (80 µg/mL). Add 900 µL of 40 mg/mL cam, remove 100 mL to pour 5 plates (200 µg/mL). Add 1 mL of 40 mg/mL cam, remove 100 mL to pour 5 plates (400 µg/mL). Add 1.5 mL of 40 mg/mL cam to the remaining 100 mL and pour 5 plates (1 mg/mL).
15. The degree of resistance to chloramphenicol can depend on many factors whose effects might not be independent of each other. The number of experiments easily skyrockets when trying to test all combinations. Here it can be helpful to use a factorial design approach (12).
16. The actual number depends on whether you pick by hand or use a picking robot. In the latter case, please refer to its manual on how far separated and how big the colonies have to be to allow good picking.

17. One would be tempted to pick only the biggest colonies, as they should represent the most soluble variants. When the selection is carefully adjusted, any bacteria that will give rise to colonies will contain more soluble variants. It is advisable to pick a good mix of differently sized colonies.

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Proside

A Phage-Based Method for Selecting Thermostable Proteins

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1. Introduction

For many applications proteins must retain their function for a long time and under a wide range of conditions, sometimes at elevated temperatures. However, the stability of natural proteins is often very low, just sufficient to ensure proper functioning under cellular conditions (**1**). It is still largely unknown how the stability of a protein is encoded in its sequence, and theoretical approaches to calculate the contributions of individual amino acids to stability are still in their infancy. Therefore, evolutionary methods for increasing protein stability are of great interest. In such approaches, large protein libraries are searched for stabilized variants by a screening or, ideally, a selection technique.

In vivo selection methods for increasing the stability of specific proteins have been successful in cases where the survival of a microorganism could be linked to the function of these proteins (**2**). In vitro, proteins with specific binding functions can be selected, e.g., by ribosome display (**3,4**) or phage display (**5**). Since ligand binding and protein stability are linked thermodynamically, these display techniques can, in principle, also be adapted to select for binders with improved stability (**6–9**).

Proside (protein stability increased by directed evolution) (**10**) is a general method for protein stabilization that does not depend on specific properties of the protein, such as enzymatic activity or ligand binding. This in vitro selection technique links the proteolytic resistance of the protein to be stabilized with a well selectable property, the infectivity of a filamentous phage. Conformational stability and proteolytic resistance are often correlated (**11**), since the folded state of a protein is normally much less sensitive to cleavage by proteases than the

unfolded state. This holds in particular for proteases such as chymotrypsin with a preference for hydrophobic regions, which are inaccessible in folded proteins.

Infection of *E. coli* by the phage fd is mediated by the gene-3-protein (g3p), which consists of three domains, N1 (68 aa), N2 (131 aa) and CT (150 aa), connected by glycine-rich stretches of 18 and 39 residues, respectively (see **Fig. 1A**). The domains of g3p must be tightly linked for the phage to be infective (**12**), and this linkage can also be provided by inserted guest proteins (**13**). Therefore, in *Proside* a repertoire of sequences coding for the protein to be stabilized is inserted between the N2 and CT domains of g3p, and the resulting pool of phages is subjected to an in vitro proteolysis step (see **Fig. 1B**). The tight linkage between these domains and thus phage infectivity is maintained only when the inserted guest protein remains intact. Variants with increased stability and increased resistance towards proteolysis can be enriched in repeated cycles of in vitro proteolysis of the phage library, infection of *E. coli*, and phage propagation. Since the proteolytic selection step is performed in vitro and fd phages are exceptionally stable, the conditions can be varied over a wide range and tailored for the protein to be stabilized. Generally, conditions are used under which the wild-type form of the guest protein is partially unfolded, either by adding a denaturant or by increasing the temperature.

The power of the *Proside* method could be demonstrated by the successful selections of stabilized variants of RNaseT1 (**10**), the β 1-domain of the staphylococcal protein G, and the cold shock protein *Bs*-CspB from the mesophilic bacterium *Bacillus subtilis* (**14**). In the case of *Bs*-CspB, six surface exposed positions were randomized by saturation mutagenesis, and many strongly stabilized variants were identified in two selections, one in the presence of the denaturant guanidinium chloride (GdmCl) and the other at elevated temperature. The best variant showed an increase of more than 28°C in the midpoint of the thermal unfolding transition (T_M), and thus surpassed the cold shock protein *Tm*-Csp from the hyperthermophilic organism *Thermotoga maritima* in stability. In addition, *Proside* was used to increase the stability of the host phage protein g3p itself. Random mutagenesis and selection yielded a variant of g3p with an increase in T_M of 13°C for domain N2 and 6°C for domain N1 (Martin et al., submitted). The use of this stabilized g3p extends the temperature range for the selection of guest proteins to 60°C.

2. Materials

2.1. Solutions and Media

1. Double-yeast-tryptone medium (dYT): 16 g tryptone, 10 g yeast, 5 g NaCl dissolved in 1 L H₂O; for plates supplemented with 1.5% agar-agar.
2. Chloramphenicol, 25 mg/mL in ethanol.
3. dYT^{cam} : dYT with 25 µg/mL chloramphenicol.

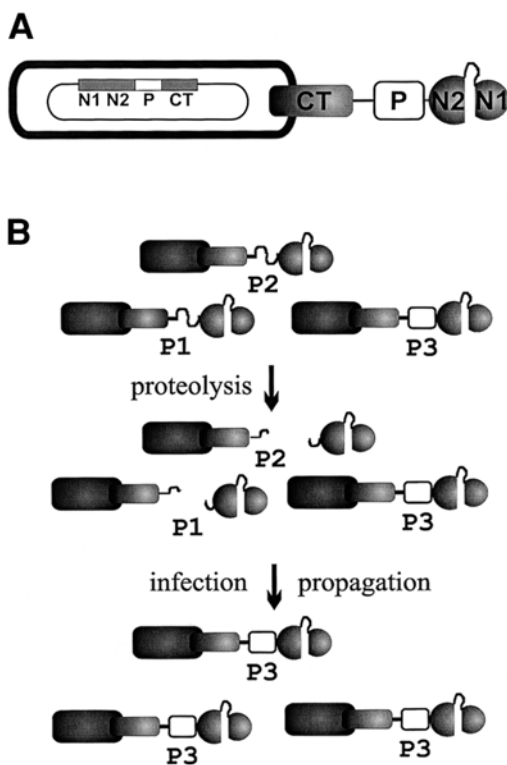


Fig. 1. (A) Schematic representation of the filamentous phage fdpro hosting a protein (P) inserted between the C-terminal domain (CT) and the two N-terminal domains (N1,N2) of g3p. (B) Outline of the *Proside* selection procedure (10). Phages hosting different variants of the guest protein (P1, P2, P3) are subjected to an in vitro proteolysis step. Phages with less stable and thus unfolded variants (P1, P2) are cleaved by the protease and lose their infectivity, whereas phages with more stable mutants (P3) remain infectious and can become enriched in repeated selection cycles.

4. Phage precipitation solution: 30% PEG 8000, 1.5 M NaCl, sterilized.
5. Phosphate-buffered saline (PBS)-buffer: 80 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄, 0.24 g KH₂PO₄ dissolved in 1 L H₂O, pH 6.9, 0.01% NaN₃, sterilized.
6. Tris-EDTA (TE)-buffer: 10 mM Tris-HCl, 1 mM Na₂-EDTA, pH 8.0, sterilized.
7. Ethidium bromide, 1% in H₂O.
8. Phenol, pH 7.5, saturated with TE-buffer.
9. Chloroform: isoamyl alcohol, 24:1 (v:v).
10. 3 M sodium acetate, pH 5.2.
11. NAD⁺, 10 mM in H₂O.
12. ATP, 10 mM in H₂O.

13. dNTP mixture, 10 mM each dATP, dCTP, dGTP, dTTP.
14. Oligonucleotide primers, 10 μ M in H₂O.
15. 100 mM potassium phosphate, 100 μ M CaCl₂, pH 8.0.

2.2. Enzymes

1. Restriction endonucleases: *Sfi*I, *Kpn*I, *Hind*III (New England Biolabs, Beverly, MA).
2. T4 DNA Ligase, 20 U/ μ L (New England Biolabs).
3. T4 Polynucleotide Kinase, 10 U/ μ L (New England Biolabs).
4. *Vent* DNA polymerase, 2 U/ μ L (New England Biolabs).
5. *Pfu* DNA polymerase, 3 U/ μ L (Promega Corporation, Madison, WI).
6. *Taq* DNA polymerase, 5 U/ μ L (Promega Corporation).
7. *Taq* DNA Ligase, 40 U/ μ L (New England Biolabs).
8. Chymotrypsin (Roche Diagnostics, Penzberg, Germany).
9. Trypsin (Sigma Chemical Co., St. Louis, MO).

2.3. Miscellaneous

1. Filamentous phage fdpro (send requests to fx.schmid@uni-bayreuth.de).
2. *E. coli* XL1-Blue and *E. coli* ABLE K (Stratagene, La Jolla, CA).
3. Agarose gels, electrophoresis equipment, UV transilluminator, electroporator.
4. DNA molecular weight standards.
5. QIAEX II gel extraction Kit (Qiagen, Hilden, Germany).
6. Flexi Prep kit (Amersham Biosciences, Freiburg, Germany).
7. MF-Millipore membrane filters, pore size 0.025 μ m (Millipore GmbH, Schwalbach, Germany).

3. Methods

The method section outlines: 1) how a guest protein is inserted into g3p of the phage fd, 2) how the library of mutants is created, and 3) how stabilized variants of the guest protein are selected from this library. As an example, the selection of stabilized variants of *Bs*-CspB is described.

3.1. Insertion of the Protein to be Stabilized into g3p

Domains N1 and N2 of g3p form a structural and functional entity in which domain CT seems not to be involved (15). Therefore, foreign proteins can be inserted between N2 and CT (see Fig. 1A) without significantly changing phage infectivity (13). It is not known whether there are limits regarding the size of the guest protein or the distance between its N- and C-termini. Disulfide bonds or cysteine residues in the guest protein might interfere with the correct folding of the g3p and should be eliminated before insertion (see Note 1). Flexible parts or unstructured linker regions between globular domains could be susceptible to the protease used in the selection step and thus should be cleared of aromatic amino acids (cleavage sites for chymotrypsin) and positively charged residues (cleavage sites for trypsin).

Our modified phage fdpro is derived from fCKCBS (**13**), which contains a *cat* gene for the selection of infected cells. The glycine-rich linker between N2 and CT is considerably shortened (see **Note 2**) and contains a *Sfi*I, a *Hind*III and a *Kpn*I restriction site for the cloning of the guest protein. The insertion of the protein results in linkers of seven amino acids (PSGAQPA) between N2 and the guest and eight amino acids (ASEAEGTP) between the guest and CT, respectively. Twelve base pairs downstream of the C-terminal end of the guest protein (between the *Hind*III and *Kpn*I restriction sites) a TAG amber codon is placed, which is used to eliminate phages coding for the wild-type gene by infection of the non-suppressor *E. coli* ABLE K (see **Subheading 3.2.**). In the suppressor strain XL1-Blue a Glu residue is incorporated at the amber codon.

Two primers are required for amplifying the guest gene before insertion. The N-terminal primer 5'-TCCGGGGCCCAGCCGCC(X)₂₀-3' codes for the *Sfi*I restriction site (*italic*) and binds to the first 20 nucleotides of the guest gene (X₂₀). The C-terminal primer 5'-CCGGGGTACCCTAAGCTTCAGAGG C(X)₂₀-3' binds to the last 20 bp of the guest gene and contains a *Hind*III restriction site (*italic*), the amber codon (**bold**), and a *Kpn*I site (*italic*) for alternative cloning with *Hind*III or *Kpn*I.

The procedure involves the following steps:

1. Amplify the gene to be inserted in a 100 μ L PCR reaction, containing 10 μ L Vent buffer, 1 μ L 100 mM MgSO₄, 4 μ L of each primer, 3 μ L dNTPs, 10 ng template and 1 μ L Vent DNA polymerase. Run an appropriate PCR program (see **Note 3**).
2. Separate the amplified gene using an agarose gel of suitable pore size (e.g., 1.6%).
3. After ethidium bromide staining, excise the bands exhibiting the right size and purify the DNA from the agarose using the QIAEX II gel extraction kit.
4. Determine the concentration of DNA.
5. Purify the double-stranded phage DNA from a 5-mL culture of *E. coli* XL1-Blue infected with the phage fdpro using the Flexi Prep kit.
6. Clone the guest gene into the phage vector via the restriction sites for *Sfi*I and *Hind*III or *Kpn*I, respectively, according to standard protocols (**16**).
7. Transform *E. coli* XL1-Blue (see **Note 4**) with the ligation product by electroporation and plate the cells on dYT^{cam} agar.
8. Screen the obtained transformants for the right insert by colony-PCR (see **Note 5**).
9. Verify the phage variants by sequencing.
10. Grow the positive clones in 5-mL cultures for phage production.
11. Prepare the phages (see **Subheading 3.2.1.**).

3.2. Creation of the Phage Library

Having a very large library of different mutants is a prerequisite for a successful search for stabilized variants. Restriction and ligation of double-stranded DNA fragments is usually not efficient enough to obtain libraries with more than 10⁵ variants. Therefore, we developed an alternative approach that consis-

tently yields libraries of more than 10^7 variants. We hybridize the mutated DNA fragments coding for the protein to be stabilized to the (+) DNA strand of the phage hosting the corresponding wild-type gene between N2 and CT, synthesize the (–) strand with *Pfu* DNA polymerase in the presence of *Taq* ligase, and use the resulting double stranded DNA chimeras for transformation of *E. coli* XL1-Blue (see **Note 6**). Next to the wild-type gene the template strand contains an amber codon, which is not present in the mutated fragments. Thus, phages with the wild-type protein (coded by the original template strand) are eliminated simply by infection of the non-suppressor strain *E. coli* ABLE K with the phages isolated from the suppressor strain XL1-Blue (see **Note 7**). The (–) strand is synthesized in the presence of *Taq* ligase to close the nick that is left when the strand is completed. This covalent closure of the mutated strand reduces the loss of mutations by mismatch repair in *E. coli* (see **Note 8**). Compared to Quick Change techniques using double-stranded plasmid DNA as a template, this fill-up reaction with single-stranded DNA is much more efficient. The library size is mainly limited by the transformation efficiency of *E. coli* XL1-Blue and ranges between 10^7 – 10^8 variants.

3.2.1. Preparation of Filamentous Phages

The phages are prepared from the *E. coli* XL1-Blue culture in the stationary phase by polyethylene glycol precipitation (**16**) (see **Note 9**).

1. Centrifuge 1.5 mL of the XL1-Blue culture at 13,000g to pellet the cells, mix 1.3 mL of the supernatant with 250 μ L phage precipitation solution and incubate for 10 min at room temperature.
2. Pellet the precipitated phages at 13,000g for 10 min and remove the supernatant carefully.
3. Resuspend the phages in 50 μ L PBS-buffer for storage and infection of *E. coli* or TE-buffer for preparation of single-stranded DNA (see **Subheading 3.2.2.**) (see **Note 10**). Phage solutions can be stored at 4°C for months without a loss in infectivity or can be frozen at –20°C.

The titers of the prepared phage solutions should be about 10^{10} – 10^{11} cfu/mL (for determination of phage titers see **Subheading 3.3.1.**).

3.2.2. Preparation of Single-Stranded Phage DNA

Single-stranded phage DNA is prepared on a small scale according to a standard protocol (**16**):

1. Mix 100 μ L of TE-buffered phage solution with 50 μ L TE-saturated phenol, vortex for 30 s, incubate for 1 min at room temperature and vortex again for 30 s.
2. After centrifugation at 13,000g for 1 min transfer the supernatant into a fresh tube, mix it with 50 μ L chloroform/isoamyl alcohol and vortex twice for 30 s.

3. After centrifugation mix the supernatant with 11 μL 3 M sodium acetate, pH 5.2, and 289 μL ice-cold ethanol. Precipitate the DNA at room temperature for at least 15 min and centrifuge for 10 min at 13,000g.
4. Wash the pellet with 200 μL 70% ethanol and dry the DNA.
5. Redissolve the air-dried DNA pellet in 20–30 μL water.

The yield should be about 2–5 μg per 100 μL phage solution.

3.2.3. Synthesis of Mutated Gene Fragments

Depending on the desired number and distribution of mutations within the gene, the fragments are synthesized by PCR using degenerate oligonucleotides or by error-prone PCR. These fragments must be extended to the g3p linker downstream of the inserted gene to abolish the amber codon present in the phage DNA with wild-type insert. The primer for this extension 5'-CCGGGT ACCAGAAGCTTCAGAGGC-3' replaces the amber codon by a Ser codon (bold) and ends right at the C-terminus of the guest gene. For introduction into the phage genome the extended gene fragments have to be phosphorylated.

1. Extend the mutated fragments in a 100 μL PCR reaction containing 10 μL *Vent* buffer, 4 μL of the linker-primer, about 400 nM of mutated fragment, 3 μL dNTPs, 10 ng template (single-stranded DNA of the phage containing the wild-type insert) and 1 μL *Vent* DNA polymerase. Run an appropriate PCR program (see **Note 3**). Depending on the length of the mutated fragment it is advisable to use a temperature ramp for the annealing step, ending at about 50°C.
2. Purify the PCR products by agarose gel electrophoresis and extraction from the gel using the QIAEX II gel extraction kit. Elute the DNA with 25 μL dH₂O.
3. The phosphorylation reaction contains 24 μL purified gene fragment (about 2–4 μg), 3 μL polynucleotide kinase buffer, 3 μL ATP and 1 μL T4 polynucleotide kinase. Incubate for 2 h at 37°C.

3.2.4. Introduction of Mutated Genes into the Phage Genome

The mutated fragments are hybridized to the (+) DNA strand of the phage hosting the wild-type gene, the (–) strand is synthesized by *Pfu* polymerase and the nick is closed by the thermostable *Taq* ligase.

1. The reaction mixture contains 4 μL *Pfu* buffer, 2 μL dNTPs, 4 μL NAD⁺, about 2 μg (+) strand phage DNA template (see **Subheading 3.2.2.**), 200–400 ng mutated gene fragment, 0.5 μL *Taq* DNA ligase, 1 μL *Pfu* DNA polymerase and H₂O to bring the total volume to 40 μL .
2. The program for the extension consists of 1 min denaturation at 95°C, 1 min hybridization at 65°C, and 1 h polymerization and ligation at 68°C.
3. Check the success of the extension reaction by loading 3 μL of the sample before and after the reaction onto an agarose gel. The bands of single-stranded DNA should ideally be doubled in their intensity. They are mostly shifted from a posi-

tion of apparent migration (19 kb) to the position of double stranded linear phage DNA (7.6 kb).

4. Desalt the sample by microdialysis on MF-Millipore membrane filters, and use 10 μL for the transformation of 40 μL electrocompetent *E. coli* XL1-Blue.
5. Add 700 μL dYT medium right after the electroporation pulse and incubate the cells for 30 min at 37°C.
6. To determine the number of transformants, prepare a dilution series of 1:10 steps (10 μL diluted in 90 μL dYT) in a microtiter plate and pipet 2 μL of each dilution step onto a dYT^{cam} plate. The total number of transformants should be in the range of 10^7 – 10^8 .
7. Pellet the transformed cells and plate them on dYT^{cam}. After growing at 37°C for 10–12 h resuspend the colonies on the plate in 5 mL dYT^{cam} and shake the culture for further phage production at 37°C for 2–3 h.
8. Prepare the phages by polyethylene glycol precipitation (see **Subheading 3.2.1**).
9. To check the titer of mutated phages (without the amber codon) mix 495 μL of an *E. coli* ALE K culture (OD 0.8) with 5 μL phage solution, incubate for 30 min at 37°C and prepare a 1:10 dilution series as described above. Only the cells infected with phages without the amber codon will grow normally on dYT^{cam} (see **Note 7**).
10. Infect *E. coli* ALE K with the phage library by adding 50 μL of phage solution to 5 mL of a culture with OD 0.8 (see **Note 11**).
11. After shaking for 30 min at 37°C add chloramphenicol to a final concentration of 25 $\mu\text{g/mL}$ and incubate the culture at 37°C for at least 7 h for phage production.
12. Prepare the phages by polyethylene glycol precipitation.

3.3. Prospective Selection of Stabilized Variants

Filamentous phages like fd show a very high resistance towards proteolysis by site-specific proteases such as trypsin, chymotrypsin or GluC (**17–19**). The wild-type phage fd remains fully infective after incubation for 1 h with 2.5 μM chymotrypsin or trypsin at 37°C, pH 8.0 or with 1 μM pepsin at 15°C, pH 4.0 (**10**), and also after incubation for 30 min with 0.25 μM chymotrypsin at 20°C in 1.75 M GdmCl, pH 8.0 (**14**). Our improved phage fdproS with the stabilized g3p retains its infectivity even after incubation for 15 min at 57.5°C in the presence of 25 μM chymotrypsin. Thus, proteases can specifically degrade proteins that are inserted into g3p without cleavage of the phage proteins.

To obtain optimal discrimination between folded and unfolded forms of the inserted protein, the type and concentration of the protease, the temperature, the solvent conditions, and the duration of the proteolysis step can be varied over a wide range, especially when using the phage with stabilized g3p. Moreover, the very slow unfolding kinetics of g3p enables a selection of the guest proteins at even stronger denaturing conditions when only short pulses of denaturation and proteolysis are used.

For every guest protein appropriate selection conditions must be determined in a preliminary screen. The selection pressure should not be too strong so that small differences in stability can be exploited in the selection. In addition, early in a selection many individual variants are poorly populated in the library and therefore, under too-stringent conditions some of them might be lost, even if they are highly stable. The partial unfolding of the guest protein can be achieved either by denaturants, such as GdmCl or urea, or by increased temperature. We prefer to select at elevated temperature, because temperature is easy to adjust and salt effects on stability (as in GdmCl) are avoided.

3.3.1. Finding Optimal Conditions for the Proteolytic Step

The equilibrium unfolding transition of the wild-type form of the guest protein provides a valuable reference for finding suitable selection conditions, although the protease in the *Proside* selection shifts the equilibrium to the unfolded state. Often, a temperature or a denaturant concentration near the mid-point of the unfolding transition are good conditions for the proteolysis step. To determine the stability of phages under specified selection conditions, the retained infectivity of the phages after incubation in the presence of the protease is compared with the infectivity of a control sample without protease.

1. For the proteolysis sample mix 17.5 μL 100 mM potassium phosphate, 100 μM CaCl_2 , pH 8.0, with 5 μL phage solution, and for the control sample mix 20 μL 100 mM potassium phosphate, 100 μM CaCl_2 , pH 8.0, with 5 μL phage solution.
2. Incubate the sample and the control at an appropriate temperature for several minutes to establish the folding equilibrium (the incubation time depends on the unfolding kinetics of the guest protein).
3. Add 2.5 μL protease (e.g., 2.5 μM chymotrypsin) to the proteolysis sample and incubate the sample and the control for 15 min.
4. Dilute 5 μL of the proteolysis sample and the control into 495 μL of an *E. coli* XL1-Blue culture (OD 0.8) and incubate at 37°C for 30 min.
5. Prepare dilution series of 1:10 steps, pipet 2 μL of each dilution on a dYT^{cam} plate and determine the number of colonies after growing for 10–12 h.

For a successful selection of stabilized variants the conditions should be optimized such that the retained infectivity of phages with the wild-type protein inserted lies between 1% and 0.01%.

3.3.2. Selection of the Phage Library

The selection procedure consists of repeated cycles of proteolysis, infection of *E. coli* XL1-Blue, phage propagation and preparation. The number of cycles that is necessary to enrich the most stable variants depends strongly on the library size, the applied selection pressure and the stability of variants. The selection pressure should be increased when, after several cycles, the retained infectivity of the phage pool approaches 50–100%.

1. Mix 35 μL 100 mM potassium phosphate, 100 μM CaCl_2 , pH 8.0, with 10 μL phage solution (about 10^8 – 10^9 phage particles).
2. Perform the selection step under the conditions identified in **Subheading 3.3.1**.
3. Use the whole sample to infect a 5 mL culture of *E. coli* XL1-Blue (OD 0.8).
4. After incubation for 30 min at 37°C add chloramphenicol to a final concentration of 25 $\mu\text{g/mL}$ and shake for at least 7 h for phage production (*see Note 12*).
5. Prepare the phages as described above and use them for the next selection cycle.
6. For every selection cycle prepare in addition two samples of 25 μL , one with and the other without the protease (*see Subheading 3.3.1*.) to determine the retained infectivity of phages in the library and to follow the development of the selection.
7. Check the library for the presence of recombinants with shortened inserts after every second or third selection cycle or when the retained infectivity increases dramatically. Determine the insert size of 10–20 isolated clones by colony-PCR (*see Note 5*). If recombinants are present, prepare the single stranded DNA from the whole library (*see Subheading 3.2.1*.) and use it as a template for PCR amplification of the guest inserts with the two linker primers described in **Note 5**, for PCR conditions *see Subheading 3.1*. Isolate the full length inserts by agarose gel electrophoresis and extraction from the gel, and re-introduce the fragments into the phage genome as described in **Subheading 3.2.4**.
8. If there is no further increase in phage infectivity after proteolysis isolate individual phage clones by plating an appropriate dilution of the infected cells on dYT^{cam}, pick single colonies, and grow the bacteria for phage production.
9. Screen the isolated phages for those with the highest proteolytic stability and determine the sequences of their guest proteins.
10. Clone the variants into a suitable expression vector.

3.4. Example: Stabilization of *Bs-CspB* (14)

To explore the role of six surface exposed positions (2, 3, 46, 64, 66, 67) for the stability of *Bs-CspB* we constructed a library of variants by using PCR and degenerate oligonucleotides allowing all 20 amino acids at the six sites. The first selection of this library was for stability towards GdmCl-induced unfolding. The phages were incubated in 1.5 M GdmCl, 100 mM potassium phosphate, pH 8.0, at 25°C (which corresponds to the conditions at the transition midpoint of wild-type *Bs-CspB*), and selected by exposing them to 0.25 μM chymotrypsin for 30 min. Only 0.006% of the phages with the wild-type *Bs-CspB* insert remained infectious under these conditions. The course of the selection that consisted of six cycles is shown in **Fig. 2**. After the third round, the resistance towards proteolysis had already reached 65 %, and therefore the denaturant concentration was increased to 1.75 M GdmCl. After the sixth round, five different stabilized variants of *Bs-CspB* were identified; they were overexpressed and characterized. All of them were much more stable than the wild-type protein ($T_M = 54^\circ\text{C}$) with increases in T_M between 13 and 22°C.

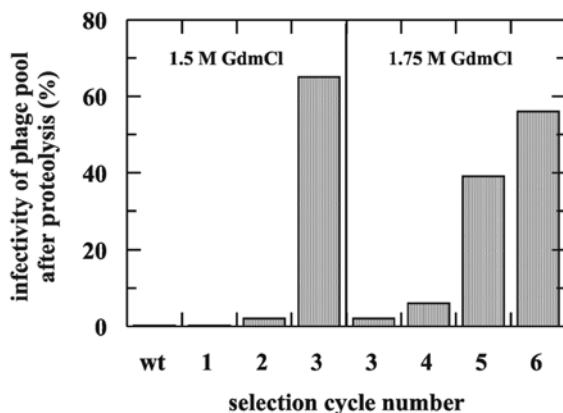


Fig. 2. Selection cycles of the *Bs*-CspB library in the presence of GdmCl (14). The relative infectivities of the phage pool after the various proteolytic selection cycles are shown. Selection rounds 1–3 were performed in 1.5 M GdmCl with 0.25 μ M chymotrypsin. Under these conditions phages hosting the wild-type *Bs*-CspB (wt) retained 0.006% of their infectivity. For rounds 4–6 the GdmCl concentration was increased to 1.75 M. The infectivities after round 3 are shown for both 1.5 and 1.75 M GdmCl.

The second selection was performed at elevated temperature. In this case the library of *Bs*-CspB was introduced into the phage fdproS with stabilized g3p. In the first six rounds of the selection the phages were exposed to 2.5 μ M chymotrypsin at 57.5°C for 15 min. The infectivity of the phage library increased during the first 4 cycles from 3 to 28% and then remained approximately constant (see Fig. 3). Therefore, we used the library present after the fourth round and enhanced the selection pressure by increasing the chymotrypsin concentration ten-fold to 25 μ M and by combining it in tandem with a 15 min proteolysis with 0.2 μ M trypsin. At 57.5°C chymotrypsin becomes markedly inactivated within 10 min. Therefore, for selection rounds eight to ten, 25 μ M chymotrypsin was added twice for 5 min each. The six variants of *Bs*-CspB that were chosen from the final pool for purification and characterization were all strongly stabilized. They showed T_M values that were 21.9–28.2 degrees higher than the T_M value of the wild-type protein.

4. Notes

1. A protein pre-destabilization by eliminating disulfide bonds (e.g., by Cys to Ala mutations) facilitates the selection for higher stability, since much milder conditions are sufficient for a comparative selection pressure on the guest protein, and the phage proteins as well as the proteases are less strained (e.g., stabilization of RNase T1 [10]).

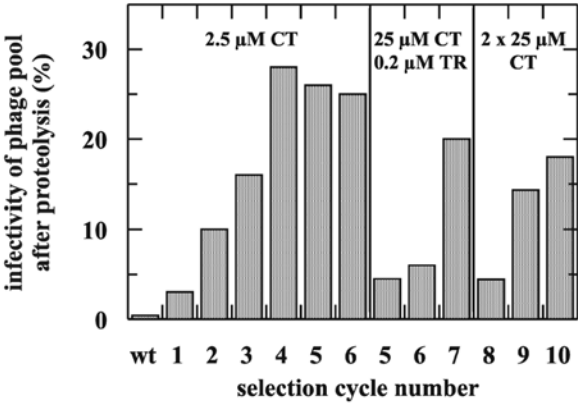


Fig. 3. Selection cycles of the *Bs*-CspB library at elevated temperature (14). The infectivities of the phage pool after the proteolytic selections at 57.5°C are shown. The first six cycles were performed with 2.5 μM chymotrypsin (CT) for 15 minutes. Phages with wild-type insert (wt) retain 0.4% of their infectivity under these conditions. Rounds 5–7 were performed with tandem 15 min proteolyses by 25 μM chymotrypsin and by 0.2 μM trypsin (TR), followed by three rounds with 25 μM chymotrypsin added twice for 5 min each.

2. The linker between N2 and CT was shortened to 15 amino acids to reduce the frequency with which inserted guest sequences are eliminated by recombination. Nevertheless, if particular protein inserts impair phage infectivity (e.g., by non-specific interactions with the domains of g3p) the linker can be again extended to 58 amino acids.
3. The following PCR program gives good yields when amplifying a fragment of 500 bp with *Vent* polymerase: 30 cycles of denaturation at 95°C for 20 s, annealing at 55°C for 20 s and extension at 72°C for 30 s. In the case of annealing problems add 1–2 mM MgSO₄ or supplement the reaction mixture with 3 U *Taq* DNA polymerase.
4. *E. coli* XL1-Blue carries the *supE44* gene, which allows suppression of amber codons by introduction of a Glu.
5. For colony-PCR prepare a mixture of 10 μL *Taq* buffer, 10 μL 10 mM MgCl₂, 2 μL dNTPs, 2 μL upstream-linker primer 5'-CCGTCCGGGGCCCAGCCG GCC-3', 2 μL downstream-linker primer 5'-CCGGGTACCAGAAGCTTCAGA GGC-3', 0.5 μL *Taq* DNA polymerase and H₂O to a final volume of 100 μL. Aliquot this mixture to 10 μL per PCR tube and transfer cells from the colonies on the plate with a pipetting tip into the PCR tube. Inoculate a second tube containing 50 μL dYT medium with the same tip to have a small pre-culture available in the case of a positive PCR result. Run a PCR program as described in Note 3.

6. A digestion of remaining single-stranded DNA template is not necessary, since it has a very low transformation efficiency and the phages that are encoded by it are eliminated by subsequent infection of the non-suppressor strain *E. coli* ABLE K.
7. In ABLE K the translation stop at the amber codon results in a g3p without the CT domain. Thus, the phages lack g3p, they are not infective and not released from the cells due to defects in the phage assembly (20). The cells grow very slowly or stop growing at an early stage.
8. The methylation of the synthesized DNA by dam methylase had no effect on the mismatch repair of transfected DNA chimeras in *E. coli* XL1-Blue.
9. We prefer to prepare phages on a small scale (5 mL cultures, precipitation in 1.5 mL), since the quality and reactivity of the single-stranded DNA prepared from these phage solutions is significantly higher than with large-scale preparations (100–500 mL).
10. The phages are more stable when stored for longer times in PBS buffer, but for subsequent preparation of DNA we resuspend the phages in TE buffer to reduce the amount of salt in the DNA sample.
11. In *E. coli* ABLE K the *recA* gene (responsible for homologous recombination) is not knocked out as it is in *E. coli* XL1-Blue. Thus, if the phage construct is susceptible to a complete or partial loss of the inserted guest gene by recombination, the probability for such loss is considerably increased when propagating the phages in the ABLE K strain. We use the infection of ABLE K to reduce the high number of phages with wild-type guest protein in the library and to facilitate the enrichment of stabilized, but rare variants. The selection itself also functions without this step, and it can be skipped to prevent recombination. Before the first selection it is, however, necessary to do a infection with the phages prepared from the transformants, because DNA chimeras were transfected and the produced phages can differ in their pheno- and genotype for the guest protein (i.e., phages can present a mutant of the protein on their surface but still contain the gene of the wild-type protein and vice versa).
12. Very low phage titers could give problems with the phage propagation after infection of 5 mL liquid cultures. In these cases pellet the cells from the liquid culture after infection and incubation for 30 min at 37°C, and plate them on a dYT^{cam} plate. After growing for 10–12 h isolate the cells from the plate with 5 ml dYT^{cam} and shake the culture for 2–3 h for further phage production.

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Minimization of Proteins by Random Fragmentation and Selection

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1. Introduction

Protein-protein interactions are involved in most biological processes and are an important target for drug design. Over the past decade, there has been an increased interest in the design of small molecules that mimic functional epitopes in protein-protein interactions. However, the design of small molecules that disrupt protein-protein interactions remains a considerable challenge (1,2). Progress has been achieved towards minimizing proteins into significantly smaller polypeptides that retain the ability to bind a partner protein (3–5). These mini-proteins represent a potential intermediate step between proteins and small molecules, and thus may facilitate the development of drugs targeted to protein-protein interfaces (6). Protein minimization has been achieved by both rational and combinatorial approaches and a large collection of proteins have now been minimized (3,7–9).

Here we describe a rapid method to identify polypeptides that retain the ability to interact with a target protein (5). The method is based on the random shearing of a DNA fragment encoding the protein to be minimized followed by the shotgun cloning of the resultant fragments into a phage display vector (see Fig. 1). The end result is a library consisting of a large collection of polypeptide segments displayed on the surface of M13 bacteriophage. Because the library is generated by random fragmentation of the gene, there is no preconceived bias for a particular segment for display, rather a large assortment of different polypeptides representing all regions of the protein are displayed on the phage surface. Functional polypeptides are selected by biopanning of the library on an immobilized target protein.

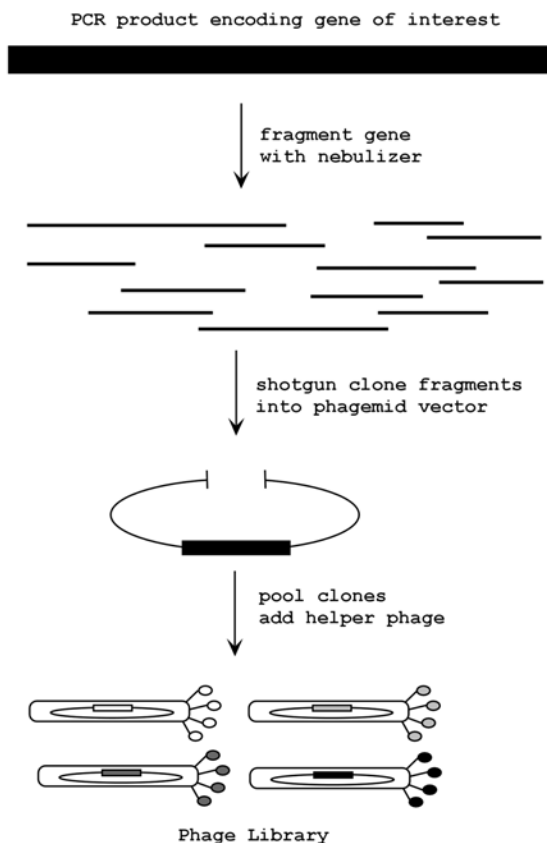


Fig. 1. Overview of the construction of a gene fragment library. The starting point is a DNA fragment encoding the gene of interest that has been generated by PCR amplification. This PCR product is randomly fragmented by high pressure using a nebulizer. The resultant fragments are cloned into an appropriate phagemid vector to construct the primary library. The clones resulting from the ligation are pooled and helper phage is added to create the phage library that can be used for biopanning.

2. Materials

2.1. PCR Amplification

1. 10X PCR reaction mixture: 500 mM KCl, 100 mM Tris-HCl, pH 9.0, Triton-X 100 (Promega).
2. *Taq* DNA polymerase (Promega).
3. 2.5 mM dNTP mix (Invitrogen).
4. 25 mM $MgCl_2$ (Promega).
5. QIAprep spin kit (Qiagen).

2.2. DNA Library Construction

1. Nebulizer (IPI Medical Products, Chicago, IL, part number 4207).
2. QS-T Plastic Nebulizer Cap (ISOLAB Inc., Akron, OH).
3. 1/4" inner diameter tubing.
4. Nitrogen gas.
5. 40% glycerol, 10 mM Tris-HCl, pH 7.5 (autoclaved). Store at room temperature.
6. 10X T4 DNA polymerase buffer (New England Biolabs, Beverly, MA).
7. T4 DNA polymerase (New England Biolabs).
8. 10X T4 DNA ligase buffer (New England Biolabs).
9. T4 DNA ligase (New England Biolabs).
10. XL1-Blue *E. coli* electroporation-competent cells (Stratagene, cat. no. 200228).
11. 1-mm electroporation gap cuvettes.
12. 2YT media: 16 g tryptone, 10 g yeast extract, 5 g NaCl per liter (autoclave).
13. Tris-buffered saline (TBS): 10 mM Tris-HCl, pH 7.5, 150 mM NaCl (**10**). Store at 4°C.
14. SOC media: 20.0 g tryptone, 5.0 g yeast extract, 0.5 g NaCl per liter. Sterilize by autoclaving. Add 20 ml of 20% (w/v) glucose (filter sterilize) prior to use.
15. Luria-Bertani (LB) media: 10 g tryptone, 5 g yeast extract, 10 g NaCl per liter (autoclave).
16. Luria-Bertani medium: LB media containing 1.5% bacto agar.
17. Appropriate antibiotics.
18. Appropriate restriction enzymes and buffers.
19. 50% glycerol (autoclaved). Store at room temperature.

2.3. Phage Display

1. VCS M13 helper phage (Stratagene).
2. 20% polyethylene glycol (PEG) 8000, 2.5 M NaCl (autoclave) (**11**). Store at room temperature.
3. 100 mg/mL Bovine serum albumin (BSA) (filter sterilized). Store at -20°C.
4. STE: 100 mM Tris-HCl, pH 8.0, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0, 100 mM NaCl (autoclave) (**11**). Store at room temperature.
5. TG1 *E. coli* (Stratagene).
6. 0.05 M Na₂CO₃ buffer, pH 9.6 (filter sterilize). Store at 4°C.
7. Wash Buffer: TBS containing 1 mg/mL BSA and 0.05% Tween-20 (filter sterilize) (**11**). Store at 4°C.
8. Blocking buffer: 1X wash buffer containing 5% non-fat dry milk. Make fresh.
9. Elution buffer: 0.1 M glycine-HCl, pH 2.2, 0.1 M KCl, 1 mg/mL BSA, 0.05% Tween 20 (filter sterilize) (**11**). Store at 4°C.
10. 1 M Tris-HCl, pH 8.0 (autoclave). Store at room temperature.

3. Methods

The methods described below outline 1) the amplification and random fragmentation of the DNA encoding the protein to be minimized, 2) the ligation of the fragmented DNA into a phage display vector to establish a library, 3) the

production of the phage library, and 4) biopanning of the library using an immobilized target protein to identify peptides that bind the target.

3.1. DNA Amplification and Fragmentation

To generate a randomly fragmented protein phage display library the gene encoding the protein of interest is PCR amplified and fragmented by nebulization. The resulting DNA fragments are then blunt end filled and ligated into the desired phage display vector.

1. PCR amplify the gene of interests to be fragmented by setting up the following PCR reaction: 1 μ L of a 0.2 ng/ μ L DNA template, 5 μ L 10X PCR reaction buffer, 5 μ L 2.5 mM dNTP mixture, 3 μ L 25 mM $MgCl_2$, 0.5 μ L each of 20 μ M forward and reverse PCR primers (*see Note 1*), 0.5 μ L Promega *Taq* DNA polymerase, and 34.5 μ L water. PCR conditions are 1X (30 s at 94°C, 60 s at 55°C, 90 s at 72°C), 25X (30 s at 94°C, 60 s at 50°C, 90 s at 72°C), 10 min at 72°C.
2. Purify the PCR product by agarose gel electrophoresis, extract DNA from the agarose using a QIAprep spin kit following the manufacture's directions (*see Note 2*). Elute the PCR products with 50 μ L of 10 mM Tris-HCl, pH 8.5. Keep sample on ice or place at -20°C for up to one week.
3. For DNA fragmentation, a nebulizer from IPI Medical Products was used (*see Note 3*). Modify the nebulizer by removing the inner plastic cylinder drip ring and cut off the outer rim of the cylinder. Invert the drip ring and place it back into the nebulizer. Seal the large hole in the top cover with a QS-T plastic cap from ISOLAB Inc. (*see Note 3*) and connect a 1/4 in inner diameter length of tubing to the smaller hole on the nebulizer cap.
4. Prepare the DNA sample for nebulization by adding 10 μ g of the purified PCR product to ice cold 40% glycerol, 10 mM Tris-HCl, pH 7.5 to make a final volume of 2 mL.
5. Add the 2 mL DNA sample to the bottom of the nebulizer cup and place the nebulizer on ice.
6. Connect the free end of the 1/4 in. tubing to a nitrogen gas tank and apply pressure to the sample at 5 min intervals (*see Note 4*). After each interval, disconnect the nebulizer from the nitrogen source and centrifuge the nebulizer containing the DNA sample at 150g for 1 min at 4°C to collect the DNA sample in the bottom of the nebulizer cup. Add ice cold 40% glycerol, 10 mM Tris-HCl, pH 7.5, to the collected DNA sample to bring the final volume back up to 2 mL (*see Note 5*).
7. Repeat nebulization and centrifugation steps until the desired DNA fragment lengths are obtained as determined by agarose gel electrophoresis.
8. Once the DNA has been fragmented to the desired lengths, concentrate and clean the nebulized DNA using a QIAquick spin kit according to the manufactures directions and resuspend in 50 μ L 10 mM Tris-HCl, pH 8.5 (*see Note 6*).
9. Blunt-end fill the sheared DNA fragments by adding the 50 μ L of the purified fragmented DNA, 8.5 μ L 2.5 mM dNTPs, 8.5 μ L 1 mg/mL BSA, 8.5 μ L 10X T4 DNA polymerase buffer, and 5 μ L (15 U) T4 DNA polymerase. Incubate at 11°C for 60 min.

10. Gel purify the desired molecular weight, fragmented DNA from an agarose gel using a QIAquick spin kit. Resuspend sample in 50 μL 10 mM Tris-HCl, pH 8.5.

3.2. Construction of Phage Display Vector Library

1. DNA ligation is performed by incubating the end-filled DNA fragments with an appropriate linearized cloning vector (see **Note 7**).
2. Ligate the randomly fragmented DNA library into the linearized blunt-ended vector by adding 3 $\mu\text{g}/\text{mL}$ linearized vector, 25 μg of fragmented DNA (25 μL of sample from **Subheading 3.1., step 10**), 8 μL 10X T4 DNA ligase buffer, 5 μL T4 DNA ligase (2000 units), and water to make 80 μL . Incubate overnight at 16°C.
3. Remove T4 DNA ligase and clean the ligation reaction using QIAquick spin kit. Resuspend recovered DNA in 20 μL 10 mM Tris-HCl, pH 8.5 (see **Note 8**).
4. Transform 5 μL of the cleaned ligation reaction into Stratagene *XL1-Blue* electroporation-competent cells according to the manufacturer. Resuspend transformed bacteria in 1 mL SOC and incubate at 37°C with shaking for 1 h.
5. Remove 20 μL of the transformed cells and add to 180 μL LB media to make a 1/10 dilution. Repeat this step but add 20 μL of the 1/10 bacterial dilution to 180 μL LB to make a 1/100 dilution. Using a sterile glass rod, spread 100 μL of each dilution on LB medium containing the appropriate antibiotics.
6. Plate the remaining undiluted transformation on LB medium with the appropriate antibiotic using 200 μL per plate.
7. Incubate plates overnight at 37°C.

3.3. Determining the Complexity of the Fragmented Library

After constructing the phage display vector library it is important to determine the complexity of the DNA library. The objective is to obtain a library that contains a large number of DNA inserts that range in size. The more diverse the initial library is, the greater the probability will be of sampling all possible peptide sequences for binding the target protein.

1. From the LB plates containing the diluted transformation reaction from **Subheading 3.2., step 5** determine the number of colony forming units (cfu) per mL (see **Note 9**).
2. Divide the number of colony forming units per mL by the number of bases found in the gene used for fragmentation. As a general rule, the number of colony forming units should be at least 50-fold greater than that of the gene size.
3. Screen 25 bacterial colonies by colony PCR to analyze DNA library inserts (**12**). For colony PCR use primers specific for the vector sequence that lie outside and near the DNA insertion site. For each PCR reaction set up the following cocktail: 5 μL 10X PCR reaction buffer, 5 μL 2.5 mM dNTP mixture, 3 μL 25 mM MgCl_2 , 0.5 μL each of 20 μM forward and reverse PCR primers, 0.5 μL Promega *Taq* DNA polymerase, and 34.5 μL water.
4. Use a sterile disposable 100–200 μL pipet tip, lightly touch a single, well isolated bacterial colony (see **Note 10**). Inoculate a PCR tube containing the PCR reaction mixture and swirl pipet tip to loosen bacteria.

5. Transfer tubes to a thermocycler using the following PCR conditions: 1X (30 s at 94°C, 60 s at 55°C, 90 s at 72°C), 25X (30 s at 94°C, 60 s at 50°C, 90 s at 72°C), 10 min at 72°C.
6. Analyze the products of the PCR by electrophoresis through an agarose gel, using markers of suitable size. Colony PCR should result in PCR products from each colony with DNA inserts ranging in size between the smallest and largest DNA fragments ligated into the cloning vector (**Subheading 3.1., step 10**).
7. Sequence PCR products to verify that the DNA inserts amplified consist of fragmented DNA from the gene of interest.

3.4. Pooling the Randomly Fragmented DNA Library

1. Pool the transformants by adding 1 mL of LB media to each Petri dish containing the transformed bacteria colonies (**Subheading 3.2., step 6**).
2. Using a sterile glass rod, swirl LB to resuspend bacteria colonies in the media.
3. Pool media to one side of the Petri dish, collect the bacterial solution, and place in a sterile 15-mL tube.
4. Repeat **steps 2** through **3** for each Petri dish and pool bacteria into the 15-mL tube.
5. Remove 5 μ L of the pooled bacteria and place in a 1.7 mL microcentrifuge tube for use in **Subheading 3.5**.
6. To the remaining pooled bacteria add sterile 50% v/v glycerol to make a final glycerol concentration of 10% v/v.
7. Vortex the culture gently to ensure that the glycerol is evenly distributed.
8. Transfer the culture to a storage tube and place at -70°C for long-term storage.

3.5. Preparing a Phage Display Library

1. Using a 125-mL flask, inoculate 25 mL of LB containing the appropriate antibiotic with the 5 μ L of reserved pooled bacteria from **Subheading 3.4., step 5 (13)**.
2. Grow the *E. coli* culture at 37°C with shaking to an OD₆₀₀ of 0.6.
3. Add 1×10^{11} VCS M13 helper phage to the culture and continue shaking at 37°C overnight.
4. Pipet the overnight bacterial culture into a 40-mL centrifuge tube and pellet bacteria at 10,000g for 10 minutes at 4°C.
5. Pour the supernatant into a fresh centrifuge tube and add 1/5 vol 20% PEG, 2.5 M NaCl and mix gently by inverting tube. Incubate at room temperature for 30 min.
6. Spin centrifuge tube at 15,000g for 15 min at 4°C.
7. Carefully and quickly pour off supernatant. At this point a white phage pellet should be visible at the bottom of the tube. Replace the lid and keep centrifuge tube inverted at room temperature for 10 min.
8. Using a Kim-wipe, carefully wipe the remaining liquid from the centrifuge tube without disturbing the phage pellet.
9. Add 0.5 mL of STE to the phage pellet and mix gently using 1-mL pipet tip until the phage pellet fully dissolves.

10. Transfer the suspended phage library to a 1.7-mL microcentrifuge tube and store at 4°C for up to 1 mo (*see* **Note 11**).

3.6. Titering Phage

1. Using a 125-mL flask, inoculate 25 mL of LB with TG-1 *E. coli* cells. Incubate at 37°C with shaking until an OD₆₀₀ between 0.5–0.7 is reached (*see* **Note 12**) (**13**).
2. Make serial dilutions of 10⁻², 10⁻⁴, 10⁻⁶, ...10⁻¹² of the phage library stock (**Subheading 3.5., step 10**) by adding 10 µL of phage stock to 990 µL of LB. Vortex tube gently for 30 s. Repeat using 10 µL of the previous dilution and adding to 990 µL of fresh LB media until a phage dilution of 10⁻¹² has been reached.
3. Add 100 µL of the 10⁻⁶, 10⁻⁸, 10⁻¹⁰, and 10⁻¹² phage dilutions to 100 µL of TG-1 mid-log phase cells in a 1.7-mL microcentrifuge tube. Mix by vortexing for 30 s.
4. Incubate tubes at 37°C for 30 min with shaking.
5. Plate each 200 µL sample on LB medium containing the appropriate antibiotic.
6. Incubate plates at 37°C overnight.
7. To determine the number of phage/mL in the phage stock, select a plate from the overnight incubation containing between 50–500 cfu and multiply the number of cfu by the phage dilution factor used to infect the plated bacteria. Generally 10⁻¹² to 5 × 10⁻¹³ plaque-forming units (PFU)/mL are obtained (*see* **Note 13**).

3.7. Enrichment of Phage Library

1. Dilute the target receptor protein and a BSA negative control (or other desired protein control) to 50 µg/mL in 0.05 M Na₂CO₃ buffer, pH 9.6.
2. For each phage library to be sorted, add 200 µL /well of the target protein and BSA control to a Nunc Maxisorb ELISA plate well.
3. Incubate overnight at 4°C or room temperature for 2 h to allow the protein to absorb to the ELISA wells.
4. Pour off protein solution by inverting plate and tapping on counter top to remove residual liquid.
5. Block coated wells with 0.2 mL blocking solution (*see* **Note 14**).
6. Incubate at room temperature for 1 h with gentle shaking.
7. During the incubation step, inoculate 25 mL LB with TG-1 *E. coli* cells and incubate at 37°C to OD₆₀₀ between 0.5–0.7.
8. Pour off blocking solution by inverting plate and tapping on counter top to remove excess blocking reagent.
9. Wash each well 10× with 1X wash buffer by adding 200 µL of wash buffer to each well, swirl gently, invert plate, tap gently on paper towels to remove excess solution, and repeat.
10. Add 200 µL of 1 × 10¹¹ PFU/mL of prepared phage solution (**Subheading 3.5., step 9**) to the target and BSA protein coated wells.
11. Incubate at room temperature for 2 h with gentle shaking.
12. Pour off phage solution and wash wells 10× with 1X wash buffer as in **step 9** (*see* **Note 15**).

13. Elute bound phage from wells by adding 200 μL of elution buffer to each well and incubating at room temperature for 30 min with gentle shaking.
14. Pipet out eluted phage solution and place in a 1.7-mL microcentrifuge tube containing 25 μL of Tris-HCl, pH 8.0.
15. Amplify the collected phage from the target protein well by adding 180 μL of the eluted phage to 1 mL of the mid-log phase TG-1 *E. coli* and set at room temperature of 30 min. Inoculate the infected bacteria cells into 25 mL of 2YT containing the appropriate antibiotic and 500 μL of 10^{11} VCS M13 helper phage. Incubate at 37°C with shaking overnight (*see Note 16*).
16. After overnight growth, collect phage as described in **Subheading 3.5., steps 4–10** followed by titering as described in **Subheading 3.6.**
17. Begin the next round of panning to further enrich for phage that bind the target protein by using the amplified, enriched phage library from the previous panning round. Monitor each round of binding and enrichment by colony PCR as described in **Subheading 3.3., steps 3–7** using bacterial colonies from **Subheading 3.8.**
18. Continue the binding and enrichment process for 3–8 rounds or until the library converges on a consensus or a single clone as determined by sequence analysis.

3.8. Titering Phage Elution

1. Add 10 μL of phage collected from the target and BSA coated wells from **Subheading 3.7., step 14** to 990 μL LB to make serial dilution of 10^{-2} , 10^{-4} , and 10^{-6} as described in **Subheading 3.6., step 2**.
2. Add 250 μL of each dilution to 250 μL of mid log phage TG-1 cells and grow shaking at 37°C for 30 min.
3. Plate 200 μL of each dilution on LB medium containing the appropriate antibiotic.
4. Incubate overnight at 37°C.
5. Count colonies to determine the number of phage eluted from target and BSA coated wells (*see Note 17*).

4. Notes

1. When selecting or designing primers for DNA amplification it is not necessary that the primers anneal within the gene of interest. During successive rounds of biopanning, DNA fragments that encode peptide sequences that do not bind the target protein will be selected against and removed from the library (5).
2. If QIAquick spin columns are not available, DNA purification can be performed using a low-melt agarose gel (10).
3. The nebulizer technique used here was modified from a protocol that can be found at the University of Oklahoma's Advanced Center for Genome Technology website (dna1.chem.ou.edu/protocol_book/protocol_partII.html). This website gives additional useful hints for fractionating DNA by nebulization along with information on the purchase of nebulizers and attachments for DNA fractionation. This site also contains detailed protocols on DNA fractionation by sonication, along with methods for preparing fractionated DNA for ligation into blunt-ended vectors.

4. Fragmentation of the DNA by nebulization is achieved by applying pressurized nitrogen gas to the DNA sample. The extent of DNA fragmentation is proportional to the duration and level of pressure applied. It is recommended that a range of pressures and time points be used to determine the settings that provide the desired results. When fragmenting small DNA products (1000 bp or less), the maximum pressure the nebulizer cup can withstand (45 psi) is recommended for up to 30 min. For larger DNA fragments a pressure of 30 psi should give the desired results.
5. During nebulization the sample volume will be noticeably reduced. Generally after 5 min the sample volume has been reduced to such a point that further nebulization will not result in further DNA fragmentation. At this point, the remaining DNA sample should be collected by centrifugation to restore the liquid volume.
6. After the last nebulization, recollect the sample by centrifugation. No additional liquid needs to be added to the DNA sample at this point. When using a QIAquick column for concentrating the DNA, only 0.8 mL of the sample can be loaded onto the column at one time. For sample volumes of more than 0.8 mL, reload the column as many times as necessary until the entire sample has been applied to the column.
7. Regardless of the binding affinity between the selected protein to be fragmented and its binding partner, it is recommended that the fragmented proteins be fused to the M13 gene VIII. Since the binding properties between the fragmented protein and the target protein cannot be predicted, increasing the number of fragmented proteins on the phage surface will increase the sensitivity of the assay by generating more binding interactions between the fragmented peptide and its target.
8. When precipitating DNA from QIAgen columns using 20 μ L of Tris-HCl, let the solution incubate on the column for 5–10 min prior to collecting the sample by centrifugation. This additional extra time allows for the Tris-HCl to fully saturate the column and release the bound DNA.
9. If there are no colonies on the plates containing the diluted transformation sample, the size and thus diversity of the library is most likely too low to continue. Generally the problem is caused by insufficient fragmented DNA in the ligation step or insufficiently-filled fragmented DNA by T4 DNA polymerase. These problems can often be overcome by increasing the amount of fragmented DNA in the ligation step by five-fold or higher or by treating the fragmented DNA for longer periods of time with a higher concentration of T4 DNA polymerase. If these steps fail to increase the size of the library, using a different *E. coli* strain for transformation such as *E. coli* TG1 may also increase the library size.
10. It is important not to be too generous with the amount of bacteria added to the PCR reaction. The addition of too many bacteria will often lead to a failed PCR reaction, or the accumulation of RNA that will obscure DNA bands when resolved on an agarose gel. In general, when inoculating a PCR cocktail, the bacterial colony should not be visible on the pipet tip. Excess bacteria can be removed from the inoculated tip by lightly touching the tip to a sterile LB plate and scraping the bacteria off.
11. If phage stocks are stored for longer than 1 mo, it is recommended that the titer of the stock be determined again before use.

12. Start the *E. coli* TG1 culture for infection at least 5 h in advance. It is recommended that a plate of TG1 cells be grown that contains several well-isolated colonies. The plate can then be placed at 4°C for up to 2 wk. Pick a single TG1 colony to inoculate the culture. Once an OD₆₀₀ between 0.5–0.7 is reached the culture can be placed at room temperature for up to three hours before infecting. Alternatively, an overnight culture can be used to start a fresh culture 2–3 h in advance.
13. During infection with M13 bacteriophage, *E. coli* will show a noticeable reduction in growth. If an insufficient number of phage are used to infect the bacterial culture or not all the bacteria become infected by the phage, the uninfected bacteria may overgrow the culture and reduce the number of phage particles produced. To overcome this potential problem, 35 µg/mL of kanamycin may be added to the bacterial culture 2 h post-infection with M13 helper phage. Since the M13 helper phage carry the gene for kanamycin resistance, only infected bacteria will grow under these conditions, which will increase phage production.
14. Although non-fat dry milk is the recommended blocking solution, Superblock (Pierce) has also been successfully used in phage display experiments with no significant background.
15. By lengthening the wash steps, the selection process can be biased for high-affinity binders. Increasing the wash time selects for tight binders by eliminating variants that possess fast off rates (14).
16. On occasion it has been noted that little or no bacterial growth occurs post-infection with the VCS M13 helper phage, resulting in a low phage titer. If after three hours post-infection the bacterial culture shows little to no growth, add 2 mL of mid-log phase *E. coli* TG1 cells to the culture. The addition of the extra bacteria appears to overcome the reduced growth state of the infected cells and results in a high phage titer.
17. The success of the biopanning experiment can be followed by determining the number of phage eluted from the immobilized target protein versus the BSA coated ELISA wells (15). After each round of binding and enrichment, the number of phage eluted from the target protein should increase while the number of phage eluted from the BSA control should stay approximately the same. Although this is a good method for monitoring the success of the panning experiment, it does not always hold true if the displayed peptides bind weakly to the target molecule. If after three rounds of binding and enrichment there is no increase in the number of phage eluted from the target protein versus the BSA control, it is recommended that sequence analysis of the recovered phage be performed. A comparison of the target protein and BSA control sequence data should indicate if a specific type of sequence is being selected.

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Evaluating a Screen and Analysis of Mutant Libraries

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1. Introduction

Directed evolution by sequential cycles of random mutagenesis and screening has proven to be useful for producing new or improved enzyme properties (1,2). The first step is construction of a mutant library, usually accomplished by random point mutagenesis with error-prone PCR (3) or by DNA shuffling (recombination) (4,5). The second, and most critical step is finding the desired mutants by screening or selection of the libraries. Screens for enzyme activity usually operate via detection of optical absorption or fluorescence. Some screens are performed directly in agar plates, where changes of color are observed by direct visualization (6,7) or by digital image analysis (8). However, most screens involve transferring individual colonies to multi-well microplates containing culture medium, growing the cells until stationary phase, and inducing protein expression. If the target protein is intracellular, a cell lysis step has to be carried out to release the cellular contents. Finally, enzymatic activity is assayed with a microplate reader spectrophotometer. For many enzymes, thermal stability (9) is conveniently assayed by making a replica of the library and measuring the ratio of the activity after heating at defined temperature and time (residual activity, R_A) to the activity measured before the heating (initial activity, I_A) (see **Chapter 11**).

Experimental limitations impose significant restrictions on the number of mutants that can be screened. Designing a screen that captures the essence of a complex chemical function and that can be repeated for thousands of mutants is the most difficult part of directed evolution. Significant experimental efforts are spent in developing, validating, and implementing screens. In a directed evolution experiment, thousands of data points are generated in each genera-

tion. These data contain information on library quality, evolvability of the parent enzyme, and precision of the high-throughput method. It is indispensable to organize and illustrate these data efficiently, so that useful information can be extracted from them, information that can be used to optimize the screening protocol and decide on a good evolutionary search strategy.

To correctly interpret the data and reduce the probability of selecting false positives, variability among individual assays must be minimized during each step of the screening process. This is achieved by performing a set of experiments designed to validate the screen. In these experiments, multiple colonies of a single clone are grown and processed in 96-well microplates, just as the mutants are going to be cultivated and assayed. In this chapter, we describe a practical approach to validating a screen for directed enzyme evolution. In addition, some aspects of library data analysis are considered, with the goal of designing effective search strategies.

2. Materials

2.1. Chemicals

1. *E. coli* clone expressing the wild type gene or a mutated version used as the parent for the next round of directed evolution (*see* **Note 1**).
2. A ligation mixture containing a mutant gene library (e.g., prepared by random point mutagenesis or recombination (*see* **Notes 1 and 2**).
3. Liquid Luria Broth (LB) and LB agar plates supplemented with the appropriate antibiotic.
4. Terrific Broth (TB).
5. IPTG (isopropyl- β -D-thiogalactopyranoside; Sigma, St. Louis, MO) or other convenient inducer for target gene expression.
6. Lysozyme (Sigma).
7. DNase I (Roche, Indianapolis, IN).
8. Solutions to assay the target enzyme activity.
9. Deep-well plates.
10. 96-well plates.

2.2. Equipment

1. Orbital shaker.
2. Centrifuge and compatible multi-well plate rotor.
3. Plate reader/spectrophotometer.

3. Methods

3.1. Validation of a Screen

The following steps are used to optimize and validate a screen. Validation is strongly recommended before attempting to analyze a mutant library. Here we provide a generic protocol for growing *E. coli* cells in a plate, lysing the cells,

and preparing samples for an enzyme assay. Details will of course change, depending on the particular system. The key idea is that the screen must be validated using a library prepared from a single sequence (not a mutant library). This allows you to identify sources of variability inherent to the screening method.

3.1.1. Experimental Procedure

1. Transform the appropriate *E. coli* host with vector DNA containing the parental gene (*see Note 3*). Bacterial clones expressing identical gene sequences are referred to as a “single sequence library”.
2. Spread appropriate aliquots on LB-antibiotic plates, in order to obtain a uniform distribution of well-isolated colonies.
3. Pick and inoculate single colonies into 300 μ L LB-antibiotic in deep-well plates.
4. Incubate the cells at 30°C in a shaking incubator for 24 h (*see Note 4*).
5. Transfer aliquots of 30 μ L of the culture into a new deep-well plate with 300 μ L TB-antibiotic in each well and containing an inducer agent for target gene expression (*see Note 5*).
6. Cultivate the cells for 18 h at 30°C in a shaking incubator (*see Note 5*).
7. Spin down the cells and resuspend in 600 μ L of 100 mM Tris-HCl, pH 8.2, containing 0.25 mg/mL lysozyme and 1.5 U/mL DNase I (*see Note 6*).
8. Centrifuge plates at 1600g for 15 min and transfer aliquots of supernatant to a 96-well microtiter plate to assay the enzyme activity.

3.1.2. Data Collection and Analysis

Practically, having basic programming skills will facilitate manipulation of the large amounts of data generated in directed evolution experiments. It is convenient to transfer data into a spreadsheet (Microsoft Excel or another computational tool) for data management. When storing library data in a Microsoft Excel spreadsheet, preparing macro codes (Visual Basic for Applications) will greatly reduce the time and increase efficiency of data manipulation. A short paragraph of code enables us, for example, to set a property threshold to avoid unusually large numbers when we calculate the ratio of two properties and to sort data by position of a mutant in the plate.

3.1.3. Quantifying Variability in the Screen

To quantify the reproducibility and uncertainty of a screen, repeated measurements on a single clone (single sequence library) must be carried out (e.g., using wild type or a parent sequence). Measuring variability of the screen is important to determine a lower limit above which an observed improvement in a targeted property can be considered significant. The variability has direct influence on your ability to identify real, improved mutants: the greater the inherent variability, the higher the rate of false positives (and negatives).

Errors should be random and can be described statistically using data obtained from the screen. A good way to estimate the variability or dispersion of data is by determining the coefficient of variance ($CV = [\text{standard deviation} \div (\text{mean}) \times 100\%]$).

When the results of screening a single sequence library are collected, the activities (or other properties) can be plotted versus the positions of the clones in a plate (*see Fig. 1*) to check how the activity varies in the different wells of the plate. This way of plotting the data helps to identify systematic errors and provides important guidance for further improvements in the screen, for example eliminating positional effects in a plate originating from differences in oxygen supply or heat interchange between the environment and medium in the plate (*see Fig. 1B*). Other times it permits one to observe biases in columns or rows that come from systematic errors, such as in pipetting.

The results of screening a single sequence library can also be presented in a histogram, which should resemble a Gaussian distribution with points symmetrically distributed around a mean value (*see Fig. 2*). Skewed distributions indicate systematic biases, the sources of which should be identified and eliminated. The wider the distribution, the greater the data dispersal, and consequently the lower the reproducibility of the screen.

You can estimate the rate of false positives from the single sequence screen using statistical analysis. False positives are clones whose fitness values, i.e., activity or thermostability, are at or beyond a certain threshold (χ_0) defined by the researcher. The rate of false positives in a population depends on the standard deviation (σ) or the CV, and the defined threshold χ_0 . Once the mean (μ) and standard deviation or CV of the data obtained from the single sequence screen have been determined, you can calculate the expected rate of false positives assuming the data points (χ) follow a Gaussian distribution. **Figure 3** illustrates the frequency distribution for fitness values in a single sequence population. The shaded area corresponds to the probability of finding values of χ at or higher than χ_0 , and it represents the rate of false positives in a screen that looks for fitness values of χ_0 or higher. This rate can be calculated from

$$\text{rate} = 1 - F\left(\frac{\chi_0 - \mu}{\sigma}\right)$$

where $F\left(\frac{\chi_0 - \mu}{\sigma}\right)$ is the probability of finding a value of $\chi \leq \chi_0$ (*see Note 7*). The threshold value of fitness can be expressed as a fraction of the mean μ . **Table 1** shows the rate of false positives you would expect to find for screening libraries with different target thresholds and variability in the measurements (CV values).

According to **Table 1**, screening 2000 clones by a screen with CV of 20% leads to one false positive. In our experience, however, the number of false

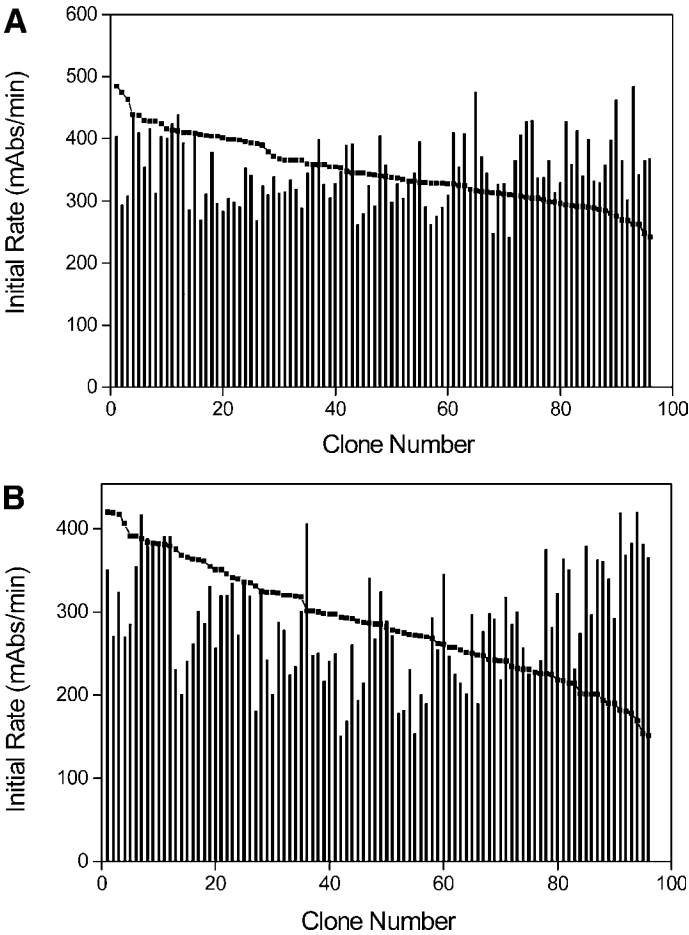


Fig. 1. Evaluating a screening method with a plate of 96 wildtype samples. (A) Enzyme activities are plotted versus well position (columns) and in descending order (squares). The CV for this screen is 15%. (B) Plotting enzyme activity versus plate position can reveal a relationship between culture activity and position in a deep-well plate. Here, the cell cultivation method should be further optimized, since the cells in the inner part of the deep-well plate obviously do not produce as much enzyme as those in the plate perimeter. The CV of this screen is 23%.

positives calculated using this approach represents the minimum—the real number in an actual experiment is usually higher. It is possible that experimental errors in screening are not well represented by a Gaussian distribution, as has been assumed in preparing **Table 1**. In practice, the CV should be as small as possible (we strive for less than 10%). In the experiment of **Fig. 1A**, the CV

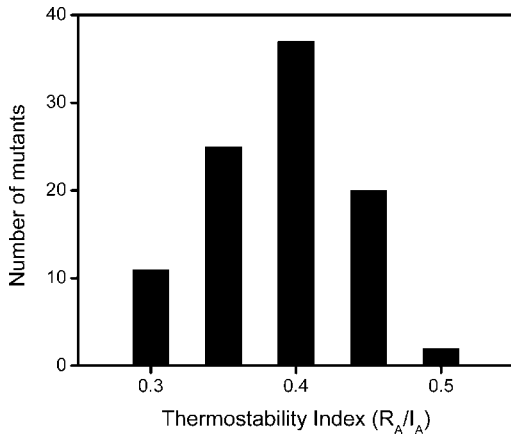


Fig. 2. Thermostability index distribution for a single sequence screen of 96 colonies cytochrome P450 BM3. The mean is 0.36 and the CV is 12.6%. The thermostability index is the ratio of enzyme activity measured after (R_A) and before (I_A) heating the plates at 57°C for 10 min. Cytochrome P450 BM3 activity was measured using ω -*para*-nitrophenoxycarboxylic acid (*p*NCA) and H_2O_2 in crude extracts, and is expressed as change of absorbance at 398 nm per min (see **Chapter 15**).

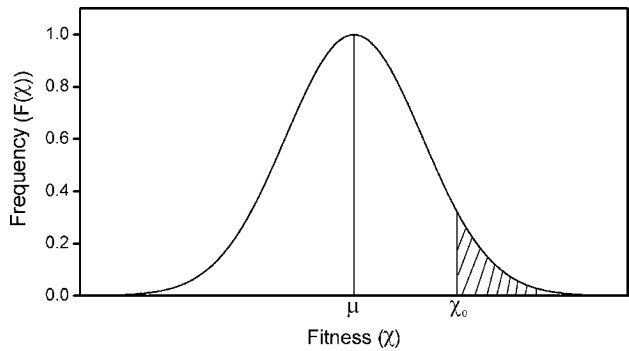


Fig. 3. Gaussian distribution for fitness values (χ) of a population of clones in a single sequence screen. The highlighted area represents the fraction of false positives and χ_0 corresponds to the defined threshold. μ is the mean for fitness values in the population.

is 15%, a variability that allows us to identify mutants with at least two-fold improved activity in a library of 2000 clones. A lower CV is required if small improvements are expected.

A screening procedure generally consists of several steps, including cell growth, gene expression, lysis, and the activity assay. The errors associated

Table 1
Rate of Expected False Positives for Variants Exhibiting Higher Than 1.5 and 2.0 Times the Mean Value (μ), for Different Values of CV

Fitness target, χ_0	1.5 μ	2.0 μ	1.5 μ	2.0 μ	1.5 μ	2.0 μ
CV	10%	10%	20%	20%	30%	30%
Fraction of false positives	2.87×10^{-7}	0	6.21×10^{-7}	2.87×10^{-7}	4.78×10^{-2}	4.29×10^{-4}

False positive rates were calculated using a Gaussian distribution function. Number of false positives = (fraction of false positives) $\times$ (number of clones screened)

with each step are reflected in the variability of the activity data finally collected. When you observe a very high CV or a skewed distribution in a single sequence screen, it is best to dissect the procedure into steps and to optimize each step, one at a time. Errors often come from differences in cell density and protein expression levels, and can be decreased by growing cells until stationary phase before protein expression induction. This operation can increase the uniformity of cell density in a 96-well plate. Another important source of error is poor design of the activity assay. Assay conditions must be adjusted for quantitative measurement, i.e., initial rate and saturating substrate concentration, for the parental enzyme as well as the potential improved variants. If substrate concentration is not high enough, very active variants may deplete the substrate at short times, and improvements can be underestimated or, in the worst case, are not observed. Also, the initial rate of an assay should not be so high as to exceed the linear range of the photometer.

It is important to keep in mind that the screening protocol may need to be optimized for each generation of a directed evolution experiment, since different sources of errors can become significant as the parent activity increases. Activity assays are often affected by this. If the activity of parental enzyme is high, the reaction will proceed faster, and substrates can be depleted early in the reaction. Also, very high initial rates in the kinetic assay could produce significant differences in activity values between the first and last wells charged in the plate. This problem, often not observed in earlier generations, can be solved by changing the conditions to slow down the reaction.

We generally screen for two types of properties. The first is a property characterized by absolute values, for example, enzyme activity. The other is a relative value of two properties, for example substrate specificity and thermostability, which are ratios of absolute values. In both cases, it is important to design a screen to have high reproducibility from well to well and to be

sensitive enough to detect the (usually low) activity levels characteristic of the first generations of directed evolution. However, since variable expression in different clones and other errors in a screening method affect relative properties less than absolute properties, screening relative properties usually requires a slightly less reproducible screen than does screening absolute properties.

3.2. Mutant Library Analysis

Once the screen has been validated and optimized to minimize the CV, you can proceed to screen mutant libraries. It is best to screen a small number (96) of clones first, to make sure the library is of good quality (mutation rate is not too high or too low). The procedure described below is generic, and details must be adapted to the specific system.

3.2.1. Experimental Procedure

1. Transform appropriate host cells with DNA of the mutant library. Plate dilutions to have approx 3 or 4 colonies per cm². Incubate 12 h at 30°C.
2. Transfer individual colonies to deep-well plates containing LB antibiotic, grow clones, induce the target gene expression, make crude extracts, and assay the interesting properties in the mutant library, following the protocol optimized according to directions described under Validation of a Screen (*see Subheading 3.1.*). Remember to inoculate a group of wells in each plate with parental clone colonies, in order to have as a reference the original activity measured under identical experimental conditions. The original plates are stored at 4°C for retrieval of the positive mutants identified in screening.

3.2.2. Analysis of Mutant Libraries

We often plot activities (or other data) for each mutant in descending order, to generate “fitness profiles.” Descending order is usually used to characterize mutant libraries for single properties (*see Fig. 4*). To identify appropriate mutagenesis conditions, we recommend that you prepare and analyze 96 clones from several libraries made under different conditions. For example, the random point mutagenesis libraries of **Fig. 4** were generated by error-prone PCR using the Mutazyme kit (Stratagene, La Jolla, CA) and three concentrations of template DNA. The fraction of inactive clones can be used to estimate the mutation rate. In **Fig. 4**, it is observed that the lower the target DNA concentration, the higher the fraction of inactive clones, owing to a higher mutation rate generated in the PCR. Although sequencing random clones is the most accurate method for determining mutation rates, measuring the fraction of inactive clones in a library is the most convenient way to set a good mutation rate for directed evolution. In our experience, 40–50% inactive clones in a library often corresponds to an average of 1–2 amino acid substitutions per gene. This is a good mutation rate for evolution by sequential rounds of point mutagenesis.

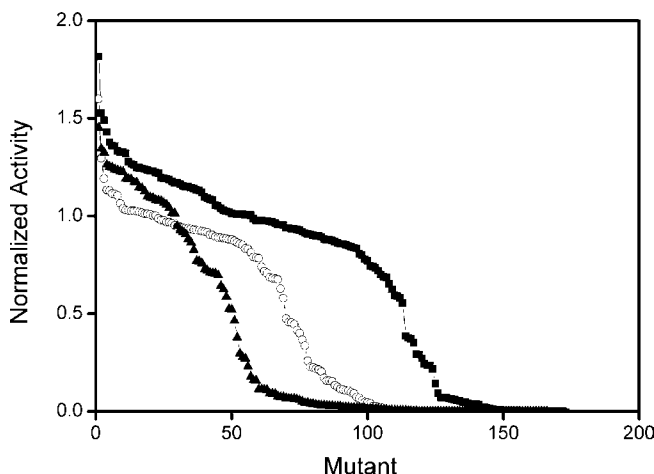


Fig. 4. Activity profiles for three cytochrome P450 BM3 mutant libraries prepared under different mutation rate conditions. For each library, activity is plotted in descending order. Libraries were constructed by random point mutagenesis, using 0.5 (▲), 5 (○) and 50 ng (■) of target gene DNA in the PCR. Activity was quantified as in Fig. 2 in the crude extracts of 170 mutant clones cultured in deep-well plates.

The number of possible variants of a protein that can be created increases rapidly with the size of the enzyme and with the mutation rate (10). If the mutation rate is too high, most clones generated will be inactive (either by increasing the frequency of stop codons or by introduction of deleterious mutations), as can be observed in Fig. 4 for the highest mutation rate library. The chances of identifying improved enzymes in libraries containing large numbers of mutations are very small. A protein with 300 amino acid residues can have 6000 single amino-acid variants. But because we use error-prone PCR to perform the random mutagenesis, owing to the degeneracy of the genetic code, only ~5.7 amino acid can be accessed on average for a given amino acid, leading to less than 2000 variants, a number can be handled easily in a microplate-based screening procedure.

Useful parameters derived from fitness profiles include the frequency and fitness of improved clones, which are directly observed if fitness data are plotted normalized to the parent. In Fig. 4, for example, approx 6% of the clones show activity at least 20% higher than the parental enzyme, and the best improvement in activity observed is about 80%. These numbers are only as reliable as the screen permits. If the CV is high, these numbers are unreliable because they can include a significant number of false positives.

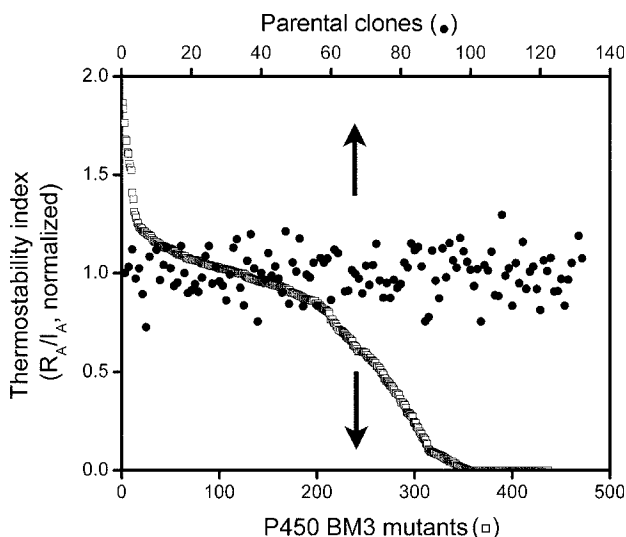


Fig. 5. Thermostability profile for mutant cytochrome P450 BM3 library. Thermostabilities for 440 variant clones are plotted in descending order. Library was constructed by error-prone PCR, and thermostability was measured as the ratio of activities after and before incubation at elevated temperature. The ratio was normalized to the parental ratio, to allow comparison among different plates. Clones with thermostability indices higher than ~ 1.5 are good candidates for rescreening to confirm the improvement in thermostability. Full circles show the thermostabilities of parental clones cultured under the same conditions, illustrating the variability in the assay ($CV \sim 10\%$).

3.2.3. Screens of Two Enzyme Properties Simultaneously

Data from screening two properties can be plotted as the ratios of the two for each clone (*see Fig. 5*). This representation clearly indicates improved ratios, and superior mutants can be conveniently identified. However, this approach ignores the absolute values of each property, which are also important in evaluation of enzyme fitness. A second useful representation is to plot one property versus another property (*see Figs. 6 and 7*). All mutants fall into four categories: mutants with improvement in one property but a decrease in the other, mutants with improvement in both properties and mutants with decreases in both properties. In **Fig. 6**, for example, it is deduced that after several generations of directed evolution, the thermostability of cytochrome P450 is more amenable to improvement than activity. In this experiment, it is difficult to identify mutants in which both properties improve simultaneously. The reason is that it is often difficult to improve two uncoupled traits (e.g., activity and stability) simultaneously with a single amino acid substitution. In contrast, coupled traits such as activity on related substrates are often improved together (*see Fig. 7*).

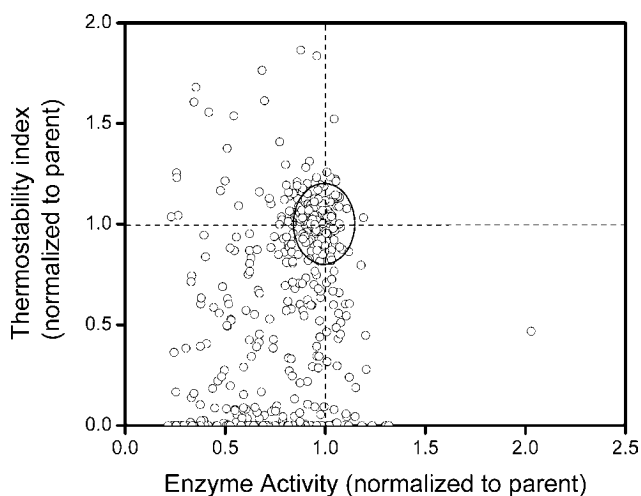


Fig. 6. Relationship between thermostability and activity in 900 cytochrome P450 BM3 variants. The distribution of parental clones measured under the same conditions is indicated by the ellipse. Most improvements in thermostability come at the cost of activity because most mutations reduce activity and many thermostabilizing mutations affect catalysis.

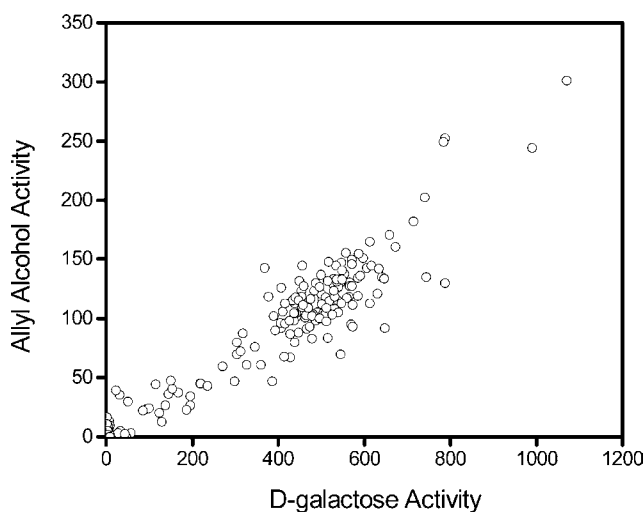


Fig. 7. Library data representation. Substrate specificities of galactose oxidase mutants can be illustrated by plotting activity on one substrate against another for each member of the library.

4. Notes

1. In this protocol, an *E. coli* expression system is used. Transcription of the cloned gene is under the direction of double *tac* promotor and is induced by IPTG. Other combinations of expression vector-host systems have been used successfully (13,14). In general, the same restrictions and requisites have to be considered for choosing the expression system as for any cloning expression experiment (15). Obviously, since no more than one milliliter of culture is grown in one well of a multi-well plate, expression levels have to be high enough to satisfy the sensitivity requirement of the activity assay. It is important that the expression system be regulated in order to avoid negative effects on growth derived from the metabolic burden or potential toxicity of the protein.
2. In this chapter, a random point mutant library is used for illustration, but the analysis can be applied to libraries made using other methods.
3. In our experience, data generated from freshly transformed colonies are more reproducible.
4. For low variability of the screen, bacterial cultures in deep-well plates have to be incubated until cells are in stationary phase (optical density at 600 nm ~ 2 under the conditions described here) before inoculating fresh media. This means that particular experiments must be optimized, depending on the bacterial strain and growth conditions used (composition of the medium, temperature and shaking speed). Maintaining high relative humidity in the shaker is recommended to reduce water evaporation. (See **Chapter 2** in the companion volume, “*Directed Evolution Library Creation*” for useful suggestions for growing *E. coli* in microtiter plates.)
5. For optimal induction of gene expression, conditions to consider include: time to start induction, inducer concentrations, time, and temperature of induction.
6. If the protein is expressed in the cytoplasm, a lysis step has to be included in the screening procedure. Buffer conditions have to be optimized depending on the specific enzyme activity and stability (see **Chapter 15** for an example of a lysis procedure).
7. The probability function of a Gaussian distribution is,

$$F(x) = \frac{1}{\sigma\sqrt{2\pi}} \int_0^x e^{-\frac{(x-\mu)^2}{2\sigma^2}} dx$$

where μ is the mean of the distribution, σ is the standard deviation, and $F(x)$ represents the probability of a random variable with values less than or equal to x (the unshaded area under the distribution in **Fig. 3**).

$$F\left(\frac{x_0 - \mu}{\sigma}\right)$$

can be calculated from the distribution function $F(x)$ or can be found in any book of general statistics.

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Screening Mutant Libraries in *Saccharomyces cerevisiae*

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1. Introduction

Functional gene expression is a prerequisite for directed evolution with *Escherichia coli* (*E. coli*), the preferred host organism. However, bacterial expression of eukaryotic genes can be impossible, or produce proteins with substantially altered properties, because of differences between bacterial and native expression systems (1). Different codon usage, missing chaperones, and posttranslational modifications like disulfide bridges or glycosylation can all cause low expression levels, misfolding, and inclusion bodies (2). Some of these problems can be avoided if these genes are expressed in a eukaryotic host whose expression machinery is more similar to the native one. Considering transformation efficiency, stability of plasmid DNA, and growth rate, *Saccharomyces cerevisiae* (*S. cerevisiae*) (3,4) is best suited for directed evolution (5–8), but the potential of this host has not been widely appreciated. Growing and manipulating *S. cerevisiae* is regarded as time consuming and more complicated than working with *E. coli*. The advantages of this host for directed evolution, such as homologous recombination that facilitates library construction (see Chapter 3 in companion volume, “Directed Evolution Library Creation”) or secretion of mutant proteins, are frequently overlooked.

Secretion of foreign proteins by *S. cerevisiae* is a well established process (9). Passage through the yeast secretory pathway leads to important eukaryotic post-translational modifications (10). Simplified screens with reduced variability result from avoided lysis steps. The low secretion level of yeast proteins increases assay sensitivity often compromised in cell lysates. In this chapter, we describe how to design a successful high throughput screen with *S. cerevisiae*.

Modifications in the screening procedures mainly reflect differences in growth characteristics and physiology between yeast and *E. coli*.

The example we use is a screen for secreted activity of laccase (EC 1.10.3.1). The typical laccase is a 59–85 kDa extracellular fungal monomeric multicopper glycoprotein with a carbohydrate content of 15–20% and several disulfide bridges (**11**). Laccases, like other ligninolytic enzymes are notoriously difficult to express in non-fungal systems. Expression in bacteria has not been reported, but yeast expression was successful for some laccases (**10,12–16**). Interest in laccases has been fueled by their potential uses in detoxification of environmental pollutants (**17**), paper processing (**18,19**), and biofuel cells (**20**). To improve laccases for these applications we have developed several assays (see **Chapter 20**) and the screening procedure described in this chapter. We hope that this protocol will correct some misconceptions and make *S. cerevisiae* accessible for directed evolution experiments.

2. Materials

2.1. Biological Materials

1. Appropriate *S. cerevisiae* strain. In the example: BJ5465 (ATCC 208289).
2. Expression shuttle vector containing the gene of interest under appropriate promoter, a signal sequence for secretion and selection markers for *S. cerevisiae* and *E. coli*. In the example: pJRoC30, Gal10 promoter, *Myceliophthora thermophila* laccase gene with native signal sequence, markers uracil, ampicillin.
3. Competent *E. coli* cells.

2.2. Chemicals

All chemicals used were reagent grade purity.

2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS).

2.3. Buffers and Solutions

1. Chloramphenicol stock solution: 25 mg chloramphenicol in 1 mL of ethanol.
2. YPAD solution*: 10 g yeast extract, 20 g peptone/tryptone, 100 mL 20% sterile glucose,** 100 mg adenine hemisulfate + dd H₂O to 1000 mL.
3. YP medium*(1.55X): 10 g yeast extract, 20 g tryptone/peptone, 650 mL dd H₂O.
4. Minimal medium*: 50 mL 20% sterile raffinose,** 5 mL 0.25% L-His, 5 mL 0.25% L-Trp, 5 mL 0.25% L-Leu, 10 mL 0.25% adenine hemisulfate, 150 mL ddH₂O, 25 mL 6.7% yeast nitrogen base, 250 µL chloramphenicol stock solution.**
5. Expression medium*: 325 mL YP medium, 25 mL 1 M KH₂PO₄-buffer, pH 6.0, 2 µL sterile 1 M CuSO₄**, *** 50 mL 20% sterile galactose,** 400 µL chloramphenicol stock solution** + dd H₂O to 400 mL.
6. SC-drop-out plates*: 6.7 g yeast nitrogen base, 50 mg L-His, 50 mg L-Trp, 50 mg L-Leu, 50 mg adenine hemisulfate, 15 g bacto agar, 100 mL 20% sterile glucose,** 1 mL chloramphenicol stock solution** + dd H₂O to 1000 mL.

7. Luria-Bertani (LB)-medium with appropriate antibiotic: 10 g tryptone/peptone, 5 g bacto-yeast extract, 10 g NaCl, 1 mL of 100 mg/mL ampicillin solution** + dd H₂O to 1000 mL. (plates: +15 g bacto agar/L).
8. Britton and Robinson (B & R) buffer: 0.1 M boric acid, 0.1 M acetic acid, 0.1 M phosphoric acid with 0.5 M NaOH to pH 6.0.
9. ABTS reaction solution: 5 mL ABTS 60 mM, 10 mL PEG 5000 50% (w/v), 50 mL B & R buffer 100 mM, pH 6.0, add ddH₂O to 90 mL. Final concentrations in the assay: 3 mM ABTS, 5% PEG 5000, 50 mM B & R buffer.

2.4. Equipment

All equipment used in the preparation of the libraries for screening is listed in **Chapter 3** in the companion volume, “*Directed Evolution Library Creation*.”

1. 96-well microtiter plates: R-96-OAPF-ICO (Rainin, Emeryville, CA).
2. Humidity shaker: ISF-1-W Kuhner (Switzerland).
3. Centrifuge for 96-well plates: Qiagen Sigma 4k15 (Qiagen, Valencia, CA).
4. Pipet robot: Multimek 96 Automated 96-Channel Pipettor (Beckman Instruments, Palo Alto, CA).
5. Spectrophotometer/plate reader (Model Spectra Max Plus 384, Molecular Devices, Sunnyvale, CA). Software Softmax Pro 3.1.1.
6. Oven: (model I540, VWR Scientific Inc.).
7. Picking robot (model QPix, Genetix).
8. 8-channel pipettor.
9. Glass beads, 6 mm.
10. Sealing film (Genemate).
11. Miniprep: QIAprep Spin Miniprep Kit (Qiagen).
12. Zymoprep: Yeast Plasmid Mini-Prep Kit (Zymo Research, Orange, CA).
13. Yeast transformation: the Gietz Lab Transformation Kit (Tetra Link, Amherst, NY).

3. Method

3.1. Screen for Activity and Stability of Myceliophthora thermophila Laccase Expressed in *S. cerevisiae* (Fig. 1)

1. Transform *S. cerevisiae* with a mutant library and plate the cells onto SC-drop out plates containing chloramphenicol using glass beads (see **Note 1**). Incubate the plates for four days at 30°C.
2. Screen: Fill an appropriate number of 96-well plates with 25 µL minimal medium per well with an 8-channel pipettor. Using the picking robot pick the colonies from the SC-drop out plates and transfer them into the 96-well plates. One column on each plate is inoculated with standard (wild-type or the best clone of the previous generation); one well is not inoculated (control) (see **Note 2**).

*Store in darkness (light sensitive).

**Added after autoclaving.

***Addition specific for the expression of the laccase gene. Not general requirement.

3. Wrap the plates in Parafilm and incubate them for 24 h at 30°C and 260 rpm in the humidity shaker at 85% humidity (*see Note 3*).
4. Remove the Parafilm and add 80 μ L of expression medium to each well using the pipetting robot. Reseal the plates with Parafilm and incubate them for 24 h under the conditions specified in **step 3** (*see Note 4*).
5. Fill new 96-well plates with 80 μ L of 10 mM B & R buffer pH 6.0 per well (stability plates). Remove the Parafilm from the culture plates and centrifuge these plates (master plates) for 5 min at 2500g at 4°C (*see Note 5*).
6. Transfer 40 μ L of the supernatants into the stability plates and mix by pipetting up and down with the pipetting robot (*see Note 6*). Transfer 20 μ L of each well of the stability plate into new 96-well plates (activity plates).
7. Seal the stability plates with sealing film and incubate them at 37°C for 48 h (*see Note 7*).
8. Activity assay: Add 180 μ L of ABTS assay solution to each well of the activity plate. Mix and measure the absorption at 418 nm in a platereader. Incubate at room temperature until green color develops. Measure absorption at 418 nm again (*see Note 8*).
9. Calculate the relative activities from the difference between initial absorption and that after the incubation divided by the incubation time (min). Adjust the activities of the mutants with the relative activities of the standard in the corresponding plate to allow a comparison between plates (*see Note 9*).
10. Screening for stability: Centrifuge the stability plates briefly at 1000g to remove condensed water from the sealing film. Remove the sealing film and transfer 20 μ L of each well of the stability plate into new 96-well plates. Follow the procedure described in **steps 8 and 9** to measure the residual activities.
11. Calculate the relative stabilities from the difference between the activities measured before and after the incubation.
12. First rescreen: Fill columns 1 and 12 of an appropriate number of 96-well plates with 20 μ L of minimal medium. Resuspend the cell pellets of the best mutants identified in the screen in the masterplate by pipetting up and down and stirring with a micropipet. Use 2 μ L of the suspensions to inoculate wells 1B-G and 12B-G of the rescreen plate, leaving one of those wells for the standard (*see Note 10*). Inoculate the standard well of all rescreen plates with the same resuspension of standard cell pellets from one of the master plates. Wrap plates with Parafilm and incubate them in the humidity shaker at 30°C and 260 rpm for 24 h at 85% humidity.
13. Fill columns 2–11 of the rescreen plates with 20 μ L of minimal medium per well. Using an 8-channel pipettor, resuspend the cultures in columns 1 and 12 and transfer 2 μ L into each of the adjacent 5 wells on the plate (*see Note 11*). Follow the screening procedure as described in **steps 3–11**.
14. Second rescreen: Resuspend the cell pellets of the best mutants identified in the rescreen in the rescreen-masterplate by pipetting up and down and stirring with a micropipette. Inoculate 3 mL of YPAD with chloramphenicol in round bottom culture tubes with an aliquot (150 μ L) of the cell suspensions and incubate at 30°C and 300 rpm for 24 h (*see Note 12*).

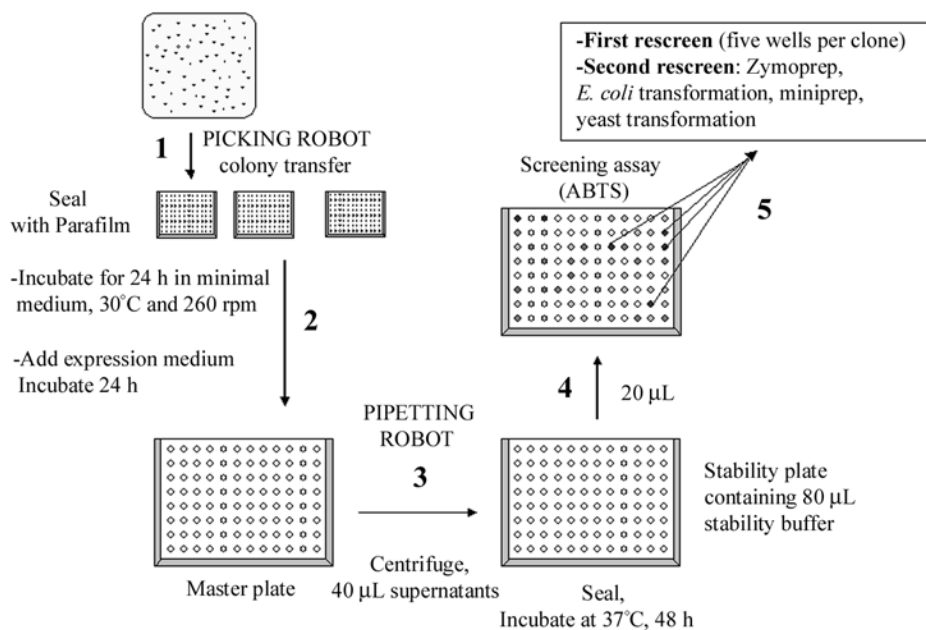


Fig. 1. Screening activity and stability of *Myceliophthora thermophila* laccase expressed in *S. cerevisiae*. (1) Thousands of colonies are transferred to 96-well plates containing 25 μ L of minimal medium. (2) After sealing, the plates are incubated for 24 h in order to allow the colonies to grow. Afterwards, 80 μ L of expression medium containing galactose (inductor) are added, and the plates are incubated for another 24 h. (3) Plates are centrifuged, and supernatants (containing the expressed laccase) are transferred to the stability plate. (4) Twenty microliters of mixture from the stability plate are transferred into the activity plate. After addition of 180 μ L of ABTS assay solution the activity/expression level can be assessed. Steps 3 and 4 are done using the pipetting robot. The stability plate is sealed, incubated for 48 h and assayed again (step 4). (5) The selected clones are confirmed with two consecutive rescreens. In the first, cells from the master plates are tested again, and in the second, freshly transformed cells are used. Rescreens are performed in quintuplicate.

15. Extract the plasmids from the 24 h culture (Zymoprep Yeast Plasmid Miniprep Kit) (see **Note 13**).
16. Transform competent *E. coli* cells with 5 μ L of the plasmid preparation. Plate the cells onto LB-ampicillin and incubate at 37°C for 14–16 h.
17. Inoculate 5 mL LB-ampicillin medium in culture tubes with single colonies and incubate them overnight at 37°C, 220 rpm.
18. Freeze samples of the overnight culture at –80°C in LB medium containing 20% glycerol. Extract the plasmids from the remaining overnight culture (QIAprep Spin Miniprep Kit).

19. Transform *S. cerevisiae* with plasmids from the selected mutants and also with the plasmid of the standard (Gietz Yeast Transformation Kit) (see **Note 14**).
20. Plate the transformed cells onto SC-drop out plates. Incubate the plates for 4 d at 30°C.
21. Fill an appropriate number of 96-well plates with 20 μ L of minimal medium per well. Pick five colonies from each transformation into five adjacent wells leaving columns 1 and 12 and rows A and H blank. On each plate cultivate up to 11 mutants and the standard.
22. Follow the screening procedure described in **steps 3–11**.
23. Plasmids from the best mutants of the second rescreen can be used to create mutant libraries for subsequent generations.

4. Notes

1. For further information about the construction of the *Myceliophthora thermophila* laccase libraries in *S. cerevisiae* as well as all the molecular biology tools used (cells, transformations, DNA purifications, etc.), see **Chapter 3** in the companion volume, “*Directed Evolution Library Creation*.” BJ5465 is deficient in proteases, which might be helpful when expressing less stable proteins (**21**). Yeast are more difficult to plate than *E. coli*. Glass beads are superior to spreaders in giving an even distribution of the cells, which is very important for the plates made for the picking robot. Chloramphenicol is added to plates and media to prevent contamination by bacteria. When observing standard rules of sterile work, chloramphenicol can be omitted.
2. The variability of the screen is significantly lower if the picking robot is used instead of toothpicks. The robot is especially useful for handling big libraries (more than 1000 clones). If the robot software does not allow the automatic incorporation of standard wells, one column of each 96-well plate is left empty (without minimal medium). When the inoculation with the mutants is complete each well of the empty column is filled with 25 μ L of minimal medium and all but one well are inoculated with standard using toothpicks. Inoculation efficiency is strongly dependent on the initial density of the culture. Yeast picking pins have to be used with the robot to transfer enough biomass. The culture volume should be as small as possible. 25 μ L drops of medium should be pipetted to one side of the wells. For the picking robot used here, the 96-well plate has to be defined as a deep-well plate to reach the appropriate inoculation depth. The offsets of the wells have to be defined so that the pins are inoculating not into the center of each well, but on one side of each well into the medium.
3. Parafilm is used to seal the gap between plate and lid. This prevents excessive evaporation of the medium, which would be uneven throughout the plate and increase the variability of the screen. The humidity shaker has the same purpose. The incubation time is essential to get a high and even cell density. The typical doubling time of haploid yeast strains in synthetic media is 140 min, and strains with even lower growth rate are often encountered. The incubation time might change depending on the features of the shaker (24–48 h).

4. The two growth phases allow the inoculation into a small volume, synchronize cell growth and restrict the growth-slowing expression of the laccase to the second growth phase. The expression uses the Gal10 gene coding for uridine diphosphoglucose-4-epimerase that is strictly regulated in *S. cerevisiae* (induced with galactose, repressed with glucose). The expression level of the laccase reaches a plateau in the late exponential phase, and is reduced when the stationary growth phase is reached. Therefore, the second incubation time should be close to 24 h in all screens.
5. The expression plasmid includes the native signal sequence of the laccase gene that directs expression into the secretory pathway of *S. cerevisiae*. Therefore, additional steps of cell lysis are not required, and all laccase expressed will be found in the supernatant after the centrifugation.
6. The good reproducibility and low variability of the screen reflect the use of the pipetting robot. We strongly encourage you to use it. The aspiration of the supernatant in the centrifuged master plates has to be done slowly, and the tips should be as far off the cell pellet as possible to avoid transfer of cells.
7. In our example, an incubation temperature of 37°C was sufficient to get mutants that were more stable over a broad range of temperatures (20–60°C). For the evolution of stability specifically at high temperatures, more stringent conditions (higher temperature, shorter incubation time) should be used.
8. The oxidation of ABTS indicates the activity/secretion level of laccase. The detection limit for this reaction is about 10 µg/L of laccase. The green reaction product (radical cation) is stable for several hours ($\epsilon = 36,000 \text{ M}^{-1}\text{cm}^{-1}$). If the activity of the enzyme and such the signal is very low, it is useful to subtract the absorption at 450 nm from that measured at 418 nm. The absorption peak of the [ABTS]⁺ species is very narrow. Thus subtraction of the absorption at 450 nm can significantly reduce the background. ABTS solution must be freshly prepared. For further information on laccase assays, see **Chapter 20**.
9. In our experience, it is not necessary to measure the cell density of the plates to include them in the calculation of the activities. Under the conditions described, cell growth variability is very low. The coefficient of variation of the screen is below 10%.
10. The outermost wells of the plate are not inoculated in the rescreens to further reduce variability produced by evaporation and uneven gas exchange. Those wells should still be filled with medium to even out the growth conditions in the inside wells.
11. The additional growth phase reduces variability by synchronizing the growth of the cultures and adjusting the cells to the change from expression medium to minimal medium.
12. As the first rescreen can contain false-positive mutants that, for example, contain more than one plasmid, a second rescreen with freshly transformed cells is carried out in order to get a correct comparison of the clones. In order to have enough of the cell suspensions, some wells belonging to every selected clone are pooled (except those in columns 1 and 12). Yeast grow faster if the ratio of surface area

to volume is high. Cultures of volume 3 mL should be grown in culture tubes of at least 18-mm diameter.

13. The plasmid preparations from yeast are impure and the concentration of the plasmid DNA is low. Therefore the yeast plasmid preparation has to be transformed into *E. coli* to obtain plasmid DNA of sufficient quality and quantity.
14. Since in this transformation only a few transformants have to be obtained the Quick and Easy protocol of the Gietz Yeast Transformation Kit can be used.

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Solid-Phase Screening Using Digital Image Analysis

Alexander V. Tobias and John M. Joern

1. Introduction

It is often possible to screen libraries of variant enzymes directly on agar plates. Colony-based solid-phase screening is an attractive option because of its relative ease and high throughput compared to liquid-phase screening in multi-well plates. As with any high throughput screening approach, a suitable colorimetric or fluorimetric assay must exist (or be developed) for the enzyme function in question. There are two major requirements that must be met for solid-phase screening to be feasible. First, the substrate must be able to be supplied as part of the growth medium, as a vapor, or within the cell itself. Second, the assay must be sufficiently sensitive that colonies expressing enzyme variants successfully evolved for the desired function will exhibit differences in color or fluorescence distinguishable from colonies expressing the wildtype/parental enzyme or unsuccessful mutants. Solid-phase assays have been successfully employed in many types of directed evolution experiments, including those involving carotenoid biosynthetic enzymes (*1–3*) and oxygenases (*4–6*).

It is often possible to identify colonies expressing successfully evolved mutant enzymes with the naked eye under proper illumination conditions. For this reason, digital image analysis is typically not necessary to identify clones of interest. Rather, digital image analysis should be regarded as a tool that permits the experimenter to 1) accurately count the number of colonies screened, 2) quantify and rank the performance of all clones in the assay by objectively assigning each colony a set of numerical color and/or intensity values, and 3) compile statistics on the performance of the entire library in the assay.

Solid-phase screening using digital image analysis begins with preparation of colonies for image acquisition. Images of prepared colonies are then acquired

using a CCD camera (for assays based on color, fluorescence or chemiluminescence) or an ordinary flatbed computer scanner (for colorimetric screening only). Finally, acquired images are processed and analyzed using image analysis software.

2. Materials

2.1. Preparation of Colonies for Solid-Phase Screening

1. Agar plates containing colonies to be imaged (*see Note 1*).
2. Two sets of small tweezers (~8–10 cm in length).
3. 70% ethanol in water in a glass dish with a removable glass lid.
4. Bunsen burner.
5. White nitrocellulose filter membranes (*see Note 2*).

2.2. Image Acquisition Using a CCD Camera

1. CCD Imaging system with appropriate light source (*see Notes 3–5*).
2. Appropriate filter(s) (e.g., from Omega Optical, Inc.; Brattleboro, VT) (*see Notes 3–5*).
3. Computer with SCSI interface.
4. Imaging software (normally supplied with imaging system).

2.3. Image Acquisition Using a Flatbed Scanner

1. Petri plates containing colonies prepared for image acquisition.
2. 100–200 sheets white copier paper, 8.5 × 11" size.
3. Color flatbed scanner connected to a personal computer (*see Note 6*).

2.4. Processing and Analysis of Digital Images

1. Image analysis software (e.g., Sigma Scan Pro from SPSS Inc., Quantity One from Bio-Rad Laboratories or Optimas from Media Cybernetics Inc.).
2. Graphics software (e.g., Adobe Photoshop or equivalent) (*see Note 7*).
3. Spreadsheet software (e.g., Microsoft Excel) (*see Note 8*).

3. Methods

3.1. Preparation of Colonies for Solid-Phase Screening

1. Dip the tips of the two sets of tweezers in 70% ethanol and flame them with a Bunsen burner to sterilize them.
2. Using the tweezers, carefully place a nitrocellulose membrane on top of the colonies on each plate. Use one set of tweezers in each hand for better accuracy in placing the membrane correctly.
3. Place the Petri plate colony face down and wait for the membrane filter to fully adhere to the agar surface. It may be necessary to use a poking motion with the tweezers to eliminate spots where a small gap remains between the filter and the agar surface.

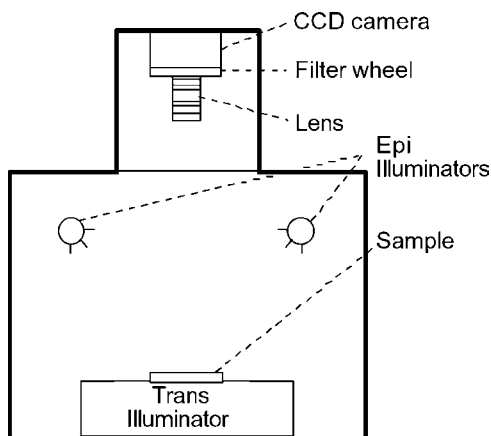


Fig. 1. Schematic section of Fluor-S Multiimager. UV and white light sources are positioned above (epi) and below (trans) the sample. Filters are attached either to the lens or between the lens and the CCD camera. Some systems have a filter wheel assembly that allows the user to select a filter through the computer interface.

4. Place the Petri plate colony face up. Using one or both sets of tweezers, lift the membrane filter slowly from the agar surface. Flip and then replace the filter back onto the agar surface such that the colonies adhering to the filter now face upwards. Again, use a gentle poking motion with the tweezers to eliminate gaps, taking care not to touch any colonies with the tweezers (*see Note 9*).
5. Store the plate colony face down.

3.2. Image Acquisition Using a CCD Camera

CCD-based imaging systems are available with both white and UV light sources and thus are useful for colorimetric, fluorometric and chemiluminescence applications. Though images appear on the screen in black-and-white, filters can be used to select a range of wavelengths for analysis. Imagers equipped with CCD cameras provide exceptional resolution, sensitivity, and reproducibility. Our experience with CCD imaging is based on the Fluor-S Multiimager from Bio-Rad Laboratories (*see Fig. 1*).

1. Configure imager with appropriate light source, filter, and lens for colorimetric (*see Note 3*), fluorometric (*see Note 4*), or chemiluminescent (*see Note 5*) imaging.
2. Position the Petri plate (*see Note 10*).
3. Using the exposure time parameter in the computer interface and the aperture ring (or f-stop) on the lens, adjust the exposure such that 1) the features are distinguishable from the background and 2) none of the pixels is saturated (*see Note 11*).
4. When the exposure time has been optimized, acquire and save a final image (*see Note 12*).

3.3. Image Acquisition Using a Flatbed Scanner

Use of a standard flatbed scanner is a simple and very inexpensive way to acquire images of colonies. This method is limited to colorimetric screens only, as wavelength filters cannot be used with a flatbed scanner. Flatbed scanners provide more than adequate image resolution for screening purposes. Often, the user can save time by imaging multiple plates at once on a flatbed scanner.

1. Place plates colony-face down onto the scanning surface. Any number of plates may be scanned at once up to the maximum number of plates that can fit on the scanning surface.
2. Place sheets of white copier paper on top of the face-down plates. This is done to help with scan quality and to give a whiter background. Experiment with different amounts and configurations of paper to find a setup that gives the best scanning results.
3. Close the scanner cover and run a preview scan. Select the plates with the selector tool on the preview screen.
4. Set the image resolution to at least 150 pixels per inch (*see Note 13*). Adjust the exposure settings on the scanner software (*see Note 14*) until there is good contrast between the colonies and the nitrocellulose filter membrane, the filter membrane appears relatively white, and the appearance of the plate on the screen closely matches its appearance under well-lit conditions. Zoom in on one plate for this step. When satisfactory settings have been found, write them down and use them for all future image acquisitions.
5. Scan the plates and save the image as a .TIFF file with a descriptive filename.

3.4. Processing and Analysis of Digital Images

1. Open a .TIFF file containing images of scanned plates. If desired, use the Text tool to write annotations beside each plate in the image (*see Note 15*).
2. Select the colonies of one plate using either a circular or rectangular selection tool, as appropriate. Ensure that the edge of the Petri dish does not appear in the selection.
3. Copy the selection and paste it into a new image having a transparent background. Save the new “single plate” image as a .TIFF file with a descriptive filename.
4. Split the image into color channels if appropriate for the type of measurement desired (*see Note 16*).
5. Filter image using image analysis software (*see Note 17*).
6. Use the “intensity threshold” function to select the colonies from the background.
7. Perform the desired measurements (i.e., intensity, size, circularity) on the colonies (*see Note 18*).
8. Export data to spreadsheet or database software.
9. Analyze data (*see Note 19*).

4. Notes

1. Colonies should be imaged as soon as they are ready so that they are as small as possible (0.5–1 mm diameter is a good size). This will improve the measure-

ments and enable better resolution of neighboring colonies. One should maximize the number of colonies on each plate up to the point where distinguishing neighboring colonies becomes problematic for the image analysis software. In our experience, this point is around 10–20 0.5-mm diameter colonies per cm², corresponding to 750–1500 colonies on a 100-mm diameter Petri dish. However, any size Petri plate, including square ones, that fits on the flatbed scanning surface should scan well. The agar layer should be thin (~5 mm recommended). A thick agar layer will cause the filter membrane to appear darker in the image when scanned, as the membrane is not perfectly opaque.

2. These are available in various sizes for use with different sizes of Petri dishes. For 100-mm diameter Petri dishes, we recommend using 82-mm diameter nitrocellulose filter membranes from Pall (Port Washington, NY).
3. Colorimetric imaging: The commercial CCD-based imaging systems we are aware of only produce black-and-white images. However, when a bandpass filter centered at the wavelength of interest is used, these systems are analogous to a single-wavelength spectrophotometer. When colony color is being assayed, an epi white light source (e.g., 400–750 nm) is used since trans-illumination will be nearly completely blocked by the colony itself (or, in some cases, the membrane supporting the colonies). Because the epi light source must be reflected from the colonies, a contrast defect usually occurs (the center of the image is brighter than the outside) (5) that must be processed away prior to quantitation (*see* **Note 17**).
4. Fluorescence imaging: In contrast to fluorescence microtiter plate readers that allow the user to tune the excitation and emission wavelengths, imaging systems excite the sample with broad-range epi UV light sources (e.g., 290–365 nm for the Fluor-S system from Bio-Rad Laboratories). The emission wavelength recorded by the camera can be tuned using a bandpass filter centered at the desired emission wavelength. An infrared cutoff filter (e.g., 660 nm) should be installed to block infrared signal from the UV bulbs.
5. Chemiluminescence imaging: Digital imaging of chemiluminescence has found use in blotting applications (7) as well as limited use in enzyme screening (8). For this application, long exposure times (>10 min) are sometimes required, and we recommend a high numerical aperture lens for faster light collection. Generally no filter is required.
6. The computer should have ≥10 gigabytes of hard disk space and a large color monitor (≥17"). Many image analysis software packages are only available for the Windows platform. Therefore, a computer running Windows is recommended. All commercial flatbed scanners come with scanning software.
7. The graphics software may be required if the image analysis software lacks features necessary for some applications such as a circular select tool, an annotation tool, or a color channel split function. Instructions in this section are written with the understanding that the user will execute them using either the image analysis software or the graphics software, as appropriate for the particular situation.

8. Database software (e.g., Microsoft Access) is optional in addition to the spreadsheet software. It is usually easier and more convenient to store and manipulate the large volumes of data generated by image analysis in a database.
9. The filters are sufficiently porous to allow transport of nutrients from the agar to the colonies. Thus, colonies will continue to grow on top of the membrane filter as long as there is no air gap between the filter and the agar surface.
10. When many Petri plates are to be imaged in the same way, imaging efficiency and reproducibility can be increased by taping a Petri dish lid to the sample stage as a rack for the dish to be imaged.
11. As more light from a certain area is collected by the CCD camera, the response to additional light slows and the pixels from this area are said to saturate. In general, the exposure is limited by saturation on the one hand, and sensitivity to the features of interest on the other. For convenience, the aperture should be adjusted such that images can be acquired in 1–10 s.
12. The image file should be saved in an uncompressed format appropriate for subsequent analysis (the “.TIFF” format is compatible with all image analysis packages and is recommended). The Fluor-S system from Bio-Rad Laboratories provides images of 1340×1040 pixels that can be saved at lower resolution if desired. Depending on the lens used, this corresponds to a resolution of 50–320 pixels/cm. As a general rule, each colony should have a diameter of at least 10 pixels.
13. A good rule of thumb for resolution is that the final uncompressed file size of the image should be 2–10 megabytes per 100-mm diameter plate in the image.
14. Different scanner software packages have different types of exposure settings. Some packages allow the user to adjust the brightness and contrast of the scanned image, while others have adjustable highlights, shadows, midtones, and color contrast settings.
15. Note that the scanned image that appears on the screen is from the perspective of an observer looking up at the plates. Therefore, the left-right arrangement of plates placed face down on the scanning surface (viewed from above) will be opposite to the left-right arrangement of the plates on the screen. After annotating the plates, it may be necessary to use a “flatten image” function in order to save the annotated image as a .TIFF file.
16. For example, if yellow intensity of the colonies is to be quantified, one should split a color image into Cyan, Magenta, and Yellow channels and perform subsequent steps on the Yellow channel (which will appear as a monochrome image). One should split color images into channels whenever it is possible to perform the desired measurement this way, as the subsequent thresholding step is easier to execute on an individual color channel than on a full color image.
17. Appropriate digital filtering using the image analysis software can sometimes improve image quantitation. Two types of image defects generally occur: salt-and-pepper noise and contrast defects. Salt-and-pepper noise is diagnosed by zooming in on the image and observing pixels with markedly different intensities than their neighbors. In most cases, this defect can easily be filtered away using the 3×3 or 5×5 pixel averaging filter function included with most image analy-

sis software. Contrast defects are characterized by readily observable differences in brightness across the image. Images acquired using a CCD camera with epi illumination often have contrast defects. In this case, the image should be processed before feature quantitation with a large kernel filter such as a Wallis filter, available with the Optimas software package. Applying a small kernel averaging filter (e.g., 3×3 pixels) before Wallis filtering can improve the results.

18. Some image analysis packages allow determination of both the colony average and the colony maximum or minimum of a particular measurement type. In this case, it is recommended that the user determine both and decide later which measurement to use for data analysis. Other useful statistics such as the colony area, circularity, major and minor axis lengths, as well as coordinates for each colony's position can be determined in many image analysis software packages. Usually, the software assigns a number to each colony so that each row of data, which corresponds to one colony, can be visually linked to the correct colony by the user.
19. At this stage, one may filter the data to exclude entries outside a certain range of size or circularity. This can help eliminate spurious measurements such as those resulting from image irregularities being mistaken for colonies. In addition, one can use circularity measurements to remove clumps of colonies (i.e., colonies that have grown into each other) from the analysis.

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Screening for Thermostability

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1. Introduction

Enzyme thermostability is a property of great importance in the era of designed biocatalysts. While enzymes are capable of catalyzing reactions with exquisite specificity and selectivity, they are often limited by insufficient stability. Improvements in enzyme activity through protein engineering often come at the cost of reduced stability. This is likely a result of both natural drift and a tradeoff that often exists between activity and stability for many single residue substitutions (**1**). However, as exemplified by thermophilic organisms (**2,3**) and demonstrated by laboratory evolution (**4-6**), it is often possible to improve enzyme stability without sacrificing activity. Thus, enzyme thermostability is an attractive optimization target in bioengineering.

To select enzymes for thermoactivity and thermostability, measuring activities at elevated temperatures might be an effective screening method. However, most high-throughput microplate readers can only reach temperatures of about 50°C, and high temperatures can cause evaporation, pH changes, and/or substrate instability. A more convenient experimental measure of enzyme thermostability is the residual catalytic activity measured at room temperature after incubation at an elevated temperature. Higher residual activity correlates with improved stability, as demonstrated by Miyazaki et al. (**4**). They generated mutant subtilisin S41 libraries, screened for high residual activity, and identified a subtilisin mutant with an increased half-life at 60°C and higher structural stability, as measured by circular dichroism.

Another important property of enzymes in biotechnology that often coincides with increased thermostability is an increase of the temperature optimum for activity (T_{opt}), which is the result of a tradeoff between the rate constant increase with temperature (the Arrhenius effect), and thermal denaturation. The

subtilisin S41 mutant described above also showed an increased T_{opt} (4). Similarly, the temperature optima of subtilisin E (6) and an esterase (7) were increased by directed evolution approaches.

Screening for thermostability requires a suitable screen for function. Many examples are described in other chapters of this volume. In this section, we describe an assay for thermostability in which enzyme activity is measured using a 96-well plate absorbance or fluorescence reader. It is applicable to systems in which the enzyme is soluble and present in a solution of cell lysate or culture broth (if secreted). Several examples of successful applications of this procedure have been reported (4,6-9). Because the thermostability screen described here compares residual activity to initial activity, it is insensitive to variations in enzyme concentration and the total activity of a sample (e.g., variations in expression or secretion level). However, the initial activity measurement should accurately reflect specific activity to prevent selection of mutants with improved stability but greatly reduced activity.

In some cases, denaturation is reversible, and heat-treated enzymes can regain activity over time when cooled. If this is the case, time becomes an important factor in screening for residual activity, and the screen is likely to lose sensitivity and perhaps reproducibility. For proteins that unfold reversibly, a better screening approach might be to perform an end-point assay at an elevated temperature, so that refolding is not allowed (*see Chapter 12* for an example).

2. Materials

Most of the materials required for screening thermostability will depend on the specific enzyme activity. The materials listed here are commonly required for screening for thermostability.

2.1. Biological and Chemical Materials

1. 96-well clear microtiter plates, flat bottom, with lid (Rainin, Emeryville, CA).
2. 96-well plates with 1 mL or 2 mL volume deep wells (Eppendorf, Hamburg, Germany, or Becton Dickinson Labware, Franklin Lakes, NJ).
3. 96-well PCR plates with V-shaped wells (HardShell 96, MJ Research, Waltham, MA).
4. Rubber sealing mats for sealing top of 96-well PCR plates (Microseal 'M', MJ Research).
5. Media (e.g., Luria Broth, Terrific Broth, sterilized by autoclaving).
6. Additional nutritional supplements for protein expression, as needed.
7. Antibiotics.
8. Cell lysis reagents (e.g., lysozyme + DNase I (*see Note 1*) or BugBuster Protein Extraction Reagent, Novagen, Madison, WI).
9. Toothpicks (sterilized by autoclaving).
10. Agar plates containing colonies from a transformed mutant library.

2.2. Equipment

1. Absorbance or fluorescence microplate reader with computer control (Spectramax, Molecular Devices, Sunnyvale, CA). Also, the corresponding software (Softmax PRO, Molecular Devices).
2. 96-channel pipetting robot with P-200 head (Multimek 96, Beckman Coulter, Fullerton, CA).
3. Multi-channel, hand-held pipette (electronic control, capable of accurate multiple deliveries in the μL range) (Biohit, Helsinki, Finland).
4. Plate shaker with humidification and temperature control (ISF-1-W, Kühner, Birsfelden, Switzerland) (*see Note 2*).
5. Thermocycler with heated lid, temperature gradient, and 96-well PCR block (PTC200, MJ Research) (*see Note 3*).
6. Centrifuge with rotor designed to spin 96-well plates (Allegra 25R, Beckman Coulter).
7. 96-pin plate replicator (V&P Scientific, San Diego, CA).

3. Methods

Important variables to consider when optimizing a screening assay include growth conditions, lysis procedure, concentrations and volumes of components used in the assay (e.g., enzyme, buffer, substrate), and the parameter(s) used to identify enzyme function (e.g., kinetics or end-point). As described below (**Subheading 3.2.**), the enzyme can be prepared as a cell lysate, or it can be used directly from the culture broth if it is secreted during growth. The methods below outline (**Subheading 3.1.**) determination of suitable thermostability screening conditions, (**Subheading 3.2.**) preparation of libraries for screening, and (**Subheading 3.3.**) screening for thermostability. Finally, **Subheading 3.4.** describes a protocol used in the application of this assay for screening a mutant library of cytochrome P450 BM-3 expressed in *E. coli*, and **Subheading 3.5.** describes thermostability screening of a mutant library of horseradish peroxidase (HRP) expressed in *E. coli*.

3.1. Determining Screening Conditions

An amount of enzyme should be used such that improvements in the initial activity at room temperature (before heat incubation) compared to the parent enzyme can be detected. When an end-point absorbance measurement is used to measure activity, an amount of enzyme should be used so that the end-point reading from parent enzyme activity has an absorbance in the range of ~ 0.3 – 0.4 . The enzyme heat treatment should be chosen so that the residual activity using the same amount of parent enzyme is about one-third of the initial activity (resulting in an absorbance of ~ 0.1 in a plate reader). With these conditions, it will be easy to identify mutants with improvements of twofold or more in either activity or stability.

To determine appropriate screening conditions:

1. Grow an entire 96-well plate of parent enzyme cultures. Follow the procedure described below for preparing libraries for screening (**Subheading 3.2.1.**), except pick colonies from a fresh transformation of the parent gene instead of from a mutant library.
2. Following expression, prepare the cell lysates as described in **Subheading 3.2.2.** Determine a suitable volume of lysate to use for screening activity, and use a pipetting robot to transfer this amount into a microplate (*see Note 4*).
3. Perform the initial activity assay for the entire parent plate (for examples of such procedures, *see Subheadings 3.5. and 3.6.*) (*see Note 5*).
4. Using a pipetting robot, transfer 50-100 μL of lysate into a PCR plate with V-shaped wells.
5. Place a rubber sealing mat over the PCR plate and insert the plate into a 96-well PCR block.
6. Program the thermocycler for heat treatment of the sample. Follow the heating period with rapid cooling to 4°C. Use a heated lid to prevent condensation that will result in variations in volume and enzyme concentration. Test various temperature and time incubation conditions to determine a suitable thermal inactivation protocol (*see Note 6*).
7. Use a pipetting robot to transfer the same volume of heat-treated lysate as was used for the initial activity measurement into a new assay plate. Use this plate to measure residual activity. Thermostability can then be estimated from the ratio of residual activity to initial activity. Choose a heat treatment that results in a residual activity of ~30% of the initial activity.

3.2. Preparation of Libraries for Screening

3.2.1. Protein Expression

1. Using sterile toothpicks, pick colonies from your transformed mutant library into 96-well master culture plates. When using an *E. coli* expression system, the master plate is typically prepared with LB media (e.g., 300 μL per well) (*see Note 7*). Place a lid over each plate.
2. Allow the master plate cultures to grow to saturation in a humidified plate shaker. Using a 96-pin plate replicator, use these cultures to inoculate another set of plates used for expression of the mutant library. TB medium enriched with additional nutrients is often used for *E. coli* expression systems (*see Note 8*).
3. Store the master plates at 4°C so you can recover those mutants with improved properties.
4. Grow the new set of cultures and induce for protein expression. After expression, pellet the cells by centrifugation using a rotor equipped with buckets for spinning plates. Be sure to follow the specifications for your centrifuge and plates to avoid cracking the plates. Spinning for 10 min at 2200g is sufficient for pelleting 500 μL *E. coli* cultures.

3.2.2. Cell Lysis/Protein Extraction

If your enzyme is secreted at appreciable and consistent levels, you can use the media directly for measuring activity and thermostability. If the enzyme is not secreted the cells should be lysed. Lysis can be accomplished in a number of ways. Detergents such as BugBuster Protein Extraction Reagent (Novagen), which releases soluble proteins by disrupting the *E. coli* cell wall, are available. The BugBuster protocol provided by Novagen can be applied to microliter- and milliliter-scale cultures. If such detergents are harmful to the assay system, lysozyme also works well. Following is a protocol for using lysozyme to lyse cell pellets from 96-well plate (deep well) cultures:

1. Freeze the pelleted cells at -20°C (see **Note 9**). Then resuspend the cells in a suitable buffer (e.g., buffer used in the screen) (see **Note 10**). Resuspension is best accomplished using a 96-channel pipetting robot.
2. After resuspension, add an additional volume (e.g., 100–300 μL) of buffer containing lysozyme plus DNase I (see **Note 1**) so that the final concentration of lysozyme in the lysate is ~ 0.5 mg/mL and the final concentration of DNase I is ~ 1.5 Units/mL (see **Note 13**). Use the robot to mix the lysates. Lysis is complete within 15 min.
3. After lysis, centrifuge the plates (2200g, 15 min) to pellet the cell debris and clarify the lysate.
4. Assay the lysates as soon as possible after lysing, to avoid enzyme inactivation.

3.3. Thermostability Assay: General Protocol

1. Prepare 96-well assay plates for the addition of enzyme lysate. Two assay plates must be prepared for each plate of lysates to be screened (one for initial activity and one for residual activity). It is useful to use multi-channel pipets or a pipetting robot for preparing the plates. For example, as described in **Subheading 3.4.**, 96-well plates are prepared by adding buffer and dimethyl sulfoxide (DMSO) containing substrate to each well.
2. Using a pipetting robot, transfer an appropriate volume of lysates to an assay plate (e.g., transfer 30 μL from each well of a lysate plate to the wells of an assay plate containing 120 μL of substrate solution). Use the robot to mix the contents of the assay plate. This is the initial activity plate.
3. Also, use the robot to transfer a larger volume (e.g., 50 μL) of the lysate into the PCR plate with V-shaped wells. Place a rubber sealing lid over the PCR plate and insert the plate into a 96-well thermocycler block.
4. Program the thermocycler for heat treatment of the sample. Follow the heating period with rapid cooling to 4°C . Use a heated lid.
5. While the plate is being heated, assay the initial activity plate at room temperature.
6. After the heated samples have been cooled, use the robot to transfer lysate from the PCR plate to the second assay plate. Use the same volume of lysate as was used for measuring initial activity (see **Note 12**).

7. Measure the residual activity of the heat-treated lysate at room temperature. Mutants with improved thermostability will have higher fractions of their initial activity retained after the heat inactivation (see **Note 13**).

3.4. Screening Thermostability of Cytochrome P450 BM-3

This specific example illustrates a thermostability screen based on the activity assay outlined in **Chapter 15**.

1. Ligate a cytochrome P450 BM-3 mutant library into the pCWOri+ (**10**) expression vector.
2. Pick colonies from the transformed library into 96-deep-well plates containing 300 μL of LB + ampicillin per well. Include on each plate six wells containing the parent enzyme. Place lids over the plates and grow for 24 h in a humidified plate shaker (30°C, 250 rpm).
3. Use 20 μL of each LB culture to inoculate new deep-well plate cultures containing 400 μL of TB supplemented with ampicillin (100 $\mu\text{g/mL}$), thiamine (1 mM), δ -aminolevulinic acid (1 mM), and trace elements (**11**). Store the LB culture plates in the refrigerator. Place lids over the TB culture plates and grow in a shaker (30°C, 250 rpm) for 3–4 h (allow the cultures to reach an OD_{600} of 0.6–0.8), induce the cultures with 0.5 mM IPTG, and continue shaking for 18 h.
4. After protein expression, centrifuge the TB culture plates (2200g, 10 min) and pour off the supernatant. Freeze the pelleted culture plates.
5. Thaw the pelleted cells and resuspend each in 300 μL of Tris-HCl buffer (100 mM, pH 8.2). Use a pipetting robot to resuspend the pellets.
6. Lyse the cells by adding 200 μL of a lysozyme solution (1.3 mg/mL lysozyme + 4 U/mL DNase I in Tris-HCl buffer) to each well. Mix with a pipetting robot for 5 min.
7. Centrifuge the lysate (2200g, 15 min).
8. Prepare microplates for the assay by adding 120 μL of Tris-HCl buffer + 10 μL of DMSO containing 4 mM substrate (12-*para*-nitrophenoxycarboxylic acid [**12**]) to each well.
9. Use the pipetting robot to transfer 20 μL of the soluble lysate to a microplate and 50 μL of lysate to a PCR plate.
10. Assay initial activity:
 - a. Initiate reactions in the microplate by adding 10 μL of 80 mM hydrogen peroxide (H_2O_2) or 10 μL of 3.2 mM NADPH to each well;
 - b. Measure the rate of *para*-nitrophenolate formation by monitoring absorbance at 398 nm in a plate absorbance reader.
11. Place the rubber sealing mat onto the PCR plate and use a thermocycler to heat these lysates to 57°C for 10 min (use a heated lid), followed by cooling to 4°C.
12. Transfer 20 μL of the heat-treated lysate to another assay microplate and repeat the activity assay measurement.
13. Choose the best mutants based on their thermostability and total initial activity (see **Note 13**).

14. Recover the improved mutant's plasmid DNA and use to generate a new library of mutants.

3.5. Screening Thermostability of Horseradish Peroxidase (HRP)

This specific example illustrates a thermostability screen based on the activity assay outlined in **Chapter 17** for HRP.

1. Ligate an HRP mutant library into the pCWori+ expression vector (**10**).
2. Pick mutant colonies into 96-well plates containing 200 μL of LB media plus ampicillin (100 $\mu\text{g}/\text{mL}$) per well. Place lids over the plates and grow for 12–14 h at 37°C in a humidity-controlled shaker at 250 rpm. Check cell density by spectrophotometer, and stop growth when OD_{600} reaches the saturation plateau. These are the master plates and can be stored at -80°C in 20% glycerol for months.
3. Using a 96-pin replicator on the master plates, inoculate fresh 96-well plates containing 200 μL of LB medium, ampicillin (100 $\mu\text{g}/\text{mL}$), δ -aminolevulinic acid (1 mM), CaCl_2 (1 mM) and trace elements (**11**). These are replica plates used for protein production.
4. Grow the replica plates at 37°C for 10–12 h, until the OD_{600} reaches ~ 0.6 . Transfer the plates to 30°C and add 1 mM IPTG to induce protein expression.
5. After expression (12–24 h, depending on the activity level desired), harvest the cells and extract the protein using BugBuster reagent. Follow the Novagen protocol (*see* **Note 14**). Before removing insoluble cell debris by centrifugation, add 100–200 μL of assay buffer (50 mM sodium acetate, pH 4.5).
6. Transfer 40 μL of the soluble protein lysate to a 96-well plate containing 100 μL of assay buffer (50 mM sodium acetate, pH 4.5). This plate will be used for the initial activity assay. Also transfer 60 μL of the soluble protein lysate to a PCR plate.
7. For the initial activity assay, add 50 μL TMB substrate (KPL) and place the plate in the microplate reader. After initiating the reaction by addition of 50 μL of H_2O_2 substrate (KPL), read the absorbance of the blue product at 650 nm (*see* (**13**) for more information on this assay).
8. Incubate the PCR plate at 62°C for 10 min followed by cooling to 4°C. Transfer 40 μL of the heat-treated enzyme sample to a fresh 96-well assay plate as described in **step 5** above.
9. Repeat the activity assay from **step 6** with the heat-treated samples. Calculate residual activity as the fraction of initial activity.
10. Select mutants based on improvements in thermostability and/or initial activity. These mutants can be re-grown from the master plates stored at -80°C (*see* **Note 13**).

4. Notes

1. We recommend using lysozyme purified by repeat crystallization and dialysis (e.g., Sigma cat. no. L6876) and DNase I purified by chromatography (e.g., Sigma cat. no. D4263) to avoid proteases and other contaminants.

2. A humidified shaker is preferred for growing cultures to prevent evaporation. Cultures in wells closest to the edge of each plate evaporate faster than the inner-well cultures, causing variations in enzyme concentration across each plate and lowered sensitivity of the screen.
3. It is also possible to perform the incubation steps in an oven. In this case, a pre-heated metal (aluminum) case should be used to contain the incubating 96-well plate for more accurate temperature control.
4. If screening is performed with standard 96-well assay plates, the final assay volume must be less than ~250 μL . However, the PCR plate that is used to heat the lysates has a 100 μL capacity, so the volume of lysate used in the assay should be less than 100 μL , and preferably less than 50 μL .
5. Make sure parent enzyme activity is consistent across the plate. The standard deviation in activity across the plate should be no more than ~10–15%.
6. Typically, 10–20 min is a good incubation time. For testing different temperatures, use the thermocycler's programmable gradient feature if available; otherwise try different temperatures in separate experiments.
7. Include the parent enzyme (i.e., the enzyme whose DNA sequence was used to generate the mutant library) in several wells of each 96-well plate for direct comparison with the mutants, to avoid difficulties with variations from plate to plate.
8. Depending on the expression level and the sensitivity of your screen, you may need to use deep-well plates (1 mL or 2 mL total volume) for increased culture volumes (up to 1 mL).
9. Freezing the cell pellet first greatly enhances lysis efficiency.
10. The volume used for resuspension depends on expression level and the amount of lysate needed for screening. For a 500 μL culture volume, the resuspension volume will probably be between 200–800 μL .
11. Note that DNase I requires divalent metal cations for activity. However, the low levels of divalent metals present in the lysate are apparently sufficient to activate the DNase I. Additional MgCl_2 can be used in the lysis reaction, but this is not necessary and may cause complications in the screen.
12. If precipitation of protein occurs as a result of the heat treatment, we recommend clarifying the lysate by centrifuging the PCR plate before assaying residual activity. This is particularly important if the volume of (cloudy) lysate used in the assay is significant enough to influence the absorbance reading. To centrifuge a PCR plate, place the plate into a 96-well microplate and centrifuge the microplate containing the PCR plate.
13. Rescreening is a crucial step in this procedure. Any potentially improved mutants should be grown in larger cultures (e.g., 10 mL) and rescreened to verify improvements.
14. Resuspension is problematic in the BugBuster protocol, since repeated pipetting of this detergent-based reagent easily creates air bubbles, thereby hindering the colorimetric screen. For 96-well plate cultures, repeated pipetting is not recommended. Resuspension can be achieved instead by stirring the cell extract with plastic needles or metal tips from a plate replicator.

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High-Throughput Screening of Mutant α -Amylase Libraries for Increased Activity at 129°C

Holger Berk and Robert J. Lebbink

1. Introduction

We describe a high throughput screening setup for measuring α -amylase activity at temperatures far above the boiling point of water. The system consists of a sealed aluminum 384-well assay plate incubated between two pre-heated aluminum blocks. Samples consisting of starch solution and α -amylase contained in the supernatants of *E. coli* cultures are rapidly and uniformly heated up to 125°C in less than 30 s. The final temperature of 129°C is reached in less than 1 min. After the high temperature incubation, the α -amylase activity is determined by measuring the concentration of released sugar moieties using *p*-hydroxy benzoic acid hydrazide. Several thousand clones from mutant libraries generated by error prone PCR techniques can be screened within a week. The described setup could be readily adapted to screen libraries of other enzymatic systems for increased thermoactivity, or to evaluate other properties of enzymes at extreme thermal conditions.

Characterization of enzymatic reactions above 100°C presents several difficulties: 1) at temperatures above 100°C assay solutions are boiling and the aqueous phase will evaporate unless the assay mixture is kept in a pressure-proof compartment, 2) the substrates for the enzymatic reactions as well as the formed products have to be stable at the elevated temperature, and 3) photometrically monitored colorimetric kinetic assays are hard to set up because of requirements for specialized optically-transparent equipment. Furthermore, the chromogenic substrates have to be heat stable and resistant to thermal quenching of the signal. To address these problems, reactors have been developed to allow the examination of enzymatic reactions at high temperature and pressure. Unfortunately, they involve expensive and custom manufactured hardware setups and are designed

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for single measurements (1–3). One effective system described by Daniel and Danson (4) can be used for activity measurements at elevated temperatures without extensive preparations. It makes use of sealed glass capillaries filled with assay mixtures immersed in a preheated oil or glycerol bath. This system is used as part of our screening procedure when only a few samples have to be evaluated, especially for re-screening of putatively positive clones.

The high temperature assay system described here allows handling hundreds of samples at once. A reversibly sealable aluminum assay plate, in standard 384-well dimensions, is placed between two preheated aluminum blocks, and is used for incubations of enzyme and substrate solutions at temperatures up to 140°C. Starch is used as substrate for heterologously produced α -amylase from the hyperthermophilic archaeon *Pyrococcus furiosus* (5,6). The oligosaccharide reaction products, as well as the substrate starch, are extremely stable, even at temperatures well above 140°C. With respect to heat transfer, the system described here is superior to other methods like peltier elements in PCR-blocks, which require >1 min to heat up from ambient temperature to 100°C. In contrast, the aluminum block system reaches 100°C in less than 10 s and 129°C in less than 1 min.

After high temperature incubation, the reducing oligosaccharide moieties released by the hydrolytic activity of the α -amylase are quantified in high throughput by using *p*-hydroxy benzoic acid hydrazide (7).

2. Materials

1. *Escherichia coli* BL21-Gold(DE3) competent cells (Stratagene, La Jolla, CA).
2. pET21b(+) (Novagen, Madison, WI) derived plasmid library carrying mutant, wild-type or truncated pyrococcal α -amylase genes.
3. SOC medium.
4. Luria-Bertani (LB) medium.
5. Ampicillin (used for both liquid and agar cultivation at a concentration of 200 μ g/mL).
6. 96-well flat-bottom microtiter plates (Rainin, Emeryville, CA) with a 3-mm glass ball in each well.
7. 20 $\times$ 20 cm 'QTray' (Genetix, New Milton, UK).
8. Colony picking equipment (sterile toothpicks or colony picker 'QPix', Genetix).
9. Moisturized styrofoam boxes or equivalent setup.
10. 96-pin replicator tool (VWR, West Chester, PA).
11. Centrifuge unit for microtiter plates.
12. Liquid handling equipment (multi channel pipettors and/or 96 channel pipetting robot 'Multimek', Beckman, Fullerton, CA).
13. Starch (Spectrum Scientific Products, Gardena, CA; the starch from this source performed best with respect to solubility at a concentration of 1% in 100 mM sodium acetate, pH 5.5).

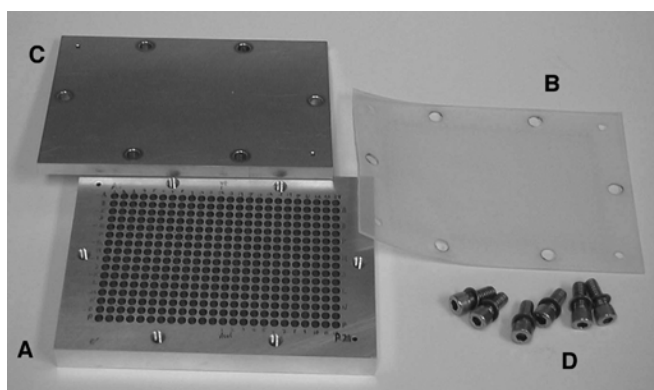


Fig. 1. High temperature aluminum assay plate in 384-well format (A) for incubation of culture supernatants containing α -amylase with starch solution at 129°C. The wells are sealed pressure proof by a silicone mat (B) that is tightly fixed by means of a lid plate (C) and screws (D).

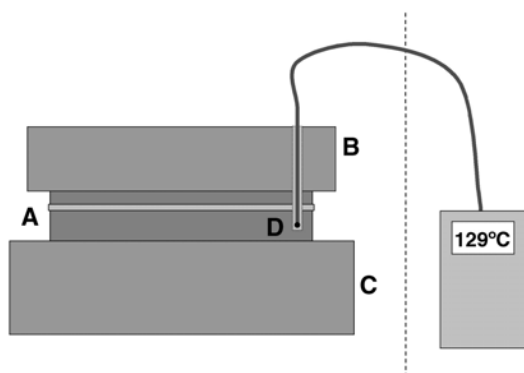


Fig. 2. Setup for rapid heating of the 384-well assay block to 129°C. The sealed assay plate (A) is sandwiched between a top (B) and bottom (C) heat transfer block that have been preheated in a temperature-controlled oven. A temperature probe (D) is inserted through an opening that penetrates the top block into a glycerol-filled hole in the assay plate. Thus, the temperature can be monitored continuously during incubation. The dashed line indicates the spatial separation of the thermometer unit (outside) and the temperature probe (inside the oven).

14. Aluminum block assembly: Assay plate in 384 well format (**Fig. 1**); lid plate and screws (**Fig. 1**); bottom and top heat transfer blocks (**Fig. 2**); heat resistant silicone mat (thickness 1.2 mm).
15. Temperature controlled oven.
16. Electronic thermometer with a type K thermocouple beaded probe (>1 m cable length).

17. *p*-Hydroxy benzoic acid hydrazide (PAHBAH; Sigma, St. Louis, MO).
18. 384-well PCR block (MJ Research, Waltham, MA).
19. 384-well PCR plates.
20. Silicone sealing mat for 384-well PCR plates ('PierceMat', E & K Scientific, Campbell, CA).
21. 384-well microtiter plates.
22. 384-well plate reader, suitable for reading absorption at 420 nm.

3. Methods

The following methods section describes 1) preparation of a pool of α -amylase variants, 2) high throughput incubation of the α -amylase reaction at elevated temperatures, and 3) determination of α -amylase activity.

3.1. Preparation of a Pool of α -Amylase Variants

Starting from a pool of mutant α -amylase genes, a library of individual enzyme variants is generated as liquid cultures of single clones in 96-well microtiter plates. Variants of the α -amylase released into the supernatants are screened for improved starch degrading activity at 129°C. The steps outlined in **Subheadings 3.1.1.** and **3.1.2.** comprise 1) transformation of *E. coli* BL21-Gold(DE3) cells with a mutant plasmid library, and 2) growth of *E. coli* clones in liquid culture for the production of the recombinant α -amylase.

3.1.1. Transformation of *E. coli* BL21-Gold(DE3) Cells with a Mutant Plasmid Library

Using the α -amylase gene from the hyperthermophilic archaeon *Pyrococcus furiosus* as template, a library of mutant genes is prepared using the error-prone PCR techniques (*Taq* DNA polymerase and MnCl_2) outlined in earlier chapters of this volume. This library is cloned into the expression vector pET21b(+) using of standard molecular biology techniques. This pool of mutant plasmids is used to transform the *E. coli* strain BL21-Gold(DE3) in order to express the mutated α -amylase gene (see **Note 1**). Analogously, a plasmid that contains the wild-type gene was constructed as a positive control. A plasmid that contains a truncated α -amylase gene, yielding an inactive clone upon transformation, serves as a negative control from which background activity of the system can be determined. The entire procedure outlined below is always performed in parallel with these two constructs. Three positive and two negative controls are included in each 96-well plate.

1. Take 2 μL of a 2 ng/ μL solution of the pET21b(+) derived mutant library of α -amylase gene to transform 50 μL of BL21-Gold(DE3) cells in 2.0-mL Eppendorf vials, according to manufacturer protocol.
2. Add 450 μL SOC medium to the suspension and incubate for 10 min at 37°C under agitation (see **Note 2**).

3. Plate the entire sample on a 20 × 20 cm 'Q'Tray filled with 200 mL LB-Ampicillin (200 μ g/mL) agar. Alternatively, standard (10 cm) agar plates can be used for manual picking.
4. Incubate the plate at 37°C until the colonies have a diameter of 0.8–1.5 mm (at least 12 h).

This transformation procedure reproducibly yields about 2000–3000 colonies in one tray, corresponding to a colony density found most appropriate for automated picking efficiency.

3.1.2. Growth of *E. coli* Clones in Liquid Culture for Protein Production

The procedure for generating the α -amylase activity in the culture supernatant of the clones from the transformation is outlined in this section.

1. Prepare the liquid culture plates by putting a 3-mm glass ball (*see Note 3*) into each well of a 96-well flat-bottom microtiter plate. Then, fill each well with 160 μ L of LB-Ampicillin (200 μ g/mL) medium.
2. Pick the colonies resulting from the transformation with a colony picker or with sterile toothpicks to inoculate the liquid cultures.
3. Incubate the plates in humidified styrofoam boxes at 37°C and 300 rpm shaking (*see Note 4*).
4. After 26 h of incubation, transfer an aliquot of each well in the 96-well plates using a 96-pin replicator tool to a new plate containing a glass ball and 160 μ L fresh LB-Ampicillin medium in each well (*see Note 5*).
5. Incubate this plate under the same conditions for another 24 h.
6. Centrifuge the plates at 1500g for 10 min at 4°C and examine the supernatant for α -amylase activity.

3.2. High-Throughput Incubation of α -Amylase Reaction at 129 °C

In order to get a quantitative readout of the α -amylase activity produced, the supernatants of the individual clones are first incubated with a starch solution at 129°C. Application of the following experimental protocol results in the decomposition of the substrate to oligosaccharides.

1. Heat the top and the bottom heat transfer plate together in the temperature-controlled oven to 143°C (monitor the temperature by inserting the beaded temperature probe from the outside of the oven into a glycerol filled hole in the bottom heat transfer plate).
2. Prepare a 1% starch solution (in 100 mM sodium acetate, pH 5.5) and place 90 μ L in each well of the 384-well aluminum block using a multi-channel pipettor.
3. Paying attention not to move the glass ball on the bottom of the wells (*see Note 6*), transfer 10 μ L of the supernatants of the 96-well microtiter plates containing the *E. coli* cultures to the 384-well aluminum assay block using a liquid handling robot or a multi-channel pipettor.

4. At ambient temperature, mix the two solutions by pipetting a volume of 20 μL repeatedly up and down (with the pipetting robot).
5. Cover the assay block with the silicone mat and the lid plate and seal it by tightening the screws (**Fig. 1**).
6. Place the preheated (143°C) top heat transfer block quickly on top of the closed assay block (this ensures that heating is faster on top than from the bottom).
7. Quickly put the whole assembly on top of the preheated bottom heat transfer block, which has been covered with 500 μL of glycerol, and slide the assembly consisting of the assay block and the top heat transfer block to the middle of the bottom heat transfer block. In this way, the glycerol gets distributed evenly between the block and assay plate ensuring uniform and rapid heat transfer. The setup is illustrated in **Fig. 2**. The system heats up to 125°C in less than 30 s and reaches the target temperature of 129°C in less than 1 min.
8. After incubation at 129°C for 10 min, cool the assay block instantly by putting it on densely packed ice. This eliminates background activity during subsequent handling since the α -amylase is essentially inactive at temperatures below 40°C (**6**).

3.3. High-Throughput Assay for α -Amylase Activity

Hydrolysis of starch by the α -amylase in the assay mix results in the formation of reducing sugars, which can be quantified photometrically using *p*-hydroxy benzoic acid hydrazide (PAHBAH) reagent. This reliable method is highly sensitive, easy to use, well suited for high-throughput screening, and does not employ toxic substances or unnatural substrates (**8,9**).

1. Prepare the PAHBAH reagent (containing 70 mM PAHBAH, 27 mM sodium citrate, 67 mM Na_2SO_3 , 0.4 M NaOH, 14 mM CaCl_2 , *see Note 7*).
2. Fill 15 μL of the PAHBAH reagent into each well of a 384-well PCR plate using a multi-channel pipettor.
3. Pipet 5 μL of the solution contained in the wells of the cooled assay plate and mix it with the PAHBAH reagent, preferably using a 96-channel pipetting robot.
4. Cover the plate with a PCR silicone mat and incubate it in a 384-well PCR block for 10 min at 91°C.
5. After incubation, transfer the yellow solutions to a 384-well flat-bottom plate by centrifugation, placing the PCR plate inverted on top of the 384-well plate.
6. Measure the absorption at 420 nm, proportional to α -amylase activity, with a plate reader.
7. Using solutions of 1 mM and 0.2 mM maltose as standards (randomly omit 2 wells in **step 3**), determine the α -amylase activity of a given sample quantitatively.

With a little bit of practice, this setup allows for screening of about 2000 clones per day. Modification of the described high temperature, high-throughput assay method could make it a useful tool for addressing fundamental questions of thermoactivity and thermostability for many enzymes of hyperthermophilic origin.

3.4. Assay for α -Amylase Activity Using Glass Capillaries

If only a few samples are to be analyzed, especially for checking putatively improved clones, 100 $\times$ 1.8-mm capillary melting point tubes (VWR) can be used following the protocol outlined by Daniel and Danson (4). In our setup, 6.7 μ L of α -amylase containing sample are mixed with 60 μ L of 1% starch solution. The assay mixture was transferred to a capillary tube by capillary forces. Subsequently, the tube is sealed on both sides with a torch. The sample is then immersed into a preheated glycerol bath (*see Note 8*), and afterwards rapidly cooled by immersion in ice-water. The activity is determined as outlined above. Although this method allows for only a few samples to be prepared at a time, it has the advantage of eliminating the residual activity occurring during the heating up phase, which can be 40–50 % of the total activity measured (data not shown), since the sample reaches instantly the intended incubation temperature.

4. Notes

1. The cloned gene containing no leader sequence targeting it for secretion into the growth medium. However, under the conditions outlined a significant amount of α -amylase activity is measurable in reproducible amounts in the supernatant of the (wild-type) cultures. The heterologous expression does not need to be induced, as there is a basal level of production of the enzyme in spite of the tightly controlled T7 RNA polymerase expression system.
2. The short incubation time of 10 min after the transformation has been chosen in order to avoid division of positive transformants, which would result in multiple colonies originating from a single clone. Thus, the library would be “diluted out.”
3. A glass ball is put into each well because, even with agitation at 300 rpm in a rotary shaker, the *E. coli* cells would settle down to the bottom of the wells, forming a cone in the center. Furthermore, the activity found in the supernatants of the cultures increases drastically upon incubation with glass balls, possibly owing to disruption of a fraction of the cells.
4. Special care has to be taken so the wells in the corners and on the edges of the 96-well plates do not lose part of their liquid, which might affect concentration of the α -amylase in the supernatant. We use styrofoam boxes (closed, aerated by air slits), kept moist with a wick of paper towels that are placed underneath the microtiter plates and repeatedly wetted during the incubation. The microtiter plates are covered such that their lids are elevated by spacers to ensure a uniform distribution of ambient air across the plate. Commercially available humidity-controlled incubation systems for microtiter plates, if available, would be a good alternative to this setup, which is rather tedious to prepare and to operate.
5. Replication of the cultures helps significantly to synchronize the growth of the individual clones. Omission of this step results in a high variation of α -amylase activity. This variation can be accounted for by the fact that very different numbers of cells from the individual colonies are transferred from the agar plate into

the well to be inoculated. Therefore, the growth phase, during which the cultures generate the α -amylase activity, late stationary phase, is reached at different points in time for the individual cultures. After the cultures have been incubated for 26 h, ideally all of them should be in the stationary growth phase and have the same optical density, although they are not likely to contain the same amount of α -amylase activity. With the replicator tool, an equal amount of culture is transferred. Thus, all replicated cultures start off with an inoculum containing similar numbers of cells.

6. The sedimented *E. coli* cells contain a significant amount of α -amylase activity (data not shown), and agitation of the glass ball might cause part of the cell mass to enter the assay mixture, which would lead to false positives.
7. First, add the appropriate amount of a 5 M NaOH solution to sodium citrate, Na₂SO₃ and PAHBAH, then add water. After complete dissolution of the material, add the CaCl₂ from a 1 M stock solution and dissolve the formed flakes by swirling. Other modes of addition might lead to turbidity of the reagent.
8. Glycerol is preferred over oil because of its water solubility, which contributes to tidiness of the working area. Furthermore, it can be disposed of without environmental counter-indications. It is imperative that during operation of this high temperature system care is taken to make use of protective equipment like a lab coat and a face shield.

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High-Throughput Carbon Monoxide Binding Assay for Cytochromes P450

Christopher R. Otey

1. Introduction

Cytochromes P450 are a superfamily of enzymes that catalyze the monooxygenation of a variety of substrates, including aliphatic and aromatic compounds (1). They contain a noncovalently bound protoporphyrin IX, giving these enzymes characteristic spectral properties. This heme has an available sixth coordination ligand that is able to bind carbon monoxide. Difference spectroscopy yields a spectral peak at approx 450 nm when comparing bound and unbound forms (2). This difference demonstrates the presence of a correctly-folded cytochrome P450. Binding of carbon monoxide in the presence of a biologically inactive form of a cytochrome P450 yields a spectral peak at 420 nm (3). Thus CO binding effectively assays for the presence of a correctly-folded cytochrome P450, an incorrectly-folded P420, or a lack of either. Carbon monoxide binding assays are typically done in single-cuvet format (4) but are easily modified for high-throughput in microtiter plates. This method is useful in quickly assaying a library of cytochrome P450s for folded and possibly functional proteins while eliminating misfolded or low expressing variants. It can also be used to rapidly determine the P450 concentration of multiple samples or the relative expression levels of individual clones in a high-throughput screen of a mutant library.

2. Materials

1. 300 mM sodium hydrosulfite in 1.3 M phosphate buffer, pH 8.0. This should be prepared fresh for each experiment.
2. 50 mM phosphate buffer, pH 7.3.
3. 96-well microtiter plates: R-96-OAPF-ICO (Rainin, Emeryville, CA).

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4. Carbon monoxide tank with an appropriate regulator that allows for slow and controlled bubbling into liquids.
5. Carbon monoxide chamber: a container in which 96-well microtiter plates can be placed and where a vacuum can be created followed by a slight pressurization. It should be a container that can be sealed and has at least one hose connection or stopcock valve. A vacuum oven will work (Fisher Scientific) as well as a desiccator made of polycarbonate. A 3-way stopcock, C-clamps and vacuum grease may also be necessary (*see Note 1*).
6. Tygon tubing and vacuum tubing.
7. Vacuum pump.
8. Spectrophotometer/plate reader (Model Spectra max Plus 384, Molecular Devices, Synnyvale, CA). Software Softmax Pro 3.1.1.
9. Benchtop centrifuge that can accommodate 96-well microtiter plates: Allegra 25R Centrifuge (Beckman Coulter, Fullerton, CA).
10. Multichannel pipettor.

3. Methods

1. Put 40 μL of the 300 mM sodium hydrosulfite solution into wells of a 96-well microtiter plate (*see Note 2*).
2. Add 160 μL of enzyme solution per well and mix (*see Note 3*). Enzyme solution can be either purified enzyme or extract from a cell lysis reaction after centrifugation and removal of cell debris. Centrifuge at 3000g to remove any bubbles.
3. Blank plate reader, taking both baseline spectra and particular wavelengths (*see Note 4*). Put plate into carbon monoxide chamber and pull a vacuum with a vacuum pump. Fill the container with carbon monoxide until a positive pressure is obtained. Continue to apply carbon monoxide at a slow rate for 8 min (*see Note 5*).
4. Remove the plate and record spectra from 400–500 nm and at specific wavelengths (*see Note 4*).
5. Using A_{450} and A_{490} , concentrations can be determined using Beer's Law: $A = \epsilon c l$ and the extinction coefficient, $\epsilon_{450-490} = 91 \text{ mM}^{-1} \text{ cm}^{-1}$. The path length will vary based on volume and should be determined using a standard cuvet assay (*see Note 6*).

4. Notes

1. The positive pressures applied are not great and this piece of equipment can be improvised. To use a desiccator, vacuum grease is applied liberally around the edge where contact is made with the lid. When a plate is placed inside, the desiccator is clamped shut with four c-clamps evenly distributed around the edges. This easily resists the positive pressure necessary for the assay. The desiccator is attached to a three-way stopcock with tubing. The three-way stopcock is connected to a vacuum pump on one end and the carbon monoxide tank on the other. This allows for switching between the vacuum pump and the carbon monoxide tank.

2. The high buffer concentration is to buffer against pH changes caused by sodium hydrosulfite, which lowers the pH of the solution. After adding the sodium hydrosulfite, time becomes more critical. Work quickly, as the enzyme may degrade more rapidly.
3. Volumes can be scaled up accordingly, but should not exceed 300 μL since the maximum well volume of a 96-well microtiter plate is 320 μL . The assay can also be applied in 384-well plate format, which have a maximal working volume of approx 110 μL . Enzyme can be diluted if necessary. The presence of P450 enzymes can be observed at 100 nM enzyme and concentrations can be acquired above 500 nM. At 100 nM, the $A_{450}-A_{490}$ is approx 0.004. Multiple plates can be assayed, but four is the suggested maximum at one time.
4. Spectra can be recorded every 10 nm from 400–500 nm or at smaller intervals. Specific points should be taken at 490 nm and at the λ_{max} of your particular cytochrome P450, typically around 450 nm (4). Ultimately, the difference in absorbance between 450 and 490 nm is used to quantitate the amount of enzyme.
5. A slow rate means typical bubbling rate used for cuvet format. This means approximately one to three bubbles per second from a Pasteur pipet attached to the carbon monoxide tank with tubing. This flow rate will quickly fill a sealed container and provide enough pressure to supply carbon monoxide to the P450s. Only a slight positive pressure is needed since a vacuum is created prior to filling the container with carbon monoxide.
6. Path lengths have been determined to be approx 0.41 and 0.61 cm for 200 and 300 μL , respectively. The path length can vary depending on the solution used owing to the meniscus that forms. It is suggested that a control of known concentration be added to a plate if determining exact concentrations.

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High-Throughput Screen for Aromatic Hydroxylation

Christopher R. Otey and John M. Joern

1. Introduction

The oxidation of aromatic compounds is important in producing chemical intermediates for the chemical and pharmaceutical industries (**1,2**). Conventional aromatic oxidation reactions are prone to byproduct formation and often require heavy-metal catalysts, extremes of temperature and pressure, and explosive reagents (**3**). In contrast, biocatalysts such as mono- and dioxygenases perform the same chemistry in water at ambient conditions, usually with higher regioselectivity than the analogous chemical process. Because of these inherent advantages, there has been increasing interest in applying aromatic oxygenases to chemical processes and bioremediation (**4–6**). Discovery and engineering of these enzymes is critical since the available natural enzymes are often not immediately suitable for these applications. Here, three colorimetric assays for hydroxylated aromatic compounds are discussed that can be implemented in high-throughput and thus are useful for biocatalyst discovery and engineering by directed evolution. These include assays employing Gibbs' reagent, 4-aminoantipyrine (4-AAP) and Fast Violet B (FVB) (**Fig. 1**). These methods should enable the optimization of oxygenases to industrially relevant substrates and realistic process conditions.

We have applied these chemistries to cytochrome P450 monooxygenases and dioxygenases. These enzymes catalyze the insertion of oxygen into an unactivated carbon and are promising catalysts for the manufacture of chiral synthons and bioremediation (**1,4,5,7**). The P450 monooxygenases are ubiquitous, heme-containing enzymes that catalyze a variety of reactions on many different substrates, including aromatic compounds (**8,9**). Dioxygenases insert both atoms of molecular oxygen into aromatics to yield *cis*-dihydrodiols with high (>97%) enantiomeric excess (*see Fig. 1D*). For the assay, these products

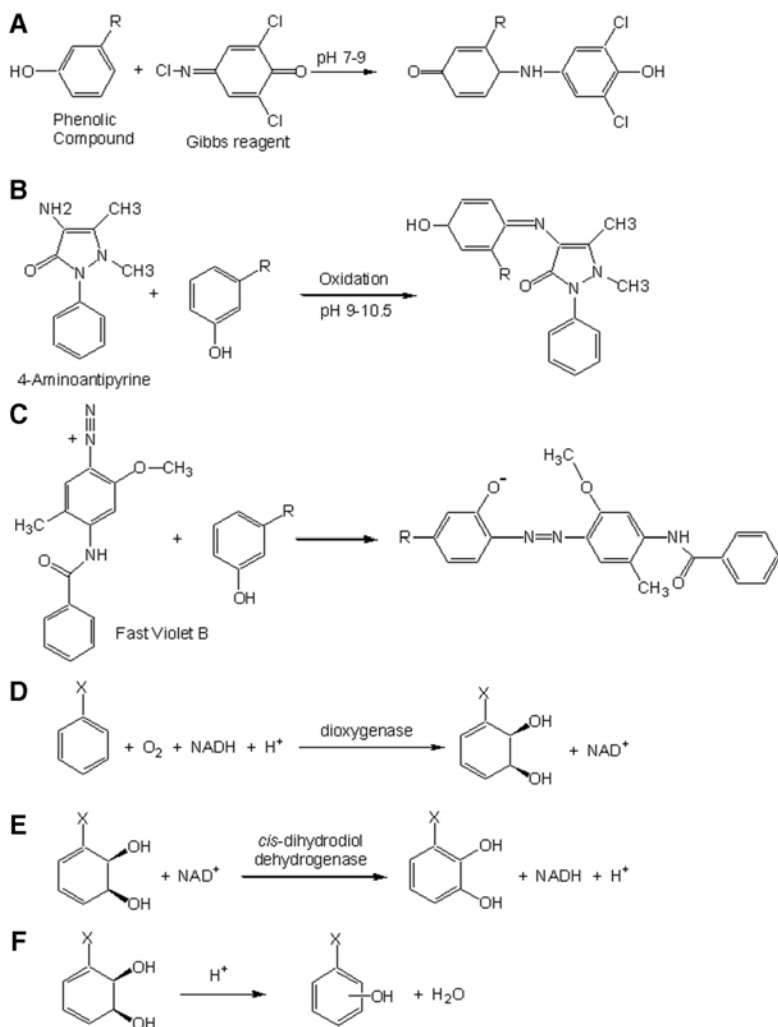


Fig. 1. Chemistry of assay methods. (A) Coupling of Gibbs' reagent to a phenolic compound (17). (B) Coupling of 4-aminoantipyrine to a phenolic compound (16). (C) Coupling of Fast Violet B to a phenolic compound (18). (D) Chemistry performed by dioxygenases to yield a *cis*-dihydrodiol. (E) Dehydrogenation of a *cis*-dihydrodiol to form a catechol. (F) Acidification of a *cis*-dihydrodiol to form phenols.

are first converted to phenols (**Subheading 3.4.**), detected using the reagents described here. The assays to be discussed are applicable to these enzymes as well as many others (e.g., oxidative dealkylases or dehalogenases). Only slight modification and optimization should be necessary from system to system.

Some issues need to be considered when applying these assays to biotransformations using whole cells or cell extracts. When a whole cell system is used, careful consideration should be given to the method of supplying substrate to the enzyme. To access the enzyme, the substrate must be soluble and must readily permeate the cell membrane. Solubility can be increased in most cases by adding a nontoxic organic solvent (**10**). Polymyxin B increases the permeability of many aromatic and non-aromatic substrates, including long chain fatty acids (**11,12**). Though Terrific Broth or Luria-Bertani-media are commonly used for whole cell growth, these rich broths contribute a significant amount of background in the assays discussed here (especially the Fast Violet B assay). This is easily remedied by using a synthetic medium such as M9 minimal medium (**13**). Supplying the substrate in the vapor phase is sometimes successful when the substrate is volatile, and is particularly convenient when screening colonies using a solid-phase format (**14**). Because every screening situation is unique, the following protocols should be adapted to your particular system.

2. Materials

1. 0.6% (w/v) 4-aminoantipyrine in ddH₂O (4-AAP). Store at 4°C and prepare fresh every 2 wk.
2. 0.6% (w/v) potassium persulfate in ddH₂O. Store at 4°C and prepare fresh every 2 wk.
3. 0.4% (w/v) 2,6-Dichloroquinone-4-chloroimide in ethanol (Gibbs' reagent). Store at 4°C and prepare fresh every 4 mo.
4. 0.25% (w/v) Fast Violet B in ddH₂O (FVB). Prepare fresh every 2–3 d.
5. 100 mM NaOH.
6. 1 M Tris-HCl, pH 8.5
7. 100 mM HCl.
8. 96-well microtiter plates: R-96-OAPF-ICO (Rainin, Emeryville, CA).
9. Spectrophotometer/plate reader (Model Spectra max Plus 384, Molecular Devices, Sunnyvale, CA). Software Softmax Pro 3.1.1.
10. Benchtop centrifuge that can accommodate 96-well microtiter plates: Allegra 25R Centrifuge (Beckman Coulter, Fullerton, CA).
11. Pipet robot: Multimek 96 Automated 96-Channel Pipettor (Beckman Instruments, Palo Alto, CA).
12. Multichannel pipettor.
13. Incubator at 37°C.

3. Methods

The 4-AAP and Gibbs' assays are very similar in the products they are able to detect (**Table 1**), as well as their sensitivity limits and coefficients of variation (see **Note 1**). Both react well with *ortho*- and *meta*-substituted phenolic compounds and with *para*-substituted compounds where the substituent is a halide or

Table 1
The Spectroscopic Signals Resulting from Coupling of Various Phenols to Gibbs' Reagent, Fast Violet B and 4-Aminoantipyrene

Compound	Gibbs' reagent assay		Fast Violet B assay		4-Aminoantipyrene assay	
	$\lambda_{\max}$	Max abs.	$\lambda_{\max}$	Max abs.	$\lambda_{\max}$	Max abs.
3-hydroxybenzaldehyde	670	0.06	n/a	< 0.05	580	0.18
2-hydroxybenzaldehyde	660	0.09	380	0.09	512	0.13
2-hydroxybenzamide	660	2.98	n/a	< 0.05	512	0.08
2,3-dihydroxybenzaldehyde	570	0.63	n/a	< 0.05	480	0.65
catechol	460	0.44	n/a	< 0.05	500	0.98
3-methylcatechol	460	0.49	n/a	< 0.05	540	0.25
3-fluorocatechol	450	0.38	n/a	< 0.05	535	0.49
phenol	630	0.10	n/a	< 0.05	510	2.57
<i>o</i> -cresol	610	0.31	n/a	< 0.05	505	1.85
<i>m</i> -cresol	620	0.17	n/a	< 0.05	500	1.76
2-aminophenol	600	0.76	440	0.08	520	1.07
3-aminophenol	570	1.61	480	0.82	470	2.61
2-chlorophenol	660	2.77	n/a	< 0.05	515	2.77
3-chlorophenol	670	1.25	n/a	< 0.05	545	0.78
1-naphthol	580	0.31 ^a	n.d.	n.d. ^b	505	1.61
2-naphthol	n.d.	n.d. ^b	520	0.47	n.d.	n.d. ^c
2,3-dihydroxynaphthalene	510	0.79	n.d.	n.d. ^b	490	0.18
4-nitrophenol	n/a	< 0.05	n/a	< 0.05	n/a	< 0.05
2-hydroxypyridine	n/a	< 0.05	n/a	< 0.05	n/a	< 0.05
3-hydroxypyridine	600	0.39	n/a	< 0.05	n/a	< 0.05
<i>o</i> -coumaric acid	650	0.42	n/a	< 0.05	520	2.39
<i>m</i> -coumaric acid	670	0.24	n/a	< 0.05	540	0.87
<i>p</i> -coumaric acid	560	0.38	n/a	< 0.05	470	0.29
3-hydroxybenzoic acid	640	0.25	n/a	< 0.05	505	0.24
3,4-dihydroxybenzoic acid	460	0.29	n/a	< 0.05	495	0.75
3-hydroxy-4-methylbenzoic acid	610	1.21	n/a	< 0.05	465	0.90
2,3-dihydroxybenzoic acid	440	0.27	n/a	< 0.05	500	0.26

^aProduct slightly insoluble.

^bProduct insoluble.

^cColor change to green that quickly fades to yellow with no $\lambda_{\max}$.

n/a, not applicable, no $\lambda_{\max}$; n.d., not determined because of insolubility.

Compounds were diluted in M9 minimal media to a concentration of 0.25 mM and assayed as described. For the Gibbs' reagent, Fast Violet B, and 4-aminoantipyrene assays, 0.1 mL of phenol solution was assayed in a 96-well microtiter plate. 30 min, 10 min, or 5 min, respectively, were allowed for the reaction to occur before recording the visible spectra using a 96-well spectrophotometer.

alkoxy group (**15**). FVB is typically less useful due to its reactivity with cells and various media. These assays are linearly dependent on concentration for all of the phenols we have examined (**14**) (see **Note 2**). The wavelength of maximal absorbance varies based on the structure of the phenol, and thus should be deter-

mined for each expected phenolic product. It may also be useful to adjust reagent concentrations to reduce background when optimizing a new assay system.

Since these assays are able to determine the products of multiple types of enzyme reactions, reaction conditions (e.g., cell growth times and temperatures, substrate concentration, cell harvesting/lysis, etc.) will vary considerably and will not be discussed here. In the assay descriptions below, “sample” refers to the solution containing the phenolic product to be determined and may be a cell extract or supernatant depending on which type of bioconversion is chosen. The absorbance value yields the total activity of the bioconversion. Times for color development are suggested below, but this is another factor that varies from substrate to substrate and should be determined on an individual basis.

3.1. Phenol Quantitation with Gibbs' Reagent

1. To 100 μL of sample add 20 μL 0.4% (w/v) of Gibbs' reagent.
2. Mix and allow 3–30 min for color development (*see Note 3*).
3. Record spectrum or wavelength.

3.2. Phenol Quantitation with 4-Aminoantipyrine

1. To 100 μL of sample add 100 μL of 100 mM NaOH (*see Note 4*).
2. Add 30 μL of 0.6% (w/v) 4-AAP. Mix thoroughly and incubate for 2 min.
3. Add 30 μL of 0.6% (w/v) potassium persulfate (*see Note 5*).
4. Mix and allow 10 min for color development (*see Note 6*).
5. Record spectrum or wavelength.

3.3. Phenol Quantitation with Fast Violet B

1. To 100 μL of sample add 10 μL 0.25% (w/v) Fast Violet B.
2. Mix and allow 10 min for color development (*see Note 3, Note 7*).
3. Record spectrum or wavelength.

3.4. Applying Phenol Detection with Gibbs' Reagent to Dioxygenases

Initial oxidation of aromatic compounds by dioxygenase results in arene *cis*-dihydrodiols, as shown in **Fig. 1D**. These compounds are difficult to detect in the background of a cell extract or supernatant, but are easily converted to detectable phenolic compounds using one of two methods. One is to convert the *cis*-dihydrodiol to a catechol by coexpressing the *cis*-dihydrodiol dehydrogenase that resides on the dioxygenase cistron. The dehydrogenase from the toluene dioxygenase cistron of *Pseudomonas putida F1* is highly expressed in laboratory strains of *E. coli*. Another method for converting *cis*-dihydrodiols to phenols is acidification, as shown in **Fig. 1F (14)**. The ratio of *ortho*- to *meta*-phenols is difficult to predict, but both types generally react with the detection reagents discussed here.

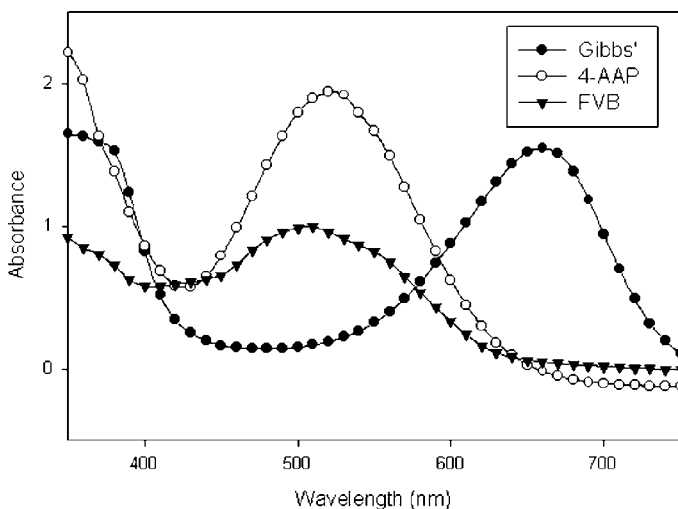


Fig. 2. Spectra of assay products. 0.25 mM solutions of *o*-chlorophenol, *o*-coumaric acid and 2-naphthol in M9 media were assayed as described using Gibbs' reagent (Gibbs'), 4-aminantipyrine (4-AAP), and Fast Violet B (FVB), respectively.

1. In a 96-well microtiter plate, combine 100 μ L of cell extract or supernatant from a biotransformation performed in M9 minimal medium (**13**) with 100 μ L of 0.1 M HCl (see **Notes 8** and **9**).
2. Incubate at 37°C for 30 min.
3. Add 25 μ L of 1 M Tris-HCl, pH 8.5 (see **Note 8**).
4. Add 20 μ L of 0.4% (w/v) Gibbs' reagent.
5. Record spectrum or wavelength after 3–30 min (see **Note 3**).

3.5. Applying Phenol Detection with 4-AAP to Cytochrome P450

Since cytochrome P450s typically perform single hydroxylation, no other preparative steps are necessary. Similar to the dioxygenases, the position of hydroxylation is not easily predicted. Reactions should be quenched in a suitable manner to allow for reproducible time points. The NaOH will provide this in some systems.

1. In a 96-well microtiter plate, following whole cell or cell extract reaction, add an equivalent volume of 0.1 M NaOH (see **Notes 4** and **9**). If using a whole cell assay centrifuge at $\sim 3500g$ for 10 min and remove 100 μ L of supernatant and transfer to a new microtiter plate (see **Note 10**).
2. Add 15 μ L of 0.6% (w/v) 4-AAP per 100 μ L. Mix and incubate for 2 min.
3. Add 15 μ L of 0.6% (w/v) potassium persulfate per original 100 μ L (see **Note 5**).
4. Record spectrum or wavelength after 10 min (see **Note 3**).

4. Notes

1. Coefficients of variation tend to be approx 8–14% for the Gibbs' and 4-AAP assays. The sensitivity limit for Gibbs' and 4-AAP is approx 10 μM . Tris has been found to give a higher background level than other buffer systems. This should be considered when the amount of product formed is near the detection limit.
2. Phenol, *o*-coumaric acid, and *m*-coumaric acid have been checked for a linear dependence of concentration on absorbance with the 4-AAP assay. 3-methylcatechol and 3-fluorocatechol have been tested with the Gibbs' assay.
3. Optimal development time depends on the phenol assayed and, in some cases, accumulation of background absorbance over time. When assaying for improved enzyme function, only the wavelength of the product is taken and not the entire spectrum.
4. A pH between 9 and 10.5 is desired since 4-AAP will react with peroxides at a lower pH and produce a red color (**16**). This also aids in solubilizing any precipitate from cell extract and/or substrate added. 4-AAP works in up to 75% DMSO in water.
5. Potassium persulfate serves to oxidize the NH_2 group of 4-aminoantipyrine, making it available for electrophilic attack by a phenol. Other oxidizing agents can be used such as ammonium persulfate and potassium hexacyanoferrate (**16**). Potassium persulfate gave the least amount of background in the P450 system.
6. The reproducibility of the 4-AAP assay is not very sensitive to incubation times beyond 10 min. It is a good idea, however, to keep the incubation time constant from screen to screen in order to minimize variance. Centrifuging between addition of 4-AAP and potassium persulfate can be useful in case there is any residual liquid on the sides of the plate-wells.
7. Increasing the pH to basic levels before addition of Fast Violet B can be useful in increasing the maximum absorbance value. It is not necessary, however.
8. For the *cis*-dihydrodiol products of dioxygenation of toluene and chlorobenzene, pH <2.5 should be reached after adding 0.1 M HCl. Incubation at low pH may or may not be required for acidification of other *cis*-dihydrodiols. Reaction with Gibbs' reagent proceeds best at pH 7–9, thus the pH should be near or above neutral after addition of Tris buffer. If media other than M9 (**13**) are used, a Gibbs' reagent-compatibility check should be made, and the amount of acid and Tris buffer added should be adjusted to match these pH ranges.
9. A pipetting robot can be useful when doing multiple 96-well microtiter plate assays, but is not necessary.
10. Removal of cell debris is not necessary in the Gibbs' and 4-AAP assays, however it increases the reproducibility of the screens. It is necessary for FVB.

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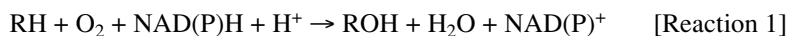
Colorimetric Screen for Aliphatic Hydroxylation by Cytochrome P450 Using *p*-Nitrophenyl-Substituted Alkanes

Edgardo T. Farinas

1. Introduction

The United States consumes approximately 16–17 million barrels of crude oil per day, and the majority is used for electricity, heating, and transportation fuel (**1**). The main constituents of crude oil are linear aliphatics. Clearly, the selective hydroxylation of alkanes to more valuable products would have a worldwide economic impact. Unfortunately, the chemical methods for the oxidation of alkanes are energy intensive, and the reagents and byproducts are hazardous to the environment (**2**). In order to overcome these shortcomings, biocatalysts such as heme (**3**) and non-heme monooxygenases (**4**) offer an attractive alternative to alkane oxidation. However, the known alkane hydroxylases are sluggish ($\sim 1\text{--}200\text{ min}^{-1}$), and high-throughput screens for aliphatic hydroxylation are expected to be useful in discovering more active clones from enzyme libraries.

P450s are monooxygenases that incorporate one of the two oxygen atoms of an O_2 molecule into a diverse range of hydrophobic substrates (RH). The second oxygen atom is reduced to H_2O (**Reaction 1**). The minimum requirements for enzymatic activity are substrate, dioxygen, and nicotinamide adenine dinucleotide phosphate (NADPH).



Cytochrome P450 BM3 is a soluble P450 found in the cytoplasm of *Bacillus megaterium* (**5–7**). P450 BM3 hydroxylates fatty acids with chain lengths between C_{12} and C_{18} at subterminal positions. Unlike other P450s that require

additional proteins as a redox partner, P450 BM3 contains both the monooxygenase and reductase domain in a single polypeptide chain. P450 BM-3 will be used to illustrate screening mutant libraries for octane oxidation.

The standard method to determine P450 activity is to measure the rate of NADPH consumption of the enzyme in presence of substrate. However, octane is a nonnatural substrate for P450 BM3, and the rate of NADPH consumption is close to the background level. Therefore, a sensitive, high-throughput assay is necessary to identify more active mutants from enzyme libraries when the activities are still very low. An assay using an octane analog, *p*-nitrophenoxyoctane (8-pnpne) was designed which fulfills this role (8–10). 8-Pnpne contains a linear C8 backbone and a *p*-nitrophenol moiety. Hydroxylation of 8-pnpne generates an unstable hemiacetal that dissociates to form *p*-nitrophenolate (yellow) and the corresponding aldehyde (**Fig. 1**). The hydroxylation kinetics or endpoint of hundreds of mutants can be monitored simultaneously in the wells of a microtiter plate using a plate reader.

Although this assay is very sensitive, it has several drawbacks. The hazard of using substrate analogs such as 8-pnpne is that the enzyme may become addicted to the screening compounds. The increased activity of surrogate substrate may not be proportional or not at all related to the target substrate. Furthermore, the assay does not directly quantify product formation. The screen measures the formation of *p*-nitrophenolate when the substrate is hydroxylated at one specific position and oxidation at other positions is not detected in this assay (**Fig. 1**).

If the activity of the mutant is sufficiently high, the rate of NADPH consumption can be measured to ensure that the activity of the mutant is increasing for the desired substrate. However, NADPH oxidation alone may not provide an accurate measure of catalytic activity since reducing equivalents from NADPH can be diverted into forming reduced oxygen intermediates (H_2O or H_2O_2) (3). Therefore, subsequent generations screened will ideally use a combination of the 8-pnpne assay, sensitive to product formation, and NADPH consumption (*see Chapter 16*) in the presence of the target substrate.

These substrate analogs can be adapted to screen for other desired enzymatic traits. For example, they can be used to screen P450s for greater organic solvent resistance, thermostability, and for the ability to utilize H_2O_2 to drive catalysis in the absence of cofactor. Changes in substrate specificity can also be investigated. For example, the *p*-nitrophenol moiety can be tethered to alkyl chains with varying length or different substituents such as fatty acids (**II**), amides, alcohols, and esters. Finally, the surrogate substrate may be useful in screening bacterial culture collections for identifying new enzymes for aliphatic hydroxylation.

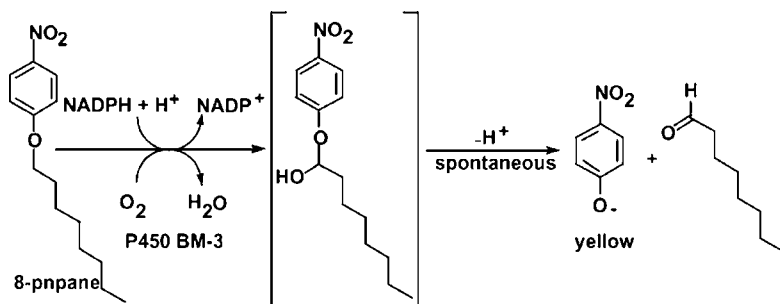


Fig. 1. The screening assay for alkane oxidation activity uses the substrate analog 8-pnpentane. Terminal hydroxylation generates the unstable hemiacetal, which decomposes to the aldehyde and *p*-nitrophenolate, monitored at 410 nm.

2. Materials

2.1. Biological and Chemical Materials

2.1.1. Synthesis of 8-Pnpentane (see Note 1)

1. Dimethyl sulfoxide.
2. 1-Bromooctane.
3. 4-Nitrophenol, sodium salt.
4. Silica gel 40 μm flash chromatographic packing (J. T. Baker, Phillipsburg, NJ).
5. Petroleum ether (EM Science, Gibbstown, NJ).
6. Diethyl ether anhydrous (EM Science).
7. Thin layer chromatography aluminium sheets: Silica gel 60 F₂₅₄ (EM Science).
8. Phosphomolybdic acid reagent 20 wt. % in ethyl alcohol (Aldrich, Milwaukee, WI).

2.1.2. Protein Induction in 96-Well Plates

1. Plasmid containing P450 BM3 gene.
2. Library efficiency DH5 α competent cells were purchased from Life Technologies (Rockville, MD).
3. LB (Luria-Bertani) medium, and LB agar (12).
4. Ampicillin (D-[-]- α -aminobenzylpenicillin sodium salt) (Sigma, St. Louis, MO).
5. TB (Terrific broth) medium* (12).
6. Trace elements: 0.5 g MgCl₂, 30.0 g FeCl₂·6H₂O, 1.0 g ZnCl₂·4H₂O, 0.2 g CoCl₂·6H₂O, 1.0 g Na₂MoO₄·2H₂O, 0.5 g CaCl₂·2H₂O, 1.0 g CuCl₂, and 0.2 g H₃BO₃ in 1 L HCl solution (90% v/v distilled water: concentrated HCl).
7. IPTG (isopropyl- β -D-thiogalactopyranoside) (ICN, Aurora, OH).
8. ALA (δ -aminolevulinic acid hydrochloride) (Sigma).

2.1.3. Colorimetric Aliphatic Hydroxylation Assay

1. Assay buffer: 0.1 M potassium phosphate, pH 8.0.
2. Lysozyme (Sigma).

3. DNaseI (Sigma).
4. MgCl_2 (Fisher, Fair Lawn, NJ).
5. 8-pnpene (**8**): 15 mM in dimethyl sulfoxide (DMSO).

2.2. Equipment

2.2.1. Synthesis of 8-Pnpene

1. 100-mL round bottom flask.
2. Water-cooled reflux condenser.
3. Heating mantle.
4. Variable autotransformer.
5. Rotary evaporator (Buchi, Flawil, Switzerland).
6. Chamber for TLC plates.
7. Heat gun.

2.2.2. Protein Induction in 96-Well Plates

1. Colony picker (Model Qpix, Genetix, Beaverton, OR).
2. 1- and 2-mL deep-well polypropylene plates* (*see Note 2*) (Becton, Franklin Lakes, NJ).
3. 300 μL flat-bottom 96-well microplate (Rainin, Emeryville, CA).
4. 96-replicator pin (V & P Scientific Inc., San Diego, CA).
5. Incubator shaker with humidity control (Model ISF-1-W, Kuhner, Farmingdale, NY).
6. Centrifuge (Model Allegra 25R, Beckman Coulter Inc., Fullerton, CA).

2.2.3. Colorimetric Aliphatic Hydroxylation Assay

1. Automatic 96-channel pipet (Model Multimek 96, Beckman Coulter).
2. 300 μL flat-bottom 96-well microplate (Rainin).
3. Repetitive pipet (Gilson, Villiers-le-Bel, France).
4. Microplate spectrophotometer (SPECTRAMax PLUS384, Sunnyvale, CA).

3. Methods

3.1. Synthesis of 8-Pnpene

1. 1-Bromooctane (1 g, 5.18 mmol) and 4-nitrophenol, sodium salt (0.92 g, 5.71 mmol) and DMSO (30 mL) are combined in a 100-mL round bottom flask fitted with a water-cooled reflux condenser. The reaction solution is refluxed at 120°C for 5 h. A heating mantle connected to a variable autotransformer controls the temperature. The reaction can be followed by thin layer chromatography (TLC) (*see Note 3*).
2. The DMSO is distilled off to near dryness using a rotary evaporator under vacuum with the round bottom flask heated to ~60°C.
3. The resulting brown residue is loaded onto a silica column and eluted with 10:1 mixture of petroleum ether and diethyl ether.

*Sterilize by autoclave before use.

4. The fractions containing the product are identified by TLC and pooled together. The organic solvent is removed on a rotavap. The resulting colorless, viscous liquid is the product.
5. The purity is verified using thin layer chromatography or by ^1H nuclear magnetic resonance spectroscopy (see **Note 4**).

3.2. Protein Induction in 96-Well Plates

The P450 BM3 gene was cloned into the expression vector *pCWOri* (+) (**13**) resulting in the plasmid pBM3_WT18-6 (see **Note 5**). The vector contains an ampicillin resistant coding region, and a double *Ptac* promoter controls expression.

1. DH5 α cells are transformed with P450 BM3 mutant library, plated on LB agar containing ampicillin, and grown for ~24 h at 37°C.
2. Individual colonies are picked with a robot (see **Note 6**) into 1 mL deep-well plates containing LB media (0.3 mL) and ampicillin (100 mg/L).
3. The plates are incubated at 30°C, 250 rpm, and 80% relative humidity (see **Note 7**) for 24 h.
4. Clones from the 24-h culture are inoculated with a 96-pin replicator into 2 mL deep-well plates containing Terrific broth medium (400 μL), trace elements (250 $\mu\text{L/L}$), ampicillin (100 mg/L), IPTG (10 μM), and ALA (0.5 mM). The plates from the preculture are stored at 4°C and used to isolate active clones identified in the assay.
5. The clones are cultivated at 30°C for 24–30 h (see **Note 8**).
6. The plates are centrifuged at 2300g, and the medium is discarded.
7. Cell pellets are frozen at –20°C (see **Note 9**).

3.3. Colorimetric Aliphatic Hydroxylation Assay

1. The frozen cell pellets are resuspended in phosphate buffer (1 mL) containing lysozyme (0.5 mg/mL), DNase I (0.1 $\mu\text{g/mL}$), and MgCl_2 (10 mM) using a pipetting robot (see **Note 10**). The solution is incubated at 37°C for 60 min.
2. The lysates are centrifuged at 2300g for 10 min.
3. The supernatant (150 μL) is transferred to 96-well microtiter plates (see **Note 11**) using a pipetting robot, and 8-pnpane (2.0 μL , 150 μM) in DMSO (1%) is added using a repetitive pipet (see **Note 12**).
4. After incubation for 5 min at room temperature, NADPH (50 μL , 200 μM) is added.
5. The absorbance at 410 nm is measured with a microplate spectrophotometer (see **Note 13**).

4. Notes

1. Dimethyl sulfoxide and 4-nitrophenol, sodium salt purchased should be of highest purity and free of H_2O . Alternatively, dimethyl sulfoxide can be distilled under reduced pressure in the presence of CaH_2 (**14**). 4-Nitrophenol, sodium salt can be dried by placing the compound in a tarred round bottom flask and dried under vacuum to a constant weight.

2. Deep-well plates with cone shaped bottoms are better than flat-bottom plates because cells and cell debris pellet more efficiently.
3. Thin layer chromatography can be used to determine the purity of the product. The retention factor (distance travelled by compound/distance travelled by solvent front) for 1-bromooctane, *p*-nitrophenol, sodium salt, and 8-pnpne are 0.9, 0.0, and 0.6, respectively, when using silica gel plates with a 10:1 mixture of petroleum ether and diethylether as the elutant. *p*-Nitrophenol, sodium salt and 8-pnpne can be visualized with ultraviolet light. Dip the plate in a phosphomolybdic acid solution, allow to dry and expose to heat using a heat gun to stain 1-bromooctane.
4. ^1H NMR (CDCl_3 , peaks at $\delta = 8.18$ (m, 2H), 6.93 (m, 2H), 4.04 (t, 2H), 1.81 (p, 2H), 1.33 (m, 10H), 0.89 (t, 3H).
5. The P450 BM3 gene was originally contained in the pT-UCS1BM3 (**9**), and expression was under the control of a temperature inducible P_{RPL} -promoter. Expression of P450 BM3 was found to be low and variable in 2 mL deep-well plates. Furthermore, the wildtype activity for 8-pnpne was low. Therefore, the gene was introduced into the expression vector *pCWOri* (+) (**13**), which was found to have higher and less variability in expression.
6. If a robot is not available, then colonies can be picked manually using sterilized toothpicks.
7. If a humidity-controlled shaker is not available then plates can be placed into Tupperware containers with damp sponges and incubated in a shaker.
8. The cell growth should be monitored during expression to determine whether the enzyme has become toxic to the cells. This can be a problem especially during later generations when the mutants become very active.
9. Freezing the cell pellets is necessary to obtain complete cell lysis.
10. If a pipetting robot is not available then an automated 8-channel pipet can be substituted.
11. The amount of lysate used in the assay needs to be adjusted as the enzyme becomes more active in further generations.
12. Other organic solvents can be substituted to dissolve 8-pnpne such as methanol. However, volatile solvents tend to drip from the pipet tip.
13. The formation of the yellow color is due to *p*-nitrophenylate, and the assay is pH dependent. For example, calculations using the Henderson-Hasselbalch equation indicate that at pH 8.1, *p*-nitrophenol is 90% deprotonated (**9**).

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High-Throughput Screens Based on NAD(P)H Depletion

Anton Glieder and Peter Meinhold

1. Introduction

Screening conditions should simulate the final application as closely as possible. This is especially a challenge when chromophore-free (e.g., aliphatic) substrates are used and no simple and reliable high-throughput method for quantitative analysis of the respective reaction product is available. The screening procedure should also be generally applicable for a certain class of enzymes. Generation of “surrogate substrates” by derivatization with chromogenic groups can have a negative effect on enzyme development by directed evolution, e.g., by causing a change in substrate specificity.

Stoichiometric cofactor dependence, a common feature of many oxidative enzymes, allows the design of high throughput assays using unmodified substrates of industrial interest. It also permits one to change to other target substrates without modifying the experimental procedure. These assays allow for exact determination of catalytic rates. However, electron transfer from the cofactor can also result in cofactor oxidation not coupled to the formation of the desired product. Therefore, assays based on cofactors should always be combined with some measure of product formation (e.g., with surrogate substrates) or with an analysis of putative products of uncoupled cofactor oxidation (e.g., peroxide formation).

To illustrate NAD(P)H depletion assays for oxidoreductases we use examples from screening of mutant libraries of the water soluble self-sufficient cytochrome P450 BM3 (*I*) for high alkane hydroxylase activity (*II*). The same procedure can also be applied to NADH-dependent enzymes. NAD(P)H depletion upon substrate hydroxylation can be monitored directly or indirectly

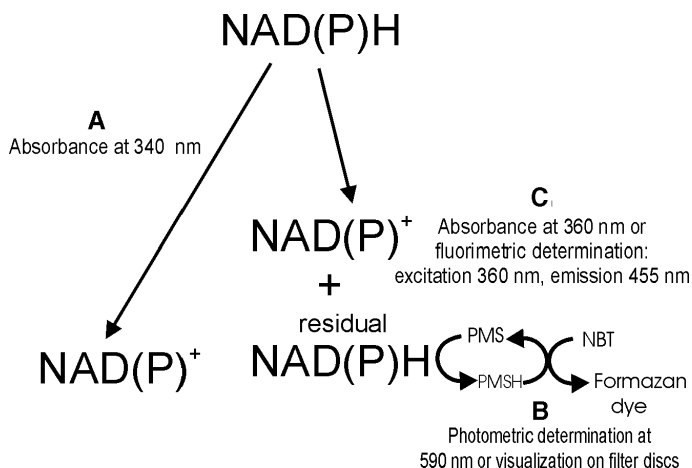


Fig. 1. NAD(P)H depletion can be measured by monitoring the decrease in absorbance of NAD(P)H at 340 nm (A). Alternatively, colorimetric analysis (B) of the concentration of residual NAD(P)H or the highly sensitive photometric or fluorometric determination (C) of the oxidized reaction product NAD(P)⁺ can be used to determine the rate of NAD(P)H depletion.

(see Fig. 1). The kinetics of the reaction can be determined directly from the decrease in absorbance at 340 nm, this being directly proportional to enzymatic activity (2). Colorimetric solid or liquid phase assays monitor NAD(P)H depletion indirectly by reduction of tetrazolium, e.g., nitroblue tetrazolium (NBT), salts to formazan dyes. Formation of formazan from tetrazolium salts by the reducing capacity of living cells can be used to monitor cell viability in cultures (3,4). Tetrazolium salts are reduced by the physiologically important reducing agents NADH and NADPH. Electron transfer from other reducing agents (e.g., dithiothreitol or 2-mercaptoethanol) is much slower than that from NAD(P)H. The efficiency of the reaction is further enhanced in the presence of the catalyst phenazine methosulfate (PMS). The reduction of tetrazolium in the presence of PMS has been described as an analytical method for assaying NADPH-dependent dehydrogenase activity (3,5) (see Chapter 19). It can also be used in a reverse assay in which residual NAD(P)H is titrated by NBT in the presence of PMS to produce visible formazan. Decreased absorption at 590 nm is used to detect the depletion of NAD(P)H (see Fig. 1) in microtiter plate screening assays or for detection of bacterial colonies which produce active enzyme on agar plate cultures. In this case, NAD(P)H reduces NBT on the whole assay filter, but areas from active colonies remain bright. Active colonies appear as white or slightly purple spots on a purple background.

Alternatively, the oxidized form of NADPH - NAD(P)^+ - can be measured. This method is based on the fact that destruction of NAD(P)H in strong alkali results in the formation of highly fluorescent products (6–8). This method has recently been adapted for high-throughput screening in microtiter plates by Tsotsou et al. (9) and is well suited for screening random libraries of NAD(P)H-dependent oxidoreductases exhibiting low activity, including P450s. The principle of this assay lies in the fact that NAD(P)H and NAD(P)^+ are differentially sensitive to destruction by acid and alkali. NAD(P)H is rapidly destroyed at low pH, which leaves NAD(P)^+ completely intact. At high pH, NAD(P)H is stable whereas NAD(P)^+ is destroyed and yields the fluorescent product.

If enzymes are screened for activity towards novel substrates, an NAD(P)H consumption assay may not be sensitive enough. In this case, the method described here is a highly sensitive alternative (detection limit = $1 \mu\text{M}$ NAD[P]^+). The standard deviation of this assay is very low, and it can also be performed using partially lysed cells (9) (not described here).

2. Materials

1. *E. coli* DH5 α .
2. 1-mL deep-well plates.
3. 2-mL deep-well plates.
4. Sterile toothpicks.
5. 8-channel multipipets.
6. Yeast extract.
7. Tryptone.
8. NaCl.
9. Isopropyl- β -D-thiogalactoside (IPTG).
10. δ -Aminolevulinic acid hydrochloride.
11. 90-mm nitrocellulose filter discs.
12. Nitroblue tetrazolium (NBT).
13. Phenazine methosulfate (PMS).
14. Trace elements: 0.5 g MgCl_2 , 30.0 g $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 1.0 g $\text{ZnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.2 g $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 1.0 g $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.5 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.0 g CuCl_2 and 0.2 g Na_3BO_3 in 1 L HCl solution (90% v/v distilled water: concentrated HCl).
15. Luria broth (LB) media.
16. Terrific broth (TB) media.
17. Color reagent: 0.5 mg NBT and 0.03 mg PMS in 1 mL 0.1 M phosphate buffer, pH 8.0.
18. Deep-well plate shaker (e.g., ISF-1-W; Kuehner, Birsfelden, Switzerland).
19. Lysis buffer: 0.1 M potassium phosphate buffer, pH 8.0, 0.5 $\mu\text{g/mL}$ lysozyme (Sigma L-6876), 0.1 $\mu\text{g/mL}$ DNase I (Sigma, 650 U/mg), 10 mM MgCl_2 .
20. Dimethylsulfoxide (DMSO) or methanol.
21. 0.1 mM Potassium phosphate buffer, pH 8.0.
22. NAD(P)H (Biocatalytics, Pasadena, CA).

23. 0.3 M HCl.
24. 9 M NaOH.
25. 96-well microtiter plates.
26. 96-well microtiter plates for fluorescence readings.
27. Microplate fluorometer or microplate spectrophotometer (e.g., Spectramax Gemini XS or 384+; Molecular Devices, Sunnyvale, CA).

Optional:

28. Colony picking robot (e.g., Qpix from Genetix, Hampshire, UK).
29. Pipetting robot (e.g., Beckmann, Fullerton, CA).
30. 96-pin replicator (V&P Scientific, San Diego, CA).

3. Methods

These screening procedures for mutants of NAD(P)H-dependent enzymes include a primary screen and rescreening of selected mutants. The procedures start with 1) the growth of cells and induction of protein expression, continue with 2) preparation of cell lysates or permeabilization of cell membranes for whole-cell assays, and end with 3) direct or indirect monitoring of NAD(P)H depletion as an indicator of the progress of the enzymatic reaction.

3.1. Cell Growth and Induction

An *E. coli* system permitting inducible expression of the *Bacillus megaterium* cytochrome P450 BM3 and variants thereof is described in **Subheading 3.1.1.**, **Subheadings 3.1.1.1.**, and **3.1.1.2.** briefly describe the generation of mutant libraries and growth of mutants in micro-scale liquid cultures (for liquid assays) and on agar plates (for solid-phase assays).

3.1.1. The Expression System

For construction of a plasmid which allows IPTG-inducible expression of the soluble and self-sufficient P450 BM3, the cytochrome P450 gene was amplified from plasmid pT-USC1BM3 (**10**) by PCR (**11**). DNA cloning was performed by standard recombinant DNA methods (**12**) using a “QiaPrep” Spin Mini Prep Kit (Qiagen, Valencia, CA) for isolation of plasmid DNA and a “QiaQuick” kit (Qiagen) for purification of DNA fragments from Tris-Acetate EDTA (TAE) agarose gels. Restriction sites for cloning of the gene behind the strong and inducible double tac promoter of *pCWOri* (+) (**13**) by *Bam*HI and *Eco*RI sites were introduced by this PCR (**11**). In order to be able to evolve the heme domain independently from the rest of the enzyme, a silent mutation was introduced to produce a *Sac*I site, 130 bases upstream of the end of the heme domain. The QuickChange (Stratagene, La Jolla, CA) protocol was followed. The resulting expression plasmid pBM3BamSacEco (**Fig. 2**) allows directed evolution of the hydroxylase domain independently from the rest of the protein (**11**).

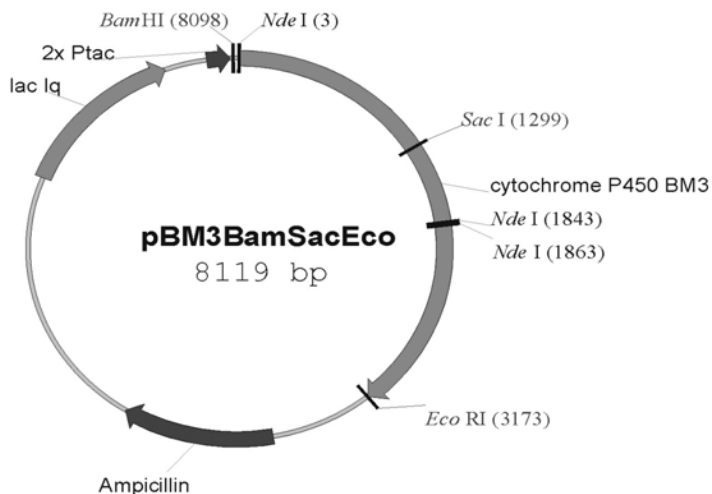


Fig. 2. The expression plasmid pBM3BamSacEco allows inducible expression of cytochrome P450 BM3 from *B. megaterium* in *E. coli*. The heme domain can be cloned and evolved independently from the rest of the protein by using the *Bam*HI and *Sac*I restriction sites for cloning.

3.1.1.1. LIQUID CULTURES

1. For screening variants by liquid NAD(P)H depletion assays, the expression plasmid pBM3BamSacEco for the wild-type P450 BM3 as well as the mutant libraries generated either by error-prone PCR or StEP recombination (**14**) are transformed into *E. coli* DH5 α (**11,15**), plated on LB agar plates containing 100 mg/L ampicillin, and grown overnight at 37°C.
2. Single colonies are picked by a Qpix (Genetix) picking robot (*see Note 1*) and inoculated into 96-well 1-mL deep-well plates containing 350 μ L LB media (100 mg/L ampicillin) (*see Note 2*).
3. These preculture plates are incubated at 30°C, 80% humidity, and 250 rpm and grown for ~ 24 h to reach a uniform cell density at the beginning of their stationary growth phase.
4. For induction of P450 protein production, 400 μ L TB-induction medium in 2-mL deep-well plates is inoculated with a 96-pin replicator from the preculture plates. TB-induction medium contains standard Terrific Broth (**12**), 100 mg/L ampicillin, isopropyl- β -D-thiogalactoside (IPTG, 10 μ M) for induction of expression (*see Note 3*), 250 μ L/L trace elements, and δ -aminolevulinic acid hydrochloride (0.5 mM) as a heme precursor (*see Note 4*). Induced cultures are cultured for 24–30 h under the same conditions as the preculture.
5. The preculture plates are kept as clone collections at 4°C or frozen at –80°C after addition of 350 μ L sterile 50% glycerol.

3.1.1.2. SOLID PHASE CULTURES

The expression plasmid pBM3BamSacEco for the wild-type P450 BM3 as well as mutant libraries are generated either by error-prone PCR or StEP recombination (**14**), transformed into *E. coli* DH5 α , and plated on LB agar plates containing 100 mg/L ampicillin, 1 mM δ -aminolevulinic acid hydrochloride as a heme precursor, and 10 μ M isopropyl- β -D-thiogalactopyranoside (IPTG) to induce expression (*see Note 3*). The solid phase NAD(P)H depletion assay can be applied directly on colonies grown overnight at 30°C.

3.2. Cell Permeabilization and Lysis

Many substrates cannot easily diffuse across the cell membrane and are therefore inaccessible to intracellular enzymes. Two approaches have been employed to overcome this problem: clear cell lysates can be prepared (**Subheading 3.2.1.**) for spectrophotometric assays or, as an alternative for colorimetric whole-cell assays, cell permeabilizers can be added to substrate solutions to facilitate substrate diffusion into the cell (**Subheading 3.2.2.**).

3.2.1. Lysis of Cells from Liquid Cultures

1. Harvest cells from induced 400 μ L liquid cultures by centrifugation at 1700g for 20 min.
2. Initiate cell lysis by resuspension of the cells in 1 mL (or less, dependent on how much enzyme is desired for the assay) of freshly prepared lysis buffer.
3. Resuspend by pipetting 100 μ L up and down until no pellets are left (approx 60 $\times$ with a 12 channel multipipette or a pipetting robot).
4. Incubate resuspended cells at 37°C for 60 min to complete cell lysis.
5. Centrifuge 200 μ L of the cell lysate in 96-well PCR plates for 20 min at 1700g. The resulting clear lysates are ready to use or prepare enzyme dilutions for spectrophotometric NAD(P)H depletion assays.

3.2.2. Cell Permeabilization for Whole-Cell Assays

Whole-cells can be used for spectrophotometric assays, especially when the absorbance of diluted cultures is low in comparison to that of the colored reaction product from the enzyme reaction. Whole cells can also be employed for kinetic assays, where a slope of absorbance increasing with time is determined in contrast to constant absorbance from the cells. However, in many cases, free substrate diffusion to intracellular proteins is not possible. Cell permeabilizers, such as detergents, salts, organic solvents, or polymyxin B, can help to get the substrate into the cells (**16**). In the procedures described here, polymyxin B sulfate is directly employed in the enzyme assays by addition to the substrate solution.

3.3. Enzyme Assays

Under the following sections, we describe high-throughput assays for oxidations by NAD(P)H-dependent enzymes. The protocols are adapted for screening cytochrome P450 BM3 libraries using alkanes or fatty acids as substrates. The protocols are also applicable to other NAD(P)H-dependent enzymes if the reaction conditions are adjusted. Depletion of NAD(P)H can be monitored directly in a spectrophotometric assay (**Subheading 3.3.1.**), or indirectly by titration of the residual NAD(P)H in colorimetric liquid or solid phase assays (**Subheadings 3.3.2.–3.3.4.**). In addition, depletion of NAD(P)H can be monitored by a sensitive fluorometric liquid phase assay measuring oxidized NAD(P)⁺ (**Subheading 3.3.5.**).

3.3.1. Direct Kinetic NAD(P)H Depletion Assay

NADH and NADPH show identical absorption maxima at 340 nm, while their corresponding oxidized forms, NAD⁺ and NADP⁺, do not absorb at this wavelength. Therefore, reaction kinetics can be assayed at 340 nm ($\epsilon_{340} = 6.22 \text{ L mmol}^{-1} \text{ cm}^{-1}$) by monitoring NAD(P)H depletion owing to substrate oxidation directly.

3.3.1.1. LIQUID ALKANE SUBSTRATES

1. Prepare substrate stock solutions (e.g., 100 mM alkanes in methanol or DMSO).
2. Transfer 50 μL of bacterial lysates, bacterial cultures for whole-cell assays, or dilutions thereof, into 96-well microtiter plates.
3. Add 100 μL of 0.1 M phosphate buffer, pH 8.
4. Add 2 μL of substrate stocks to get a final concentration of 1 mM alkane and 1% organic solvent (*see Note 5*).
5. Transfer the microtiter plate immediately into a plate reader (e.g., Spectramax 384+ from Molecular Devices) (*see Note 7*).
6. Start the reaction by addition of 50 μL of 0.8 mM NAD(P)H in 0.1 M phosphate buffer, pH 8.0 (*see Note 6*), and follow the decrease in absorbance at 340 nm for 1–3 min (*see Note 7*).
7. Prepare another plate with the same enzyme samples, but omit the substrate from the solution and make the measurement as described above. This measurement gives the background activity (e.g., uncoupled cofactor oxidation or oxidation of the solvent or components of the cell lysate).
8. Clones which show higher rates of NAD(P)H depletion with a low background rate are selected and streaked out on fresh LB agar plates (100 mg/L ampicillin) from preculture plates for further characterization by a rescreening procedure.

3.3.1.2. GASEOUS SUBSTRATES

Substrate solutions of gases (e.g., small alkanes) can be prepared by bubbling the substrate into buffer solutions up to saturation (e.g., alkane concen-

trations of ~1.5 mM methane, 2.1 mM ethane, 2.7 mM propane, and 6.6 mM butane (17) can be reached this way).

1. Prepare substrate solutions by bubbling the respective gaseous substrate into 0.1 M phosphate buffer for ~30 min (1–2 bubbles/s, using a glass Pasteur pipet).
2. Transfer 30 μ L of bacterial lysates (or bacterial cultures for whole-cell assays) into 96-well microtiter plates.
3. Add 170 μ L of the saturated substrate solution.
4. Transfer the microtiter plate into a plate reader (e.g., Spectramax from Molecular Devices).
5. Start the reaction by addition of 50 μ L 0.1 M phosphate buffer containing 0.8 mM NADPH and follow the decrease in absorbance at 340 nm for 1–5 min (see **Note 10**).
6. Prepare another plate with the same enzyme samples but omit the saturation of buffer with the gaseous substrate and repeat the measurement as described above. This measurement gives the background activities (e.g., uncoupled cofactor oxidation or oxidation of components of the cell lysate).
7. Clones which exhibit higher rates of NAD(P)H depletion with a low background rate are selected and streaked out on fresh LB agar plates (100 mg/L ampicillin) from preculture plates for further characterization by a rescreening procedure.

3.3.2. Indirect Colorimetric NAD(P)H Depletion Assay

A colorimetric assay for NAD(P)H depletion can be directly applied for assays with bacterial lysates (**Fig. 3**) and also for bacterial cultures containing whole-cells or with substrate-soaked filters for bacterial colonies grown on agar plates. In the case of whole-cell assays, substrate diffusion to the intracellular enzyme is facilitated by addition of a cell permeabilizer. NAD(P)H is depleted by hydroxylation of the substrate and residual reduced cofactor is titrated by formation of formazan from the reducible color dye NBT (see **Note 8**).

3.3.2.1. LIQUID ASSAY FOR CELL LYSATES

1. Add substrates (e.g., 1–5 mM alkanes) to 0.1 M phosphate buffer, pH 8.0. Insoluble substrates can be emulsified by sonication. For substrate solutions containing gaseous substrates, use a Pasteur pipet and bubble the substrate into the same solution for ~30 min (1–2 bubbles/s).
2. Transfer 50 μ L of bacterial lysates into 96-well microtiter plates.
3. Add 100 μ L of substrate solution.
4. Start the reaction by addition of 50 μ L of 1.6 mM NAD(P)H in 1 mL 0.1 M phosphate buffer, pH 8.0 (see **Notes 9** and **10**).
5. Add 50 μ L of color reagent (0.5 mg NBT and 0.03 mg PMS in 0.1 M phosphate buffer, pH 8.0) to stop the reaction by reaction of residual NAD(P)H.
6. Mix well and make an endpoint measurement after ~3 min at 590 nm using a microtiter plate reader. Enzymatic activity is inversely proportional to the final absorbance.

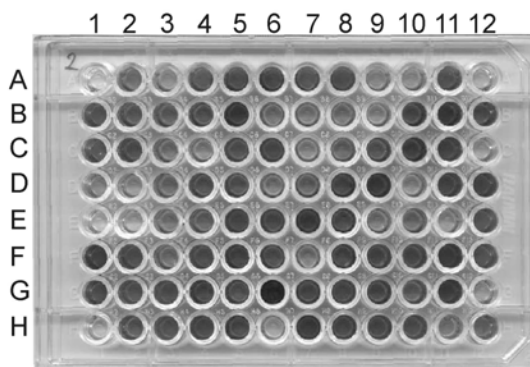


Fig. 3. Colorimetric NAD(P)H depletion assay for octane hydroxylation by mutants of cytochrome P450 BM3. Bacterial lysates of a mutant library of P450 BM3 were incubated with octane and NAD(P)H in phosphate buffer. NAD(P)H was consumed for substrate hydroxylation, and after 5 min a color reagent containing nitroblue tetrazolium (NBT) and phenazine methosulfate (PMS) was added. NBT was reduced by residual NAD(P)H and formed a dark purple dye. Lysates with high hydroxylase activity consumed most of the NAD(P)H and remained light after addition of the color reagent. Lane 3A–3F contains positive controls from the previous generation. Wells that are lighter than the controls from the previous generation (3A–3F) indicate improved enzyme variants. Wells 3G and 3H were controls for cross-contamination during cell growth and were not inoculated in the culture plates.

7. Select improved clones and streak out selected clones from preculture plates for single colony formation on LB agar plates containing 100 mg/L ampicillin.

3.3.2.2. COLORIMETRIC WHOLE-CELL LIQUID ASSAY

1. Add substrates (e.g., 1–5 mM alkanes) to 0.1 M phosphate buffer, pH 8.0, containing 100 μ M polymyxin B sulfate as a cell permeabilizer. Insoluble substrates can be emulsified by sonication (*see Note 11*). For solutions containing gaseous substrates use a Pasteur pipet and bubble the substrate into the same solution for ~30 min (1–2 bubbles/s).
2. Transfer 50 μ L of bacterial cultures into 96-well microtiter plates.
3. Add 100 μ L of whole-cell substrate solution and proceed as under **Subheading 3.3.2.1**.

3.3.2.3. COLORIMETRIC SOLID-PHASE ASSAY

1. Add substrates (e.g., 1–5 mM alkanes) to 0.1 M phosphate buffer, pH 8.0, containing 100 μ M polymyxin B sulfate as a cell permeabilizer and 1.6 mM NADPH (*see Note 10*). Insoluble substrates can be emulsified by sonication (*see Note 11*). For solutions containing gaseous substrates use a Pasteur pipet and bubble the substrate into the same solution for ~30 min (1–2 bubbles/s).

2. Soak nitrocellulose membranes (e.g., 90 mm discs for standard Petri dishes) with the substrate solution for 1 min and place it directly on top of the bacterial colonies on agar plates.
3. Keep the soaked membranes on the agar plates for ~2–15 min. Sensitivity of the assay can be regulated by varying of the incubation time.
4. Visualize depleted NAD(P)H indirectly by the addition of 1 mL color reagent (0.5 mg NBT and 0.03 mg PMS in 0.1 M phosphate buffer, pH 8.0) on top of the nitrocellulose filter disc. Residual NAD(P)H reacts with NBT. Active colonies used NAD(P)H for substrate hydroxylation. Therefore, they appear as white or lighter areas on a purple background.
5. Lift the filter and pick colonies under the white areas to streak them out on fresh agar plates for single colony formation.

3.3.3. Detection of Alkali Product of NAD(P)⁺

This method describes the destruction of residual NAD(P)H with acid after the enzymatic reaction followed by conversion of the NAD(P)⁺ to a highly fluorescent alkali product, measured spectrophotometrically or fluorometrically

1. Transfer 30 μ L of cell lysates or dilutions thereof into 96-well microtiter plates.
2. Add 170 μ L of the substrate solution (*see Subheading 3.3.2.1.*) for preparation of the substrate solution).
3. Start the reaction by addition of 50 μ L 0.1 M phosphate buffer containing 1.6 mM NADPH (*see Notes 6 and 12*).
4. Incubate both the reaction mixture containing the substrate and the control without substrate for an appropriate amount of time (*see Note 13*) at room temperature.
5. Transfer an aliquot of 70 μ L of the reaction mix to a new microtiter plate and add 70 μ L of 0.3 M HCl (*see Note 14*).
6. Incubate for at least 10 min (*see Note 15*).
7. Transfer 80 μ L of the HCl-treated sample to a new microtiter plate previously filled with 270 μ L of 9 M NaOH (*see Notes 16 and 24*), immediately mix thoroughly (*see Notes 17 and 18*) and place in dark (*see Note 19*).
8. Incubate for at least 2.5 h (*see Note 20*) in the dark at room temperature (*see Note 21*).
9. Measure the fluorescence with an excitation wavelength of 360 nm and an emission wavelength of 455 nm (*see Notes 21 and 22*) or measure the OD spectrophotometrically at 360 nm (*see Note 23*).
10. The NAD(P)⁺ concentration can be calculated according to a NAD(P)⁺ standard curve. It has been found that depending on the substrate there might be deviations in the slope of this curve. Therefore, it is best to make a NAD(P)⁺ standard curve under conditions used for the screen.

3.4. Rescreening of Improved Mutants

Since false positives from the primary screen can be expected, selected mutants should be rescreened. Therefore they are streaked out on fresh media to obtain pure single clones and then grown again in deep-well plates for liquid screening.

	Plate#1												
	Vmax	1	2	3	4	5	6	7	8	9	10	11	12
A		-147.1	-294.9	-188.9	-497.3	-328.4	-462.3	-392.5	-333.3	-190.2	-154.1	-303.4	-306.7
B		-133.4	-313.1	-186.5	-459.3	-308.9	-445.8	-371.4	-326.9	-178.7	-155.4	-299.4	-285.3
C		-131.3	-295.5	-160.1	-484.7	-332.0	-479.7	-391.4	-373.4	-195.9	-42.60	-310.7	-227.7
D		-150.3	-315.9	-163.9	-482.3	-303.3	-460.3	-394.9	-320.3	-187.6	-162.7	-282.3	-229.9
E		-118.4	-278.0	-73.13	-252.1	-349.1	-320.9	-152.4	-128.2	-211.0	-187.8	-293.1	-445.5
F		-133.8	-287.1	-87.13	-223.6	-357.9	-308.8	-197.1	-137.1	-231.7	-164.1	-300.9	-467.0
G		-126.6	-284.6	-82.53	-234.8	-339.7	-306.0	-170.1	-102.9	-196.1	-175.9	-312.6	-396.9
H		-107.5	-263.1	-99.00	-233.5	-334.1	-338.8	-184.5	-138.1	-202.6	-168.9	-305.3	-407.8

Fig. 4. Direct monitoring of NAD(P)H depletion in a plate reader at 340 nm. Four colonies of each clone selected during the primary screening were grown in deep-well plates. Wells **3A–3D** and **3E–3H** contain positive controls from the previous generation. 200 μ L of bacterial lysates in 0.1 M phosphate buffer, pH 8.0 and 0.2 mM NADPH were incubated with 2 μ L octane stock (100 mM octane in DMSO), and the decrease in absorbance was determined at 340 nm.

3.4.1. Rescreening Procedure

Select the clones from the primary screening that show high rates of NAD(P)H depletion at low background rates.

1. Take four single colonies of each selected clone for inoculation in LB medium for a 96-deep well culture, as for the primary screen.
2. Proceed as described for the primary screening procedure (see **Fig. 4**).

4. Notes

1. If more than ~1000 clones are screened per day, it starts to be worthwhile to employ a robotic system (e.g., a colony picking robot and/or a pipetting robot) for screening. Because of setup and service time, such automated systems are not helpful for fewer clone numbers.
2. Usually one lane contains clones with known (positive control) and no hydroxylase activity (negative control). As a positive control, usually the best clone of the previous generation is used. Two wells of this lane, which are not inoculated, serve as a control for cross-contamination during cultivation.
3. Advanced generations of evolved P450 BM3 libraries showed cytotoxic effects under conditions where 1 mM IPTG was added at the beginning of the main culture. Because of these expression problems, clones selected during the screening procedure did not show the expected high activity in shake flask experiments.

Weaker induction with 10 mM IPTG or short induction (1–4 h) with 1 mM IPTG at the end of the growth phase helped to reduce cytotoxicity effects.

4. Trace elements and δ -aminolevulinic acid hydrochloride can be omitted from the media, but this may reduce expression of the P450 BM3 variants.
5. Different concentrations of substrate stocks (50–250 mM) can be used for the kinetic plate reader assays to get an indication of whether there is a significant change in the K_m of the mutants.
6. If a stock solution of 1.6 mM NADPH is used, then NAD(P)H consumption at 340 nm and NADP⁺ production (endpoint at 360 nm or fluorometrically) can be determined sequentially. But higher concentrations of NAD(P)H cause high initial absolute absorbance at 340 nm and therefore a higher standard deviation of the background.
7. For the described assay conditions, the plate reader should be able to make at least one measurement every ~ 10 s. As an alternative, plate readers that add NAD(P)H in the reader (e.g., Fluostar from BMG, Germany) can be used to allow kinetic measurements within the first few seconds of the reaction.
8. It is advisable to make a preliminary experiment to determine the optimal incubation time. Therefore, dilutions (1:2, 1:5, 1:10) are made from lysates with known oxidoreductase activity. 50 μ L of these dilutions, and as a negative control, also 50 μ L of a lysate with no hydroxylase activity are pipetted into a 96-well microtiter plate (wells 1–4). Each sample can be repeated eight times (A–H). Repeat the whole series two times in the same microtiter plate (5–8 and 9–12). Start the reactions at the same time by addition of 50 μ L of a 0.8–1.6 mM NAD(P)H stock. Stop the three dilution series after different reaction times (e.g., 2 min, 5 min, and 15 min) by visualization of residual NAD(P)H by formation of the dye formazan. This allows an estimation of the optimal reaction time to see significant differences between samples that show variations in hydroxylation activity. Another possibility to regulate the sensitivity of the assay, when incubation times should be constant, is to change NAD(P)H concentrations (0.1–5 mM, but not higher than substrate concentrations).
9. The final concentration of NAD(P)H in whole-cell assays should not be lower than 0.2 mM, since reduction of NBT to formazan by viable microorganisms is also used to monitor cell viability. Therefore, variations in cell viability (e.g., because of toxicity of overexpressed proteins) of *E. coli* clones can significantly influence the assay at low NAD(P)H concentrations.
10. Since the highest rates may be observed during the first few seconds, it is critical to add NAD(P)H rapidly, either using a pipetting robot or using at least an 8-channel multipipet.
11. Alternatively, substrate stocks can be prepared by solubilization in organic solvents such as methanol or DMSO. The final concentration of the solvent should be 0.5–1% (v/v). Another possibility to regulate the sensitivity of the assay, when incubation times should be constant, is to change NAD(P)H concentrations (0.1–5 mM, but not higher than substrate concentrations).
12. The NAD(P)⁺ formation assay works best at a relatively high NAD(P)H concentration of 1.5 mM (this would yield an absorbance of 9.33 at 340 nm). In order to

couple this assay to the kinetic NAD(P)H consumption assay, a concentration of 320 μM (OD ~ 2) can be chosen. NAD(P)H consumption is used for screening libraries with relatively high activity. If no activity can be measured this way, then use the NAD(P)⁺ formation assay.

13. The ratio of the alkali product concentration in a reaction mixture with substrate to the alkali product concentration in a reaction mixture without substrate depends on reaction time, substrate concentration and catalytic efficiency (9). When screening mutant libraries the reaction must be stopped before all NAD(P)H is consumed, generally within the first 10 min.
14. The pH should now be around 2, at which remaining NAD(P)H is completely destroyed.
15. The enzymatic reaction should be stopped at exactly the same time for each plate by adding acid. Also, as NAD(P)H is relatively stable at high pH, it must be completely destroyed prior to adding alkali.
16. The pH should now be around 14.8.
17. The use of a pipetting robot will significantly lower the standard deviation and reduce hands-on time.
18. The yield of fluorescent product increases almost linearly with NaOH concentration from 0.05 M to 6 M , even though the nucleotides are destroyed completely over the whole range (7). As highly concentrated NaOH is viscous and has a higher density than water, insufficient mixing will result in pH gradients and the assay will become irreproducible. Therefore the sample should be added to the alkali (not vice versa) and mixed immediately.
19. The fluorescent product is light sensitive and thus all samples should be incubated in the dark.
20. Formation of product is complete after 2.5 h. The reaction can also be carried out overnight, as there is no detectable decrease in fluorescence for at least 15 h.
21. Formation of the alkali product of NAD(P)⁺ is temperature dependent. If different plates are to be compared with each other, it is therefore important to carry out all screens at the same temperature.
22. Several companies sell black or white plates with lower background fluorescence than normal, clear plates.
23. Fluorescence measurements are more sensitive ($\sim 1 \mu\text{M}$ NAD[P]⁺) than spectroscopic measurements ($\sim 10 \mu\text{M}$). However, the fluorescence measurement is only linear up to a NAD(P)⁺ concentration of 10 μM because of absorption of the exciting light by the product (7). The spectrophotometric measurement is linear up to a NAD(P)⁺ concentration of at least 2 mM . Thus the method used depends on the activity on the enzyme.
24. The 9 M NaOH should be prepared on ice. NaOH at this concentration is very corrosive.

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High-Throughput Tetramethylbenzidine (TMB) Screen for Peroxidases

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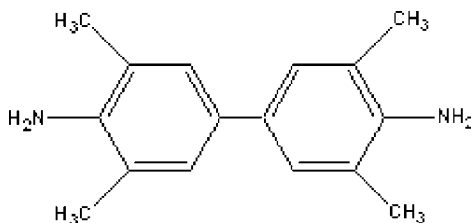
1. Introduction

Peroxidases catalyze the decomposition of hydrogen peroxide coupled to the oxidation of a variety of organic and inorganic substrates. Produced by a broad variety of natural sources, most peroxidases use heme or vanadium as a cofactor at the redox active site, while some bacterial peroxidases function without a metal cofactor (**1,2**). Peroxidases catalyze a wide variety of oxidative reactions, some of which are used in industrial and biotechnological applications. An activity assay that can be used in a high-throughput fashion becomes important for the engineering of new and improved peroxidases.

One of the most studied peroxidases is horseradish peroxidase (HRP), an enzyme produced in large quantities by the root of the horseradish, *Armoracia rusticana*. The most abundant of the horseradish isoenzymes is the C isoenzyme (HRP-C), the subject of this screening chapter. It is not clear how the HRP enzyme is used in the plant environment, but the commercial uses of HRP are significant, particularly in the medical diagnostics field (**3**).

HRP-C is a relatively stable and highly active enzyme with wide substrate specificity that allows activity measurements by colorimetric, fluorometric, or chemiluminescent methods. Among the numerous colorimetric assays available, one of the most sensitive uses the colorless benzidine-based substrate, 3,3',5,5'-tetramethylbenzidine (TMB, *see* structure drawn on next page). Upon oxidation by HRP-C, a blue charge-transfer complex is quickly formed by reaction with H_2O_2 , followed by a final oxidation to a stable yellow product (**3,4**).

This assay can be used as a kinetic assay, by measuring the absorbance of the blue complex with time, or in an end-point assay where the absorbance of the final product is measured. Low background and good reproducibility make this



assay a particularly good choice for high-throughput experiments such as screening HRP-C mutant libraries for mutants with increased activity, wider substrate specificity, or higher resistance to peroxide (5).

The TMB peroxidase assay described here is a room-temperature assay based on the screening of cell lysate from a culture of *E. coli* cells expressing a recombinant version of HRP-C. The assay also works at elevated temperatures, up to 70°C, which allows screening for thermostable HRP-C mutants.

2. Materials

2.1. Biological and Chemical Materials

1. 96-well clear microtiter plates, flat bottom with lid (Rainin, Emeryville, CA).
2. Luria-Bertani (LB) growth medium, sterilized by autoclaving (6).
3. Ampicillin, sterilized by 0.20 μm filter (A0166, Sigma, St. Louis, MO).
4. Antibiotic/agar plates (from sterilized LB medium and ampicillin).
5. Isopropyl- β -D-thiogalactoside (IPTG), sterilized by 0.20 μm filter.
6. Cell lysing reagent (such as BugBuster™ Protein Extraction Reagent, Novagen Madison, WI).
7. Wood toothpicks (sterilized by autoclaving).
8. Expression plasmid containing the HRP-C gene, under the control of an inducible promoter.
9. XL10 Gold Ultracompetent *E. coli* cells (Stratagene, La Jolla, CA).
10. TMB assay kit consisting of the TMB reagent and H_2O_2 reagent (KPL Inc, Gaithersburg, MD).

2.2. Equipment

1. 96-well microplate reader with absorbance measuring capabilities (Model Spectramax 250, Molecular Devices).
2. Computer software for microplate reader control (Softmax PRO, Molecular Devices).
3. 96-channel pipetting robot with P-200 head (Model Multimek 96, Beckman Coulter, Fullerton, CA).
4. Multi-channel hand-held pipet with electronic control, capable of accurate multiple deliveries in the μL range (Biohit, Helsinki, Finland).
5. Shaking incubator capable of 250 rpm at 30–42°C (Model Innova 4000, New Brunswick Scientific, Edison, NJ).

6. Incubator capable of 30–62°C (Model Thelco, Precision Scientific, Winchester, VA).
7. Lab rotating platform (Model 1314, Lab-Line Instruments Inc., Melrose Park, IL).
8. Plate shaker with temperature control (Model ISF-1-W, Kühner, Birsfelden, Switzerland).
9. Centrifuge equipped with rotor for spinning 96-well plates and 50 mL plastic tubes (centrifuge model GS-6R, rotor model GH-3.8, Beckman Coulter).
10. 96-pin plate replicator (V&P Scientific, San Diego, CA).

3. Methods

3.1. Expression Vector: pCWOri HRP

The expression vector used for the expression of HRP-C is based on the pCWOri (+) vector (7,8). This system is inducible by IPTG, but leaky protein expression is also observed in the absence of IPTG. Taking advantage of the unique restriction sites present in pCWOri (+), the pCWOri HRP vector was constructed by cloning a cDNA copy of the HRP-C gene into the pCWOri (+) vector using standard molecular biology techniques (6). Two copies of a *pelB* sequence were placed directly upstream of the HRP-C coding sequence (Fig. 1) to direct protein production to the periplasm (9).

3.2. Cell Strains Used

Throughout this protocol, the *E. coli* cell strain used for growth and expression is XL10-Gold. These cells are appropriate for high-efficiency transformation of the HRP-C plasmid DNA (see Note 1).

3.3. Protein Production

3.3.1. Protein Induction

Initially, XL10 Gold Ultracompetent cells are used for transformation of the pCWOri HRP expression vector. Cells are then plated on LB agar plates containing ampicillin and allowed to grow at 37°C overnight (see Note 2).

1. Using sterile toothpicks, pick colonies transformed with the HRP expression plasmid into 96-well plates that contain 200 μ L LB medium and 100 μ g/mL ampicillin (see Note 3). Grow the plates for 12–14 h at 37°C in a humidified shaker at 250 rpm. Check cell density with the microplate reader, and stop growth when OD₆₀₀ reaches the saturation plateau. These plates become the master plates and can be stored at –80°C in 20% glycerol for months.
2. Using a 96-pin replicator on the master plates, inoculate fresh 96-well plates containing 200 μ L LB medium, 100 μ g/mL ampicillin, 1 mM δ -aminolevulinic acid, 1 mM CaCl₂, and trace elements (10). These are replica plates and are used for protein production.
3. Grow the replica plates at 37°C for 10–12 h, until OD₆₀₀ reaches ~0.6. Transfer the plates to 30°C and add 1 mM IPTG to induce protein expression. Continue induction for 12–24 h, depending on the activity level desired (see Note 4).

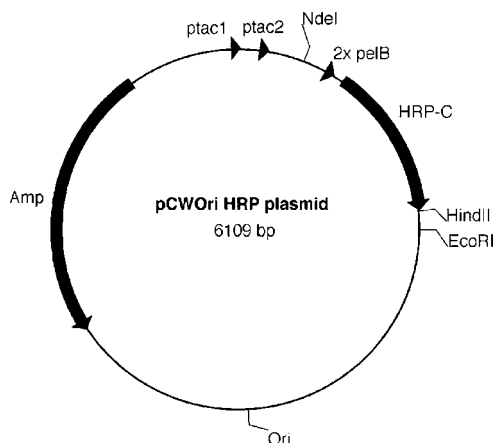


Fig. 1. Plasmid used for expression of recombinant HRP-C in *E. coli*. As shown in the diagram, a double *pelB* signal sequence is used to direct the protein expression to the periplasmic space. Two *ptac* promoters are used to control transcription of HRP-C mRNA (7).

3.3.2. Cell Lysis/Protein Extraction: BugBuster Protocol

It is possible to use a hydrophobic reagent such as BugBuster to lyse the bacterial cells. This detergent-based reagent is used to resuspend pelleted *E. coli* cells from overnight cultures, which releases proteins present in the cytoplasm and periplasm. The following protocol can be used with good results on both μL - and mL -scale cultures (adapted from Novagen):

1. After protein expression, use the GS-6R centrifuge to pellet the cells by high-speed centrifugation for 10–15 min (1900g for 96-well plates, 3000g for tubes). Pre-weigh the empty culture tubes to determine the wet cell mass (see **Note 5**).
2. Discard the supernatant and invert the tubes/plates to allow the cell pellet to drain. Using the pre-determined tube weights, determine the wet pellet mass.
3. Resuspend the wet pellet in room temperature BugBuster reagent. To make sure that the BugBuster reagent reaches the entire sample, it is important to completely resuspend the pellet by pipetting or vortexing (see **Note 6**). Use 5 mL BugBuster reagent per gram of wet pellet, or 1/20th of the original volume in the case of 96-well plate cultures.
4. Incubate the mixture for 10–20 min on a rotating platform at room temperature. Here, several volumes of assay buffer can be added to each sample to increase the total volume, which helps the last transfer step.
5. Pellet the cell debris again by high-speed centrifugation for 20 min at 4°C (see **Note 7**).
6. Carefully transfer the supernatant to a fresh tube. This is the soluble protein extract and can be stored at 4°C.

3.4. Horseradish Peroxidase Activity Assay

1. After HRP expression is complete, extract the protein using the BugBuster protocol from **Subheading 3.3.2.1**. Do not forget to add 100–200 μL assay buffer to each well before the final high-speed spin (assay buffer is 50 mM sodium acetate, pH 4.5).
2. From the lysed samples, transfer aliquots (40–50 μL) of the HRP-C soluble protein extract to a fresh 96-well plate containing 100 μL assay buffer.
3. For the room-temperature assay, add 50 μL of TMB substrate and place the plate in the microplate reader. After initiating the reaction by addition of 50 μL of H_2O_2 substrate, read the absorbance of the blue product at 650 nm (*see Note 8*).
4. Analyze the kinetic data by using the Softmax PRO software and calculate initial rate values for each well. If the wells contain colonies expressing mutant HRP-C enzyme, the initial rate values can be used to select mutants with higher peroxidase activity. However, the selected mutants need to be re-screened in mL-scale cultures to verify the results of the high-throughput screening method.

4. Notes

1. Transformation of the pCW Ori HRP vector works well with many *E. coli* cell strains. However, for transformation of HRP-C mutant libraries created by low-yield mutagenic amplification, fresh XL10 Gold Ultracompetent cells are necessary to achieve a sufficient number of transformants.
2. Keep track of the size of the fast-growing XL10 Gold colonies, since the larger colonies are more likely to give rise to satellite colonies that do not carry the desired plasmid.
3. When screening mutant HRP-C libraries, include the wild-type enzyme (i.e., the enzyme whose DNA sequence was used to generate the mutant library) in several wells of the 96-well plate. This allows direct evaluation of each mutant's activity level against the wild-type, avoiding expression variation from plate to plate.
4. Protein production continues for 24–48 h, which causes a problem with the activity screen. Long expression periods usually cause an increase in the assay variation because of changes in the ratio of dead cells to live cells.
5. To ensure that the right volume of BugBuster is used, the wet cell mass needs to be determined when growing cells in mL-scale tubes/flasks. This step is not necessary for 96-well plates, where a fixed volume (20–30 μL) of BugBuster is enough to lyse the cells in each well.
6. Resuspension is a problematic step in the BugBuster protocol, since repeated pipetting of this detergent-based reagent easily creates air bubbles that seriously hinder colorimetric screening protocols. For mL-scale cultures, manual pipetting can be done with the tip of the pipet completely immersed in the liquid, such that no air bubbles are introduced. For 96-well plates, repeated pipetting is not recommended, since custom automated pipetting devices such as multi-channel pipets or robots can introduce air bubbles. Good resuspension can be obtained instead by stirring the cell extract with plastic needles or metal tips from a plate replicator.

7. Many swing-out bucket - type centrifuges have an upper limit of 3000–4000 rpm. Even at this low rotational speed, bacterial cells can be easily pelleted from liquid culture.
8. Verify the experimental consistency by first assaying an entire 96-well plate that contains nothing but wild-type HRP-C colonies. The standard deviation in activity across the plate should be no more than ~10–15% of the total level. This is easier to achieve by diluting the soluble enzyme extract such that the initial enzymatic rates are 0.2–0.3 OD U/min.

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Screen for Oxidases by Detection of Hydrogen Peroxide with Horseradish Peroxidase

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1. Introduction

Oxidases remove hydrogen from substrates, transferring it to molecular oxygen to generate hydrogen peroxide or water. Since oxidases catalyze fundamental synthetic transformations, including alcohol-aldehyde and aldehyde-carboxylic acid conversions, they have attracted attention as targets of directed evolution for further improvement (1,2). The co-product hydrogen peroxide can be detected by various methods (3) and provides a basis for screening oxidases in a high throughput fashion.

This chapter focuses on the screening of galactose oxidase (GOase, D-galactose: oxygen 6-oxidoreductase, EC 1.1.3.9), a copper radical enzyme (4–7). Galactose oxidase oxidizes primary alcohols to generate the corresponding aldehydes. Because GOase shows fairly broad substrate specificity as well as strict regioselectivity and stereoselectivity, it has been used widely in chemo-enzymatic synthesis, analytic chemistry, process monitoring and clinic analysis (8–10). Here, we describe a 96-well plate-based method and a solid-phase high-throughput method for screening GOase libraries.

2. Materials

2.1. Biological and Chemical Materials

1. *E. coli* strain BL21(DE3) (Novagen, Madison, WI).
2. pGAO-36 plasmid containing GOase gene.
3. dNTPs (Life Technology).
4. Restriction enzymes (New England Biolabs, Beverly, MA), T4 DNA ligase (Life Technology) and *Taq* DNA polymerase (Perkin Elmer, Boston, MA).
5. Sodium phosphate (NaPi) buffer: 0.1 M, pH 7.0.

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6. CuSO_4 (40 mM).
7. Lysozyme (1mg/L in NaPi buffer).
8. Horseradish peroxidase (HRP) (60 U/mL in NaPi buffer).
9. ABTS (2,2'-azino-bis[3-ethylbenzothiazoline-6-sulfonic acid]) (13 mg/mL in NaPi buffer).
10. D-galactose (100 mM in NaPi buffer).
11. Ampicillin (100 mg/mL).
12. Sodium dodecyl sulfate (SDS) (10% w/v in NaPi buffer).
13. Luria-Bertani (LB) broth and LB-agarose-ampicillin plates.
14. PCR purification kit (Qiagen, Chatsworth, CA).
15. Plasmid mini-prep kit (Qiagen).
16. SOB broth (for the preparation of competent cells for electroporation): 20 g of tryptone, 5 g of yeast extract, 0.585 g of NaCl, 0.19 g of KCl, 0.95 g of MgCl_2 , 1.2 g of MgSO_4 , and 3.6 g of D-glucose in 1 L of water.

2.2. Equipment

1. Electroporation equipment (Gene Pulser II, Biorad, Hercules, CA).
2. 96-well deep well plates (well volume: 2 mL, Becton Dickinson Labware, Lincoln Park, NJ) and 96-well assay plates (Rainin Instrument, Emeryville, CA).
3. 96-well plate reader (Molecular Devices, Sunnyvale, CA).
4. Multichannel pipeters (Biohit, Helsinki, Finland).
5. Multimek automated 96-channel pipettor (Beckman Instruments, Fullerton, CA) (optional).
6. Toothpicks.

(The following materials are for solid-phase assay only.)

7. Nylon transfer membrane (Millipore, Bedford, MA) and filter paper (No. 3) (Waterman, Maidstone, England).
8. Forceps.

3. Methods

3.1. GOase Gene Expression System

The vector pGAO-036 (2) carrying GOase gene is a derivative of pUC-18 vector with double *lac* promoters. The GOase gene was ligated into the plasmid by using the restriction enzymes *Hind*III (N-terminal) and *Xba*I with insertion of an ATG initiation codon. Plasmid pGAO-036 can be transformed into various *E. coli* hosts (DH5 α MCR, BL21(DE3), KY-14478) to functionally express GOase.

3.2. GOase Activity Assay

GOase catalyzes the oxidation of the 6-hydroxyl group of D-galactose to generate the corresponding aldehyde with the production of hydrogen peroxide. Activity can be monitored by measuring the production of hydrogen per-

oxide. We use the HRP-ABTS- H_2O_2 reaction (**II**) to measure the hydrogen peroxide generation because this method is simple, quantitative and sensitive. HRP catalyzes the polymerization of ABTS in the presence of hydrogen peroxide. The polymerization products can be monitored by the absorbance at 410 nm. For plate readers with discrete wavelength, absorbance at 405 nm can also be used. Moreover, a membrane-based solid phase screening method using the above reaction can be applied to identify active mutants in a large library.

3.3. Screening GOase Libraries by a 96-Well Plate-Based Method

1. Plasmid pGAO-36 containing the GOase variants are transformed into *E. coli* strain BL21(DE3) (see **Note 1**).
2. The transformed *E. coli* cells are spread on LB-agarose-ampicillin plates (see **Note 2**).
3. The *E. coli* cells are incubated at 30°C overnight (see **Note 3**).
4. Single colonies are picked and inoculated into 200 μ L LB-ampicillin in deep-well plates. The parents are also used in each plate as references.
5. The cells are incubated at 30°C in a shaking incubator for 12 h (see **Note 4**).
6. 10 μ L of the culture are transferred into a new deep-well plate with 300 μ L LB-ampicillin in each well. The cells are cultivated for 12 h at 30°C in a shaking incubator (see **Note 5**). The original plates are stored at 4°C for retrieval of the mutants identified in screening.
7. The cells are spun down and resuspended in 300 μ L of sodium phosphate (NaPi) buffer (pH 7.0, 0.1 M) containing 0.4 mM $CuSO_4$ (see **Note 6**). After adding 0.5 mg/mL lysozyme, the cells are incubated at 37°C for 30 min. The cell lysate are incubated with 2.5% (w/v) SDS at 4°C overnight (see **Note 7**).
8. Aliquots of the cell lysates are mixed with HRP (5.6 U/mL) and ABTS (2 mg/mL). Then D-galactose is added and GOase activity is measured (see **Note 8**).
9. Selected mutants (e.g., with more than twofold improved activity relative to the parent) are inoculated from the master plates into 3 mL LB-ampicillin media and cultivated at 30°C overnight to purify the plasmids.
10. Typical profiles of GOase activity are shown in **Fig. 1**.

3.4. Screening GOase Libraries Using a Membrane-Based Method

1. *E. coli* strains BL21(DE3) harboring GOase variants in plasmid pGAO-036 are spread on LB-agarose-ampicillin plates (see **Note 9**).
2. The *E. coli* cells are incubated at 30°C for 12 h.
3. The *E. coli* cells are replicated on membranes (see **Note 10**).
4. The membranes with cells are placed on LB-agarose-ampicillin plates and incubated at 30°C for 12 h.
5. Solutions containing lysozyme (0.5 mg/mL), D-galactose (100 mM), ABTS (2 mg/mL), HRP (10 U/mL), and $CuSO_4$ (0.4 mM) are poured into a petri dish to just cover the bottom. Filter paper is put on the top of the solutions, and the paper will absorb enough solution for cell wall disruption and GOase activity detection.

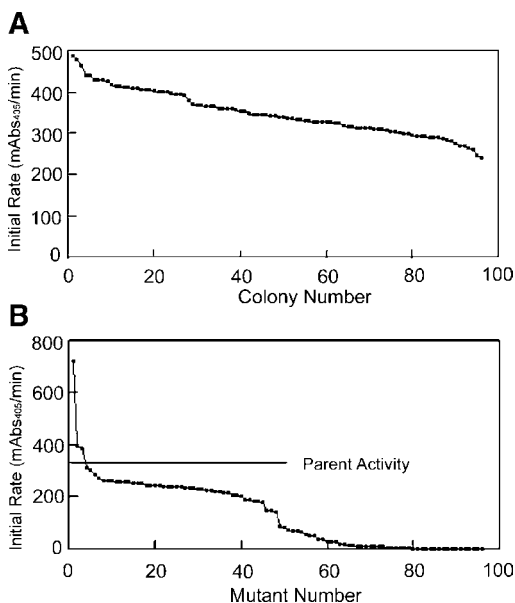


Fig. 1. (A) Variability of the 96-well plate screening method is examined by cultivating and assaying the wildtype GOase as described. Data are plotted in order of descending activity. The CV of the assay is 15%. (B) Typical activity profile of 96 clones from a mutant library.

6. The membranes with cells are placed on filter paper. Colonies with active GOase exhibit purple color (*see Note 11*).

4. Notes

1. An efficient transformation method is advantageous to obtain a sufficient number of clones for library screening, because ligated products are usually transformed with low efficiency. Generally, electroporation provides better transformation efficiency compared to chemical methods. However, the transformation efficiency is also dependent on the quality of the competent cells. The transformation efficiency of commercially available competent cells for chemical transformation methods is comparable to that of the electroporation (more than 2000 clones for 1 μ L ligation mixture).
2. Several tests might be required to obtain the appropriate number of colonies on a plate by spreading transformants in several dilutions. If a colony picker will be used, the instructions on the manual usually provide sufficient information. Generally, having less than 200 single colonies (2 mm in diameter) on a regular petri dish with uniform distribution is required for picking single colonies by hand.
3. The incubation time and temperature vary with applications. They are adjusted to obtain the right colony size (*see Note 2*). For some enzymes, low temperature is

required for functional expression. In all the cultivation steps, no IPTG was used because the amount of GOase produced without induction is sufficient for the activity measurement.

4. It is necessary to cultivate the cells in each well of a deep-well plate to a uniform cell density. Enzyme activity variations in a plate are primarily affected by the oxygen supply, which leads to a fluctuation in cell density and protein expression. At this stage, obtaining uniform cell density in a deep-well plate is very important, because the equivalent amount of cells is to be transferred into the other deep-well plates at the next step (**step 6**).
5. In **steps 4** and **5**, several methods can be applied to reduce variability. An orbital shaker can provide greater shaking diameter and therefore is better than a regular bench-shaking incubator. Usually, the cells in the outside wells of a deep-well plate show higher activity than the cells in the center, because they have a greater supply of oxygen. The variation can be decreased either by taping the lids and the deep-well plates together or using membrane lids with a hole on each well. Using a secondary container can also reduce the variation when cultivating the cells in a regular bench-shaking incubator.
6. High centrifugal forces should be avoided because they lead to difficulty in resuspending the cell pellets. In this experiment, centrifugation at 4000g for 10 min was usually employed. The automated 96-channel pipettor can be programmed to resuspend the cell pellets. However, using multichannel pipeters to suspend the cell pellets after soaking the cell pellets in the buffer for 1 h is more efficient than using the robot.
7. Lysozyme works more efficiently at 37°C than other temperatures. If SDS can denature the enzyme, other cell lysis reagents can be used, for example, Bugbuster (Novagen, Madison, WI). Chemicals that permeabilize cell walls can also be used. It takes at least 2 h for apo-GOase to bind copper ion at 4°C.
8. If SDS precipitates, the plates can be incubated at 30°C for 30 min to dissolve the SDS. The amount of cell lysate and the concentration of the substrate D-galactose required in the assay are determined by the initial rate. An initial rate of the parent between 500–1000 Abs/min is desired in order to sensitively measure the increased activity of a good mutant, which is usually 2–3 times higher than the parent. The amount of ABTS and HRP are much more than required to ensure rapid detection of hydrogen peroxide formation.
9. This solid-phase screening is not as sensitive as the 96-well plate screening method. However, this method allows one to screen thousands of variants in one day. It is a complementary method to the 96-well plate screening method and can be used to efficiently identify the active mutants in a large library.
10. Membranes are gently placed on and removed from the LB-agarose-ampicillin plates using forceps to avoid smearing the cells. Marking both the plates and the membranes is necessary to recover the cells with GOase activity.
11. It takes 20 min or longer to see the color, depending on the activity. The assay reagents are light-sensitive, and using aluminum foil to cover membranes to block light can increase the accuracy of assay.

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Colorimetric Dehydrogenase Screen Based on NAD(P)H Generation

Kimberly M. Mayer

1. Introduction

Dehydrogenases are studied for a variety of reasons. Several dehydrogenases are of use in industrial processes to produce pharmaceuticals and fine chemicals. Scientists are trying to enhance the performance of such enzymes by limiting substrate inhibition (1) and eliminating the need for expensive activators (2). In addition, dehydrogenases are involved in alcohol metabolism (3), and in mammalian cell growth (4) and cell death (5). Various dehydrogenases have been implicated in diseases and cancers (6–9) and have therefore become important targets for antiparasitic and anticancer drugs (10). High-throughput screens for dehydrogenase activity are expected to be useful in many of these studies.

The dehydrogenase used to demonstrate the colorimetric assay described in this chapter is *Escherichia coli* 6-phosphogluconate dehydrogenase (6PGDH). The second enzyme in the pentose phosphate pathway, 6PGDH catalyzes the NADP-dependent transformation of 6-phosphogluconate to ribulose-5-phosphate, producing NADPH and CO₂ in the process. The NADPH created in the reaction provides the cell with energy for biosynthesis reactions and protects the cell from oxidative damage. In addition, the 6PGDH-catalyzed reaction allows the cell to later produce ribose that can be used for nucleic acid synthesis.

The activity of 6PGDH (and other dehydrogenases) is commonly measured by following the production of NADPH at 340 nm, an unsuitable approach for high-throughput screening in cell lysates. The background absorbance of 96-well plates tends to be high in the UV-range, and reproducibility of measurements is low. Plates are available for screening in the UV-range, however,

their cost is prohibitive to large screening experiments. In addition, cell lysates themselves often have a moderate absorbance at 340 nm.

The assay described here is a colorimetric alternative using nitroblue tetrazolium (NBT) which, in the presence of phenazine methosulfate (PMS), reacts with the NAD(P)H produced by dehydrogenases to produce an insoluble blue-purple formazan. Endpoint assays taking advantage of this reaction have been used to detect lactate dehydrogenase activity on nitrocellulose membranes (**11**), alcohol dehydrogenase activity in liquid phase (**3**), and 6-phosphogluconate dehydrogenase activity in electrophoretic gels (**12**). We describe a version of this assay suitable for determining the kinetics of 6-phosphogluconate dehydrogenase catalysis in crude lysates of bacterial cells prepared in 96-well plates (**13**). The NBT/PMS assay promises to be generally applicable for measuring the activity of dehydrogenases, the only requirement being that the enzyme use NAD(P) as a cofactor.

2. Materials

2.1. Biological and Chemical Materials

All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise indicated.

1. Appropriate *E. coli* strain(s).
2. Plasmid containing dehydrogenase gene under appropriate promoter.
3. LB* (Luria Broth) and LB agar plates* (**14**).
4. TSS* (Transformation and Storage Solution): 10% w/v PEG6000, 5% dimethyl sulfoxide (DMSO), 50 mM MgCl₂ in LB, pH 6.5.
5. Liquid nitrogen or ethanol + dry ice.
6. Appropriate antibiotic(s).
7. H₂O.*
8. Appropriate PCR primers.
9. Proofreading polymerase (such as *Pfu*; Stratagene, La Jolla, CA).
10. dNTPs (Boehringer-Mannheim, Indianapolis, IN).
11. Restriction enzyme(s) (New England Biolabs (NEB), Beverly, MA).
12. Klenow fragment (NEB).
13. T4 DNA ligase (NEB).
14. 10X EP-PCR buffer:* 70 mM MgCl₂, 500 mM KCl, 100 mM Tris-HCl, pH 8.3, and 0.1% (w/v) gelatin.
15. *Taq* polymerase (Boehringer-Mannheim).
16. Agarose.
17. PCR purification kit (Qiagen, Chatsworth, CA).
18. Plasmid Mini-prep kit (Qiagen).
19. Toothpicks.

*Sterilize before use

20. 96-well (300 μ L well volume) microtiter plates (Rainin, Emeryville, CA).
21. Glycerol*.
22. Bugbuster (Novagen, Madison, WI).
23. Dilution buffer:* 50 μ M Tris-HCl, pH 8.0, and 0.13% gelatin.
24. Reagent solution: 300 μ M NADP, 300 μ M 6-phosphogluconate (6PG), 300 μ M nitroblue tetrazolium (NBT), 30 μ M phenazine methosulfate (PMS) in dilution buffer.

2.2. Equipment

1. Shaking incubator (capable of 250 rpm, 30–42°C; Model Innova 4000, New Brunswick Scientific, Edison, NJ).
2. Centrifuge (capable of spinning 96-well plates at 3000 rpm; Model CS6R, Beckman Coulter, Fullerton, CA).
3. Microcentrifuge (Model Eppendorf 5417R, Brinkmann Instruments, Westbury, NY).
4. Thermocycler (Model PTC200, MJ Research, Waltham, MA).
5. Incubator (capable of 30–62°C; Model Thelco, Precision Scientific, Winchester, VA).
6. Spectrophotometer (capable of reading 96-well plates in the range of 340–600 nm; Model Spectramax 250, Molecular Devices, Sunnyvale, CA).
7. Computer attached to spectrophotometer.
8. Softmax Pro software (Molecular Devices).
9. MS Excel software.
10. Agarose gel running system.
11. Tupperware, sponges, elastic straps for growing cells in 96-well plates.

3. Methods

3.1. Plasmids

The heat-inducible cI857 system was chosen for expression of 6PGDH in *E. coli* (see **Note 1**). The system uses the bacteriophage lambda *pL* promoter under control of the mutant cI857 repressor protein (**15**). The cI857 protein binds the operator when the cells are at low temperature (<30°C), but falls off and cannot re-attach when the cells are placed at high temperature (>37°C). The system is somewhat leaky, with low levels of expression occurring at 30°C (**16**).

3.1.1. Expression Plasmid Construction

Plasmid pNB106R (**15**) is a heat-inducible expression plasmid that contains the *Bacillus subtilis* pNB esterase gene, a gene for tetracycline resistance, and the cI857 repressor gene. The pNB esterase gene was removed from pNB106R by restriction digestion following standard molecular biology techniques (**14**).

The 1407-bp *gnd* coding region was amplified from pMN6 (**17**) using primers 5' *gnd*NdeI (5'-GCGCATGCATATGTCCAAGCAACAGATCGGC-3') and 3' *gnd*BamHI (5'-CCGGATCCGCCCGGTGCAATATACGCC-3'). Each 100 μ L reaction contained 5 U *Pfu* polymerase, 1X *Pfu* PCR buffer, 2 mM each

dNTP, and 50 pmol each primer. Thirty cycles of 94°C 1 min, 40°C 1 min, 72°C 3 min were followed by a final elongation at 72°C for 10 min. Following amplification, the PCR product was gel-purified then digested with *NdeI* and *BamHI* using standard techniques (14).

The final 6PGDH expression plasmid (pKMM-1) was constructed by ligating the PCR-generated *gnd* gene into the pNB106R expression plasmid using standard techniques (14). Sequencing revealed that a single G to T mutation at nucleotide 19 was unintentionally introduced during amplification, causing amino acid seven to change from glycine to cysteine (see Note 2).

3.1.2. Empty Plasmid (Negative Control Plasmid)

For use as a negative control in screening, the empty plasmid pKMM-0 was generated by excising the *gnd* coding region from pKMM-1 using *NdeI* and *BamHI*, treating the linearized plasmid with Klenow fragment to generate blunt ends, then ligating the ends using T4 DNA ligase to recircularize the plasmid using standard molecular techniques (14).

3.2. E. coli Cell Strains

3.2.1. For Plasmid Preparation

Commercial DH5 α cells were used to grow the pMN6 and pNB106R plasmids. Individual transformed colonies were picked from agar plates then grown overnight at 30°C (see Note 3) in LB containing antibiotic. Cells were harvested by centrifugation and plasmid was extracted using the Qiagen mini-prep kit.

3.2.2. Chemically Competent Cells for Protein Expression

To eliminate background activity from endogenous *gnd* gene product (see Note 4), strain RW181 (17) was used for 6PGDH expression. RW181 is deleted for *gnd*, *edd*, and *zwf* (F⁻ *trpR lacZ trpA kdgR* Δ (*edd-zwf*)22 Δ (*attP2H*(P2 *c5 nipl-sbcB-his-gnd-rfb*)1. Chemically-competent RW181 cells were generated by inoculating 250 ml LB with 2.5 mL of an overnight culture. Cells were grown at 37°C to OD600 of 0.4 then harvested by centrifugation at 1900g at 4°C for 10 min. The pellet was gently resuspended in sterile ice-cold TSS then placed on ice for 20 min. Competent cells were frozen on liquid nitrogen as 200 μ L aliquots and stored at -80°C. Cells were grown in LB containing 20 mg/L tetracycline unless otherwise noted.

3.3. Protein Induction and Sample Preparation in 96-Well Plates

3.3.1. Protein Induction

RW181 cells were transformed with 6PGDH expression plasmids, plated on LB agar containing tetracycline, and grown ~36 h at 30°C. Individual colonies

were picked with toothpicks into wells of 96-well plates filled with 200 μL of LB with tetracycline. The plates were placed into tupperware containers with damp sponges and grown at 30°C with shaking until saturation (~36 h) then replica-plated into fresh LB with tetracycline. The master plates were stored as 20% glycerol stocks frozen at -80°C. The replica plates were grown at 30°C with shaking until saturation (~36 h), then the *gnd* gene was induced by raising the temperature to 42°C for 15 min followed by 3 h at 37°C.

3.3.2. Sample Preparation

1. After induction, divide the cells into two aliquots of 100 μL in separate plates. Dividing the cells into two aliquots allows direct comparison of the initial activity at room temperature (RT) (A_i) of each clone to its residual activity after heating (A_r) to screen for thermostabilized variants (see **Chapter 11**).
2. Heat one plate at 62°C for 10 min then cool on ice for 10 min. Leave the other plate resting at RT.
3. Pellet cells by centrifugation at 4000g for 20 min.
4. Remove media and add 50 μL of Bugbuster.
5. Shake plates gently at RT for 20 min.
6. Add 200 μL of dilution buffer and gently shake plates briefly to mix.
7. Pellet debris by centrifugation at 4000g for 20 min.
8. Use aliquots (50 μL) of the supernatant in activity assays (see **Notes 5 and 6**).

3.4. Colorimetric Dehydrogenase Activity Assay

In the presence of a dehydrogenase, NAD(P), and phenazine methosulfate (PMS), nitroblue tetrazolium (NBT) is reduced to a formazan (see **Fig. 1**) that when scanned spectrophotometrically in the range of 450–700 nm shows maximal absorption at ~550 nm. We measured 6PGDH activity by following the increase in formazan production at 580 nm in a microplate spectrophotometer.

1. Prepare reagent solution. Keep at RT in the dark.
2. Set up the spectrophotometer to read kinetics at 580 nm for 3 min and shake before reading.
3. Set up the software template to include blank(s), control(s), and unknown(s).
4. Aliquot 50 μL of diluted crude cell lysate into 96-well plates.
5. Add 150 μL of reagent solution to wells.
6. Immediately start reading absorbance.
7. Save file when finished.
8. Reduce readout to contain only the linear part of the reaction (for 6PGDH under these conditions, at least the first 30 s is linear, see **Note 7**).
9. Have software provide V_{max} and subtract blank (from cells containing negative control plasmid).
10. Transfer data to MS Excel for analysis (see **Notes 5 and 8**). Alternatively, activity can be screened non-colorimetrically in UV plates at 340 nm. In this case, NBT and PMS would not be included in the reagent solution.

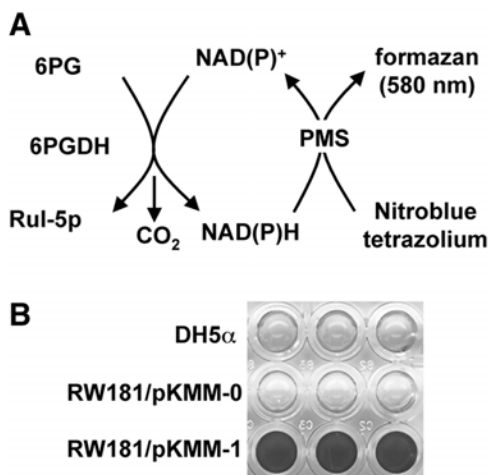


Fig. 1. (A) Colorimetric assay for 6PGDH activity. 6PGDH cleaves the substrate 6-phosphogluconate (6PG) into ribulose-5-phosphate (Rul-5p) and CO₂, reducing NADP to NADPH in the process. Phenazine methosulfate (PMS) catalyzes the conversion of nitroblue tetrazolium (NBT) into a purple formazan that is detectable at 580 nm. (B) RW181 cells were transformed either with the empty vector (pKMM-0) or with the vector containing the G7C *gnd* gene (pKMM-1) as indicated. DH5α cells were untransformed and show endogenous activity under conditions of the assay.

4. Notes

1. Other expression vectors have been shown to work well with dehydrogenases. For example, Ldh has been successfully expressed from pKK223-3 (1) and dihydroorotate dehydrogenase from pET15b (10). Both are commercially available expression vectors and place the gene under IPTG induction control.
2. The G7C mutation has little or no effect on the enzyme activity. The k_{cat}/K_m values for wildtype and G7C are very similar ($\sim 1.48 \times 10^3 \text{ s}^{-1}$).
3. Cells must be grown at 30°C for stock DNA preparations of the heat-inducible plasmid. Growing under non-inducing conditions prevents peculiar rearrangements from occurring within the plasmid (18,19)
4. Most importantly, always include proper controls when screening libraries. Empty vector is essential, as is wildtype (or parent), and these must be included on every 96-well plate. Determining the cause of and eliminating as much background activity as possible is highly recommended. Controls that allow remaining background activity to be subtracted from measurements are necessary as well. These controls may well be mutants in which the studied gene is deleted since this will allow any non-specific dehydrogenase activity (or “nothing dehydrogenase,” [12]) to be detected. For example, when RW181 (Δgnd) is transformed with the “empty” vector pKMM-0, no 6PGDH activity is detected. This

provides a control that allows us to subtract out the low levels of background noise arising from the cell lysates themselves in our assay.

5. We found the NBT/PMS assay to be reproducibly consistent in bacterial cells grown and prepared in 96-well plates (13). Plates containing cells expressing the G7C *gnd* gene showed good consistency across wells (13–18% CV) when measuring initial activity over the first 30 s of the reaction. The variation over the same plate when measuring residual activity was much higher (43–47% CV).
6. Note that when screening for thermostability, the cells are heated, not the lysate, because 6PGDH precipitates and becomes inactive when heated in the presence of Bugbuster. This may not be true for all dehydrogenases, but should be tested experimentally. Thermostability screens may be more or less variable if performed on lysates as opposed to intact cells. Other sample preparation methods, such as sonication, freeze/thaw, or lysozyme treatment could be used in place of Bugbuster.
7.
 - a. The conditions under which the assay remains in the linear range over time during screening must be experimentally determined.
 - b. Confirm experimentally that the assay is reasonably specific for the chosen dehydrogenase by testing with and without substrate.
 - c. Determine whether any of the assay components negatively affect dehydrogenase activity by testing each separately and in various combinations using partially purified enzyme and measuring NADPH production directly (non-colorimetrically at UV_{340}).
 - d. Extinction coefficients are: NBT-formazan ($\sim 12,300 M^{-1}cm^{-1}$); NADPH ($6220 M^{-1}cm^{-1}$).
8. To test the ability of the assay to reliably detect variants with improved performance, we generated a small library of ~ 400 variants by error-prone PCR and screened them using the NBT/PMS assay (13). The cells were divided into two aliquots so that initial activity at room temperature (A_i) of each clone could be compared directly to residual activity after heating at $62^\circ C$ for 10 min (A_r). Initial activity was the rate of the reaction for the first 30 s and ranged from 0–1163 mOD/min. Residual activity was determined from the lysates prepared after heating cells to $62^\circ C$ for 10 min. Thermostability was calculated as the ratio of residual activity to initial activity (A_r/A_i) and ranged from 0–1.2. Average parent (G7C) activity was 307 ± 16 mOD/min and thermostability was 0.11 ± 0.09 . Approximately 30% of the clones showed no activity at room temperature, as expected for a library of random mutants containing 2–3 base substitutions per gene (20).

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Colorimetric Assays for Screening Laccases

Miguel Alcalde and Thomas Bulter

1. Introduction

In this chapter, we describe colorimetric methods for screening laccases using natural and nonnatural substrates. Laccases (EC 1.10.3.1) are blue-copper enzymes that oxidize phenols, polyphenols, and anilines (**1,2**). The catalytic capabilities of laccase can be greatly enhanced by the addition of suitable mediator compounds. In the presence of some of its primary substrates (such as 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid [ABTS] or 1-hydroxybenzothiazole [HBT]), laccase can catalyze the oxidation of nonnatural substrates, including polycyclic aromatic hydrocarbons (PAHs), a class of highly toxic organic pollutants widely distributed in terrestrial and aquatic environments (**3–7**). The mechanism of oxidation by laccase-mediator systems (LMS) is still under discussion. In spite of enhancing the range of compounds amenable to oxidation by laccases, mediators have several disadvantages: they are expensive, poisonous, and show side reactions with substrates and products, leading to reduced yield and impurity of the products. Inactivation of laccase by free radicals of the mediators is an additional drawback (**8,9**). To optimize laccases for mediated applications or make them independent of mediators using directed evolution requires mediator-dependent screens.

The first assay described here has been used to screen mutant libraries of *Myceliophthora thermophila* laccase expressed in *S. cerevisiae* for increases in total activity (see **Chapter 9** in this volume and **Chapter 3** in the companion volume “*Directed Evolution Library Creation*”). It is based on the oxidation of ABTS and the concomitant formation of a radical cation (**10**).

The other colorimetric assays presented in this chapter are mediator-dependent assays. Two assays for laccase-catalyzed degradation of PAHs are presented, based on previous studies of the oxidation of PAHs by fungal laccases from

Trametes versicolor and *Myceliophthora thermophila* (11). Using a water-soluble sodium borohydride solution, we could reduce the single product of laccase-catalyzed anthracene biooxidation into the orange-colored 9,10-anthrahydroquinone, quantifiable spectrophotometrically. A second assay utilizing a polymeric dye (Poly R-478) as a surrogate substrate for lignin degradation by laccase in the presence of mediator is also presented (12–14). The decolorization of Poly R-478 was correlated to the oxidation of PAHs mediated by laccases. This demonstrates that a ligninolytic indicator such as Poly R-478 can be used to screen for promising PAH-degrading laccases.

We have also developed a mediator-dependent assay that utilizes the oxidation of iodide to iodine by laccase (15). The *in situ* generation of iodine for industrial and medical sterilization by laccase could have an advantage over peroxidase-based systems because it uses dioxygen instead of peroxide. To achieve a reasonable reaction rate, ABTS has to be added to the laccase-catalyzed reaction. The system would only be useful if the mutagenic ABTS can be omitted, a possible target for directed evolution.

These assays are suitable for 96-well plate format and are appropriate for laccase evolution. Laccases which work with nonnatural substrates and in the absence of mediators will have applications not only in PAH bioremediation but also in other fields such as in the pulp-kraft bleaching industry or in biofuel cells.

2. Materials

All chemicals used were reagent grade purity. Novo Nordisk kindly provided concentrated and partially purified laccase from *Trametes versicolor* (TvL) and laccase from *Myceliophthora thermophila* (MtL). Pure laccases, obtained by anion exchange chromatography on Q-Sepharose FF (Pharmacia), were used in iodide and PAH colorimetric assays.

1. Enzyme diluent: 1% Triton-X-100, 5% PEG 5000.
2. Britton and Robinson (B & R) buffer: 0.1 M boric acid, 0.1 M acetic acid, 0.1 M phosphoric acid with 0.5 M NaOH to the desired pH.
3. 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid [ABTS] reaction solution: 5 mL 60 mM ABTS, 10 mL 50% (w/v) PEG 5000, 50 mL 100 mM B & R buffer pH 6.0, add ddH₂O to 90 mL. Final concentrations in the assay: 3 mM ABTS, 5% PEG 5000, 50 mM B & R buffer.
4. 100 mM acetate buffer. Adjust to pH 5.0 with acetic acid.
5. Anthracene stock solution: 5 mM anthracene in 100% ethanol.
6. Mediator stock solution: 60 mM HBT in 20% ethanol.
7. PAH reaction solution: 2 mL Tween 20, 4 mL anthracene stock solution, 3 mL mediator stock solution + 100 mM acetate buffer pH 5.0 to 100 mL. Final concentrations in the assay: 1% Tween 20, 100 μ M anthracene, 2.3% ethanol, 0.9 mM mediator (HBT), 50 mM acetate buffer. Store in darkness.

8. Sodium borohydride water-soluble solution (SWS): 3.7 M NaBH₄ in 40% NaOH.
9. Poly R-478 stock solution: 0.2% poly R-478 in ddH₂O.
10. Poly R reaction solution: 13.5 mL Poly R-478 stock solution, 2 mL mediator stock solution + 100 mM acetate buffer pH 5.0 to 100 mL. Final concentrations in the assay: 0.02% Poly R-478, 0.3% ethanol, 0.9 mM mediator (HBT), 75 mM acetate buffer.
11. Iodide assay solution: 5 mL 1 M NaI, 25 mL 1 M sucrose, (5 mL 0.2 mM ABTS), 40 mL 100 mM B & R buffer, pH 4.1, add ddH₂O to 90 mL. Final concentrations in the assay: 50 mM NaI in 40 mM B & R buffer, pH 4.1, 250 mM sucrose with or without 10 μ M ABTS.
12. 96-well-microtiter plates: R-96-OAPF-ICO (Rainin, Emeryville, CA).
13. Pipetting robot: Beckman Multimek 96 Automated 96-Channel Pipettor (Beckman Instruments, Palo Alto, CA).
14. Spectrophotometer/plate reader (Model Spectra max Plus 384, Molecular Devices, Sunnyvale, CA). Software Softmax Pro 3.1.1.
15. Multi channel pipettor.
16. Sealing film (Genemate).

3. Methods

3.1. ABTS Assay for Screening Laccase Activity

Standard laccase activity is determined by oxidation of ABTS at room temperature (*see Note 1, Fig. 1A*).

1. Dilute the laccase sample with B & R-buffer, pH 6.0, or enzyme diluent, if necessary (*see Note 2*).
2. Add 20 μ L of the laccase sample into microtiter plate wells.
3. Add 180 μ L of ABTS solution to every well. Mix sample and reagent thoroughly using the plate reader or a pipettor (*see Note 3*).
4. Follow the oxidation of ABTS by measuring the absorbance increase at 418 nm (*see Note 4*).

One unit laccase activity is the amount of enzyme that oxidizes 1 μ mol ABTS/min under these conditions.

3.2. Assays for Mediator Dependent Activity of Laccases

3.2.1. PAH Biodegradation Assay

The oxidation of anthracene (conversion into 9,10-anthraquinone) by laccases can be followed colorimetrically (*see Fig. 1B*) via the reduction of 9,10-anthraquinone in the presence of a sodium borohydride water soluble solution (SWS) (*16*) (*see Note 5*).

1. Dilute the laccase sample with acetate buffer, pH 5.0, or enzyme diluent, if necessary (*see Note 2*).
2. Into microtiter plate wells: add 50 μ L of laccase sample. Add 50 μ L of PAH reaction solution (*see Note 6*).

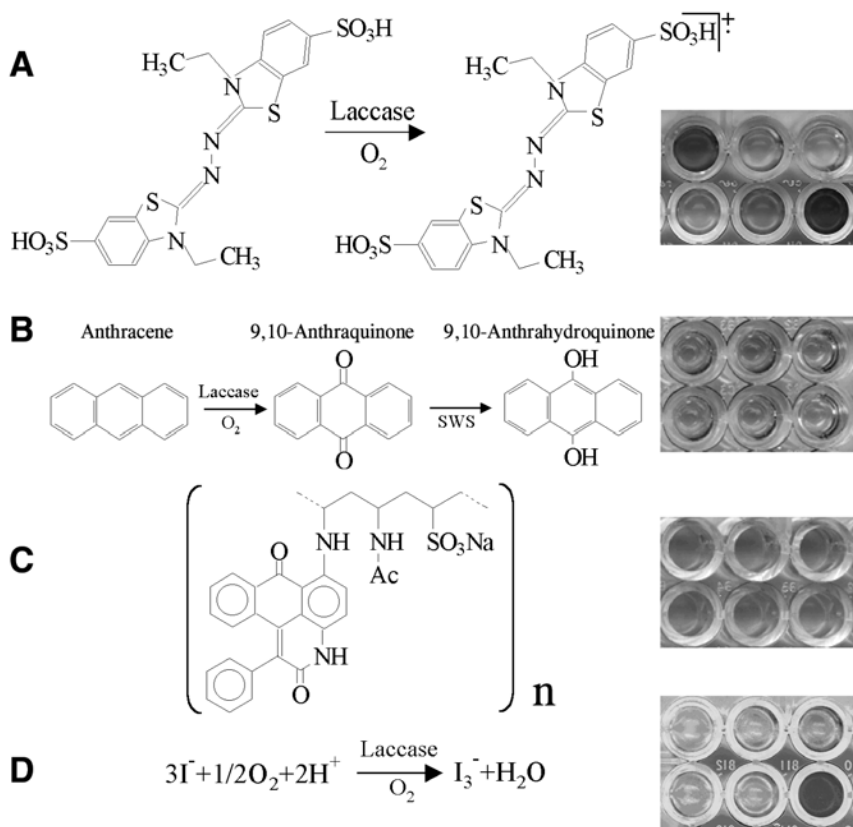


Fig. 1. Colorimetric assays for screening laccases. **(A)** ABTS oxidation assay used in the screening of mutant libraries of laccase from *M. thermophila* expressed in *S. cerevisiae*. The radical cation produced is stable for hours and has a high molar extinction coefficient. **(B)** Method for quantification of 9,10-anthraquinone. Anthracene is oxidized to 9,10-anthraquinone by laccase. 9,10-anthraquinone is reduced by a sodium borohydride water soluble solution (SWS), giving the orange product 9,10-anthrahydroquinone. **(C)** Structure of Poly R-478 (lignin model compound). **(D)** iodide oxidase reaction yielding triiodide (I_3^-) that produces yellow color (orange at high concentration).

3. Carry out controls with anthracene and mediator to check the PAH autooxidation (same amounts as **step 2** but without laccase).
4. Mix the reaction mixture by pipetting up and down with a multichannel pipettor or using the pipetting robot. Alternatively use the mixer of the plate reader.
5. Seal the wells with sealing film and incubate the plate in darkness at room temperature for 24 h (see **Note 7**).

6. After incubation mix the reaction mixture with 100 μL of 100% ethanol (*see Note 8*).
7. Add 20 μL of SWS. Mix SWS and the reaction mixture by pipetting up and down with a multichannel pipettor or using the pipetting robot. Alternatively, use the mixer of the plate reader (*see Note 9*).
8. Seal the plate and incubate at room temperature for 15 min. Measure the absorbance at 419 nm (*see Note 10*).

One unit of PAH-activity is the amount of enzyme that produces 1 μmol of 9,10-anthraquinone/min under the described conditions.

3.2.2. Decolorization Assay (*see Note 11*)

A second colorimetric assay based on the correlation between PAH biodegradation and polymeric dye decolorization was developed. Poly R-478 is a lignin model compound related to PAHs (*see Fig. 1C*), and both types of molecules can be degraded by the laccase mediator system (*see Note 12*).

1. Dilute the laccase sample with acetate buffer, pH 5.0 or enzyme diluent, if necessary (*see Note 2*).
2. Into microtiter plate wells add 50 μL of laccase sample. Add 150 μL Poly R reaction solution.
3. Carry out controls with Poly R-478 and with/without mediator to check the Poly R-478 autodegradation (same amounts of **step 2** but without laccase).
4. Mix by pipetting up and down thoroughly with a multichannel pipettor or using the pipetting robot. Alternatively, use the mixer of the plate reader.
5. Measure the initial absorption at 520 nm in the plate reader.
6. Seal the wells with sealing film and incubate the plate at room temperature for 15 min–6 h depending of the amount of enzyme used.
7. Measure the decreased absorbance at 520 nm in the plate reader (*see Note 13*).

One unit of decolorization activity is the amount of enzyme that degrades 1 μmol of Poly R-478/min under the corresponding conditions.

3.2.3. Iodide Assay (*see Note 14*) (**Fig. 1D**)

1. Dilute the laccase sample with enzyme diluent, if necessary (*see Note 15*).
2. Prepare the iodide assay solution (*see Note 16*). Add ABTS to the solution if mediated activity will be assessed.
3. Add 20 μL of the laccase sample into microtiter plate wells.
4. Add 180 μL of iodide assay solution to every well.
5. Mix samples and the assay solution by pipetting up and down with a multichannel pipettor or using the pipetting robot.
6. Measure the absorption at 353 nm in the plate reader (*see Note 17*).
7. Seal the plate with sealing film and incubate it at RT in the dark for 1–6 h (*see Note 18*).
8. Remove the sealing film and measure the absorption at 353 nm again.

9. Calculate the relative activities from the difference between initial absorption and that after the incubation divided by the incubation time (min) (*see Note 19*).

One unit of laccase activity is the amount of enzyme that oxidizes 1 μmol iodide/min under these conditions.

4. Notes

1. The oxidation of ABTS has been used for screening laccase total activity (*see Chapter 9*). The detection limit for this reaction is about 10 $\mu\text{g/L}$ of laccase. The green reaction product (radical cation) is stable for several hours ($\epsilon = 36,000 \text{ M}^{-1}\text{cm}^{-1}$) (**Fig. 1A**). The ABTS assay gives linear absorption increases over a wide range of enzyme concentration. As a rough guideline the activity of the sample should be between 1 and 20 mU/mL if initial activities are measured.
2. In highly diluted solutions, oxidoreductase enzymes in general are unstable. The enzyme diluent with PEG 5000 and Triton X-100 stabilizes the enzyme protein, as has been reported elsewhere (*17*).
3. The PEG makes the reagent mixture viscous and therefore more difficult to mix. If the pipetting robot is used for the addition of the reagent it should also be used to mix by pipetting up and down a few times. Alternatively, a multichannel pipettor or the microplate mixer in the plate reader can be used. ABTS solution must be prepared fresh. ABTS is mutagenic. Wear gloves and work carefully.
4. If the enzyme samples are of an appropriate concentration and the reaction solution is mixed properly, the increase in absorption is linear for at least 5 min. If low enzyme concentrations have to be used, an endpoint assay should be performed by measuring the absorption before and after an incubation time. For the endpoint mode, the sensitivity limit of the ABTS assay is 5 nU/mL.
5. Anthracene is selected as the substrate for a PAH-biodegradation assay because the molar yield of anthraquinone formed per mole of anthracene eliminated is 1.0 (*11*). The product, 9,10-anthraquinone, is not further degraded in the system during 24 h incubation. Anthracene has the same arrangements of fused aromatic rings as more complex carcinogenic PAHs.
6. The oxidation of anthracene by laccases is performed with 2% ethanol and 1% Tween 20 to increase solubility of the substrate and product. HBT, as mediator, improves PAH biodegradation considerably. However both laccases (MtL and TvL) are also able to oxidize anthracene in absence of mediator, a good starting point for directed evolution towards this application.
7. The reaction mixture is incubated in darkness to avoid the photooxidation of PAHs.
8. SWS is stable indefinitely and can be used directly (at room temperature) for reduction of carbonyl compounds. SWS is more soluble in methanol than in ethanol, but since it reacts with methanol at an appreciable rate, ethanol is preferred as a reaction cosolvent.
9. SWS solution contains highly concentrated NaOH. Wear gloves and work carefully. The solution is also highly viscous, which makes mixing more difficult.
10. The reduction of 9,10-anthraquinone into the deep orange-colored product 9,10-anthrahydroquinone, provides the basis for the PAH biodegradation assay. The

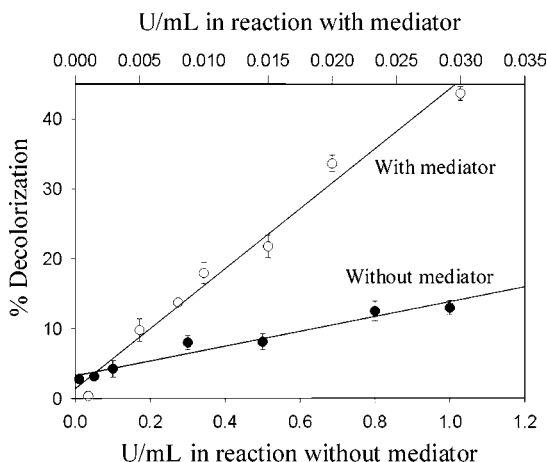


Fig. 2. Linearity of decolorization assay. Different amounts of *Trametes versicolor* laccase were assayed in the presence and absence of HBT. Each point represents triplicate experiments (○ with HBT, ● without HBT).

9,10-anthrahydroquinone generated by reduction by sodium borohydride exhibits an absorbance maximum at 419 nm with a molar extinction coefficient of $4000\text{ M}^{-1}\text{ cm}^{-1}$. The sensitivity limit of this colorimetric assay is $25\text{--}50\text{ }\mu\text{M}$ of anthraquinone. The limit of detection for laccase units is 0.01 ABTS U/mL. The reduction reaction is reversible with vigorous stirring and O_2 . However, sealing the plate keeps the solution stable at least for 1 h.

11. The biodegradation of PAH can be correlated to the extent to which laccases decolorize Poly R-478. The validity of using Poly R-478 as a surrogate substrate for PAHs was examined by plotting the benzo[*a*]pyrene oxidation data from HPLC analysis (*II*) against Poly R-478 decolorization data. Reactions using the two substrates show a very similar time course and the same mediator effect (*II*).
12. Poly R-478 decolorization is strongly affected by the action of the mediator. With *T. versicolor* laccase, in the presence of HBT, decolorization of the dye is complete after 24 h. The enzyme alone degraded the dye to a small extent, but sufficient for the evolution of laccase with this nonnatural substrate and in absence of mediators.
13. The decolorization is accompanied by a decrease in absorbance at 520 nm associated with oxidation of the dye (*18,19*). The absorption at 420 nm increased during the oxidation, and this led to a change in color from pink to orange. The detection limit for laccase units is 0.0003 and 0.01 ABTS-U/mL with and without HBT, respectively (see Fig. 2). Poly R-478 is stable and readily soluble. It has a high extinction coefficient and low toxicity toward white rot fungi, yeast, and bacteria, allowing its application in a solid-phase assay format.
14. The iodide oxidation is catalyzed by *Myceliophthora thermophila* laccase with a K_m of 0.16 M and a k_{cat} of 2.7 min^{-1} . Addition of mediator (ABTS) enhances the

catalysis (K_m , 6.4 μM and k_{cat} , 860 min^{-1}). Iodide oxidase activity was also detected with other laccases (15).

15. The sensitivity limit of this assay depends on whether it is an endpoint assay or if initial rates are determined. For the assay without mediator the sensitivity limit for initial rate measurement is 1 U/mL. In the endpoint assay 0.2 U/mL can be measured. For the assay including ABTS, sensitivities are 1 mU/mL and 0.2 mU/mL for rate and endpoint measurement, respectively. For the rate determination, enzyme concentration should be less than 20-fold the concentration of the detection limit.
16. Iodide-containing solutions are sensitive to light. To avoid autoxidation of this substrate NaI should be added to the assay solution shortly before use, and the solution should be kept in the dark afterwards. Sucrose was added to the reaction solution because it stabilizes and activates the enzyme. Addition of PEG and bovine serum albumin (BSA) did result in precipitation.
17. For high enzyme activities and when measuring the faster, mediated reactions, a kinetic measurement of the initial activity should be performed. The coefficient of variation (CV) of the rate determination is 2%. The slope of the increase in absorption is linear for at least 5 min. If low enzyme concentrations have to be used, an endpoint assay should be performed by measuring the absorption before and after an incubation time, as described in the method. The CV of the endpoint assay is 3%.
18. The assay plates were sealed to limit evaporation. The reaction was incubated in the dark to avoid the photooxidation of iodide.
19. The extinction coefficient of the product I_3^- is 28,000 $M^{-1}\text{cm}^{-1}$ (20).

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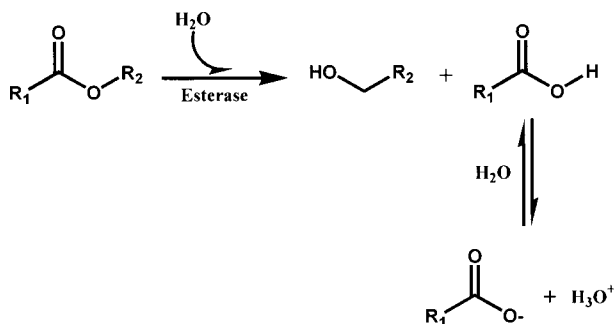
pH Sensing Agar Plate Assays for Esterolytic Enzyme Activity

Karl E. Griswold

1. Introduction

Lipases and esterases are some of the most extensively used enzymes for biotransformations (*1–3*). This is in part due to their widely applicable chemistry, often exceptional regio- and stereo-selectivity, lack of required cofactors and their functionality in organic as well as aqueous media. These enzymes catalyze the hydrolysis of a vast array of ester substrates. However, the process of identifying a native enzyme with the desired activity against a target substrate can be an arduous task. Directed evolution is a facile strategy for engineering substrate specificity and other properties of a candidate enzyme via beneficial mutation of key residues (*4*). The rate limiting step in directed evolution experiments is typically the development of a functional assay for the targeted activity. Owing to the significant investment of time and effort required to develop useful enzyme screens, general assays that may be applied to more than one enzyme or more than one substrate are of particular importance in the field of directed evolution.

The hydrolysis of esters by carboxylesterases represents one of the rare cases where development of a truly universal assay has been possible. The chemistry of ester hydrolysis is amenable to design of generalized screens by virtue of the fact that, for most substrates at neutral pH, this reaction results in the formation of a common product, the hydronium ion (**Scheme 1**). The availability of chromogenic and fluorogenic molecules able to detect changes in hydronium ion concentration allows the enzymatic hydrolysis of ester substrates to be tracked through the decrease in pH that accompanies this reaction in weakly buffered solutions (*5–7*).



Scheme 1. Enzyme catalyzed hydrolysis of a generic ester followed by ionization of the resulting carboxylic acid.

Protein expression in *E. coli* is a well developed field (8), and is ideally suited for directed evolution experiments. A number of techniques for the screening of libraries expressed in *E. coli* have been developed. Plate screens, whereby catalytic activity is directly monitored on agar plates, are suitable for the screening of libraries of up to a few hundred thousand clones (9). This assay format is particularly convenient because no preparation or purification of the cells or the recombinant enzyme is required. The commercial availability of a wide variety of chromogenic pH indicators permits the development of agar plate assays for catalytic hydrolysis of targeted ester substrates. Colonies expressing highly active enzyme variants can be identified by the color change in the solid media resulting from a decrease in the pH of the local environment around active clones (10).

When designing a recombinant expression system for *E. coli*, the protein of interest may be expressed either in the cytoplasm, the periplasmic space or anchored onto the cell surface. In our experience, the incubation time for color development is highly dependent on the nature of the expression system. Cells expressing enzymes in the cytoplasm give a color change on indicator plates only after prolonged incubation which results in partial lysis of a portion of the cells within the colony. In contrast to the inner membrane, the outer membrane of Gram-negative bacteria is readily permeable to hydronium ions. Therefore, in the case of periplasmic expression, the time required for color development depends on the diffusivity of the esterase substrate through the outer membrane. Display of proteins on the cell surface circumvents this limitation and results in a fast assay development time compared to expression in other cellular locations. Proteins can be displayed on the surface of *E. coli* via a variety of methods (11), but we prefer fusions to Lpp-OmpA that have been successful for the surface anchoring of numerous proteins (12).

As a model system we used three constructs of the fungal enzyme cutinase that were expressed in 1) the cytoplasm, 2) the periplasmic space, and 3) on the

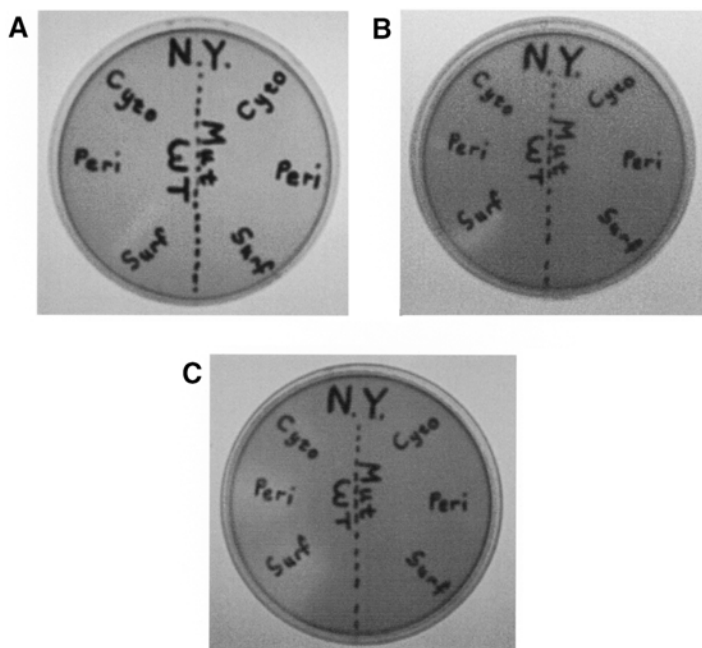


Fig. 1. pH sensing plate assay of wild-type (WT, on left) and S42-A mutant (Mut, on right) cutinase. Agar plates contain 1% tributyrin as substrate and $10 \mu\text{g} \times \text{mL}^{-1}$ nitrazine yellow (N.Y.) as indicator. Cyto = cytoplasmic constructs, Peri = periplasmic constructs, Surf = surface displayed constructs. (A) Photograph of plate after 1 h incubation at 30°C . (B) Photograph of plate after 6 h incubation at 30°C . (C) Photograph of plate after overnight incubation at 30°C .

cell surface using the Lpp-OmpA fusion system. The Ser42-Ala point mutant (~ 220 -fold less active than wild-type cutinase, [13]) was used as a low catalytic activity control. Colonies expressing cutinase in the cytoplasm, periplasm, or on the cell surface were grown on terrific broth (TB) plates, replica plated onto indicating minimal media, and activity was distinguished by the halo of altered color around the cells. The time for assay development was compared for the various constructs using this methodology. Cells expressing cytoplasmic cutinase typically required overnight incubation, periplasmic expression resulted in noticeable color change after approx 4 h, while cells expressing surface displayed enzyme required a development time of 30 min to 1 h (Fig. 1). The response time for periplasmic and cytoplasmic constructs may be accelerated by use of the chloroform vapor lysis technique described in the notes (see Note 13). However, in our experience, the surface displayed construct was the most amenable to screening by this type of plate assay.

2. Materials

1. pMoPac2 (Dr. Andrew Hayhurst, Georgiou Laboratories, University of Texas at Austin).
2. *Sfi*I (New England Biolabs [NEB]).
3. Buffer 2 (New England Biolabs).
4. 100X bovine serum albumin (BSA) (New England Biolabs).
5. Shrimp Alkaline Phosphatase (SAP) (Roche).
6. Low DNA Mass™ Ladder (Invitrogen).
7. T4 Ligase (Gibco BRL).
8. 5X T4 Ligase buffer (Gibco BRL).
9. QIAquick Gel Extraction Kit (QIAGEN).
10. 0.025mm mixed cellulose of ester filter membrane (Millipore, 10 ft roll, cat. no. VSWP00010).
11. Electrocompetent ABLE *C. E. coli* C lac(LacZw-) [Kan^r McrA- McrCB- McrF- Mrr- HsdR (rK- mK-)] [*F'*proAB lacI^qZDM15 Tn10 (Tet^r)] (Stratagene).
12. 0.1-cm electroporation cuvettes (BIORAD) and apparatus for electroporation.
13. SOC (Difco).
14. Terrific Broth (TB) (Difco).
15. Agar.
16. D-Glucose.
17. L-(+)-Arabinose.
18. M9 Minimal salts (Sigma).
19. Thiamine hydrochloride.
20. Ammonium chloride.
21. Sodium chloride.
22. 0.5 M Sodium hydroxide solution.
23. Isopropyl β-D-thiogalactopyranoside (IPTG), dioxane free.
24. Chloramphenicol.
25. Indicator grade-soluble Nitrazine Yellow, Bromothymol Blue, or Phenol Red (Acros Organics).
26. Dimethyl sulfoxide (DMSO) (Fisher Scientific).
27. Tributyrin (Fluka) (or other ester substrate of interest).
28. Sterile felt and apparatus for replica plating.
29. Sterile toothpicks.

3. Methods

3.1. Construction of Surface Displayed Enzyme (see Note 1)

3.1.1. Preparation of Vector

1. Add the following to a sterile 0.5-mL eppendorf tube: 50 μL of a ~50 ng/μL solution of pMoPac2, 6.5 μL of 10X NEB Buffer 2, 6.5 μL of 10X NEB BSA, 2 μL of NEB *Sfi*I.
2. Mix the contents and incubate the solution at 50°C for 4 h.
3. Add 1 μL of SAP to the solution, mix, and incubate at 37°C for 2 h.

4. Separate the digested and dephosphorylated product by electrophoresis on a 1% agarose gel, cut out the 4305 bp band, and purify the DNA using a QIAGEN QIAquick Gel Extraction Kit (*see Note 2*).
5. Quantitate the isolated DNA by electrophoresis and comparison to Low DNA Mass™ Ladder.

3.1.2. Preparation of Insert DNA

1. *Sfi*I sites may be installed on the 5' and 3' ends of the target gene using standard PCR amplification with the appropriate primers and template (*see Note 3*).
2. Add the following to a sterile 0.5-mL eppendorf tube: 50 μ L of a $\sim$ 50 ng/ μ L solution of the target gene with 5' and 3' *Sfi*I sites installed, 6.5 μ L of 10X NEB Buffer 2, 6.5 μ L of 10X NEB BSA, 2 μ L of NEB *Sfi*I.
3. Mix the contents and incubate the solution at 50°C for 4 h.
4. Separate the digested product on a 1% agarose gel, cut out the band corresponding to the desired product and purify the DNA using a QIAGEN QIAquick Gel Extraction Kit (*see Note 2*).
5. Quantitate the isolated DNA by electrophoresis and comparison to Low DNA Mass™ Ladder.

3.1.3. Ligation and Transformation

1. Add the following to a sterile 0.5-mL eppendorf tube: 2 μ L of 5X T4 ligase buffer, sufficient vector solution for $\sim$ 100 ng of vector DNA, sufficient insert solution for a 3:1 molar ratio of insert to vector, 0.5 μ L of T4 DNA ligase, sterile ddH₂O to 10 μ L.
2. Mix the contents and incubate the tube at 16°C for 16 h.
3. Heat inactivate the ligase by incubating the solution at 65°C for 10 min.
4. Desalt the ligation mixture by floating a 1 $\times$ 1 cm square of 0.025 μ m mixed cellulose of ester filter membrane shiny side up on 20 mL of sterile ddH₂O and carefully pipetting the ligation solution onto the filter membrane. Allow the droplet of ligation mixture to dialyze for 30 min at room temperature. A slight increase in volume will be noted.
5. Carefully remove the ligation mixture from the membrane by pipet, chill on ice, and combine with 40 mL of freshly thawed electrocompetent ABLE C cells (*see Note 4*) in a pre-chilled 0.1-cm cuvet.
6. Pulse the cells at 1.80KV, 200 Ω , 25 μ F, immediately dilute with 950 mL of 37°C SOC, transfer to a sterile 15-mL conical tube, and incubate cells with agitation at 37°C for one hour.
7. Spread the cells on TB plates containing 30 μ g $\times$ mL⁻¹ chloramphenicol (Cm) and 2% glucose in dilutions adequate to allow resolution of individual colonies. Incubate the plates overnight at 30°C (*see Note 4*).
8. Individual colonies may be assayed for the presence of insert by PCR. Clones showing insert of the correct size should be sequenced prior to preparation of frozen stocks.

3.2. Preparation of TB Master Plates

1. Prepare a stock solution of 100 mM IPTG and sterilize by filtration (*see Note 4*).
2. Prepare a stock solution of 30 mg/mL chloramphenicol in absolute ethanol (*see Note 4*).
3. Combine 47.6 g TB media, 15 g agar, 4 mL glycerol, and ddH₂O to 1 L. Sterilize by autoclaving.
4. Cool the autoclaved solution to 50°C. Aseptically add 1 mL of IPTG stock solution, 1 mL of Cm stock solution and mix thoroughly.
5. In a sterile manner, pour the agar solution into sterile Petri dishes. Allow the agar to solidify at room temperature for 30 min.

3.3. Preparation of Indicating Minimal Media Plates

1. Prepare the following stock solutions and sterilize by autoclaving: 240 mg/mL MgSO₄ (anhydrous powder) (*see Note 5*), 11.1 mg/mL CaCl₂.
2. Prepare the following stock solutions and sterilize by filtration: 20% arabinose adjusted to pH 8.0 with 0.5 M NaOH (*see Note 4*), 5 mg/mL thiamine hydrochloride, 10 mg/mL nitrazine yellow (*see Note 6*), 100 mM IPTG.
3. Prepare a stock solution of 30 mg/mL chloramphenicol in absolute ethanol.
4. Combine the following: 0.1128 g of M9 minimal salts (*see Note 7*), 1.0 g of NH₄Cl, 5.5 g of NaCl (*see Note 8*), 960 mL of ddH₂O.
5. Adjust the pH of this solution to 8.0 with 0.5 M NaOH.
6. Add 15 g of agar to the solution and sterilize by autoclaving.
5. Cool the autoclaved solution to 50°C. Move to the next step and complete quickly.
6. While the agar mixture is still liquified, add the following in a sterile manner: 1 mL of MgSO₄ stock, 1 mL of CaCl₂ stock, 1 mL of Thiamine stock, 1 mL of nitrazine yellow stock, 1 mL of IPTG stock, 20 mL of arabinose stock, 10 mL of tributyrin.
7. Mix the final solution thoroughly and aseptically pour into sterile Petri dishes. Allow the agar to solidify at room temperature for 30 min. Plates should have a dark blue or purple color.

3.4. Library Screening

1. Prepare a stock solution of 20% glucose and sterilize by filtration (*see Note 4*).
2. Prepare a stock solution of 30 mg/mL chloramphenicol in absolute ethanol.
3. Combine 47.6 g TB media, 4 mL glycerol, 900 mL ddH₂O and sterilize by autoclaving.
4. Cool the autoclaved solution to 50°C. Aseptically add 100 mL of glucose stock solution, 1 mL of Cm stock solution, and mix thoroughly.
5. Inoculate 50 mL of the above TB media with sufficient frozen library stock to yield an initial OD₆₀₀ of ~0.1.
6. Grow the culture, with shaking, at 30°C to OD₆₀₀ = ~0.6.
7. Dilute portions of culture into glucose free media such that plating 50–100 μ L aliquots allows resolution of individual colonies (*see Note 9*).
8. Spread 50–100 μ L aliquots of culture on TB plates containing 30 μ g/mL chloramphenicol and 100 μ M IPTG. Grow the plates overnight at 30°C (*see Note 10*).

9. Transfer the colonies from the TB master plates to the indicating minimal media plates using autoclaved velvet. When engineering substrate selectivity, colonies may be replica-plated onto multiple indicating plates with differing substrates while maintaining the orientation of the master and indicating plates.
10. Incubate the indicating minimal media plates at 30°C until a yellow halo can be identified around the active clones (*see Note 11*).
11. Clones may be cultured directly from the minimal media plates by touching a sterile toothpick to the center of the yellow halo and inoculating TB/Cm 30 µg/mL/2% Glucose media with the toothpick. Alternatively, clones may be cultured from the TB master plate (*see Note 12*).
12. The selected culture should be grown at 30°C with shaking to an OD₆₀₀ of ~0.6. The culture may then be used to make glycerol stocks or subjected to a second round of screening if necessary.
13. Screening of cytoplasmic and periplasmic libraries may be facilitated by use of chloroform vapor lysis (*see Note 13*).

4. Notes

1. This procedure is for cloning into pMoPac2 for surface display in *E. coli*. For library construction, please see chapters in the accompanying volume.
2. For extraction of DNA to be used in ligations, it is useful to include the optional 500 µL QG buffer wash step, and to incubate the column for 3–5 min with the PE wash buffer prior to pulling the PE buffer through the column. Elute the DNA in sterile ddH₂O in a volume sufficient to yield a solution of at least 20 ng/µL (normally 20–50 µL).
3. In order to ensure that the target gene is in frame with the Lpp-OmpA fusion partner, the *Sfi*I extension of the 5'-primer should read: 5'-CAACGCGGCCAG-CCGGCCATG...-3' where the ATG encodes the start codon of the target gene. To preserve the His6 and myc tags in frame, the *Sfi*I extension of the 3'-primer should read: 5'-GAATTCGGCCCCCGAGGCCCCXXX...-3' where "XXX" encodes the reverse complement of the C-terminal amino acid of the target enzyme.
4. pMoPac2 utilizes chloramphenicol as the antibiotic marker, and growth of cells is optimum at 30°C. The *lac* promoter is IPTG inducible, and expression may be repressed with glucose via the *lac*I repressor. We have found that the ABLE C cell line generally is optimal for surface display of proteins using pMoPac2. The appropriate antibiotic, inducer, repressor, and incubation temperature should be employed for other expression systems and cell lines. In these studies, arabinose was used as the carbon source in order to induce expression of the periplasmic and cytoplasmic constructs. Alternative carbon sources may be substituted when applicable.
5. Caution: dissolving anhydrous magnesium sulfate is a highly exothermic process. Preparation of the solution is best accomplished by slow addition of the MgSO₄ powder while cooling the stirring solution in an ice bath.
6. We have found that nitrazine yellow, bromothymol blue, and phenol red provide sufficient contrast for discrimination of positives from negatives in our model sys-

tem. Nitrazine yellow was found to be the preferred indicator. Brilliant yellow was found to be ineffectual as an indicator for plates over the pH range of 4.5–8.0. Other groups have made use of crystal violet and neutral red for plate assays (14). Alternative indicators are also commercially available, but should be evaluated for compatibility with the system and pH ranges of interest.

7. This M9 salt content resulted in sufficient buffering capacity for visible halo generation by single colonies in approximately three hours with the wild-type cutinase surface displayed construct and tributyrin. Optimization of the media buffering capacity may be necessary for alternative substrates or enzymes.
8. The sodium chloride content of the solution may be altered in order to maintain an approximately isotonic solution. This recipe is specifically for plates made with 1% tributyrin as a substrate. Different substrate concentrations, substrates of differing molecular weights, or alterations in the general recipe will require more or less sodium chloride (Isotonic solutions contain ~300 mM molecules and/or ions).
9. When the density of positive clones is high, the colored regions surrounding positive colonies may overlap making differentiation of the respective clones difficult. Thus, analysis of plates containing a higher density of colonies may require replating of neighboring colonies and rescreening.
10. Plates should be grown for a time sufficient to yield colonies at least 1 mm in diameter.
11. The incubation time required for formation of the yellow halo will be dependant on the enzymatic activity and the size of the colonies that are transferred from the master plate (larger colonies exhibit a signal more rapidly than smaller colonies).
12. When the time to halo formation is less than that required for growth of a new colony, it is possible that inactive clones in the vicinity of a positive halo will be inadvertently harvested along with the positive clone because distinct colonies are not yet visible. This problem is readily addressed by performing a second round of screening on the resulting culture of mixed cells that will be substantially enriched for the positive clone, or plating the resulting culture and performing an alternative secondary assay with individual colonies. When individual colonies are sufficiently separated, harvesting positive clones from the master plate may be facilitated by additional incubation of the plate sufficient to re-grow identifiable colonies.
13. For rapid detection of cytoplasmic and periplasmic enzymes, cells may be lysed directly on the agar plates by removing the Petri dish cover and inverting the plate on a wire mesh suspended ~1 in over a small volume of chloroform in a recrystallization dish. With the cutinase model system, cytoplasmic constructs yielded detectable signal after 20 min of incubation in the lysis chamber and a subsequent 30 min incubation at 30°C. Caution: Avoid direct contact with chloroform or inhalation of vapors (refer to MSDS for additional precautions). Plates in the lysis chamber should be watched closely as the chloroform vapors will begin to dissolve the Petri dish during extended incubations.

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A pH-Indicator-Based Screen for Hydrolytic Haloalkane Dehalogenase

Huimin Zhao

1. Introduction

Microbial hydrolytic haloalkane dehalogenases catalyze the cleavage of halogen-carbon bonds of a variety of aliphatic halogenated compounds, including a broad range of chlorinated (C_2 – C_6) and brominated (C_2 – C_8) alkanes, with water as the sole co-substrate, resulting in the production of halide ions, protons, and alcohols (**1,2**). Based primarily on substrate specificity and sequence homology, these enzymes have been classified into two general classes that are represented by the enzymes from *Xanthobacter autotrophicus* GJ10 and *Rhodococcus rhodochrous* (**3**). The study of these enzymes has been motivated largely by their potential use in waste treatment, bioremediation and industrial biocatalysis (**4,5**). A substantial amount of mechanistic and structural information is available for these enzymes (**6–9**).

The haloalkane dehalogenase from *Rhodococcus rhodochrous* (*RrDHL*) is of particular interest because it is capable of selectively converting several industrially important commodity chemicals, including 1,2-dichloropropane (DCP), 1,2,3-trichloropropane (TCP), and 1,2-dichlorobutane (DCB), into more valuable chlorohydrins. Traditional chemical catalysts cannot do this. These chemicals are generated at a scale of several hundred million pounds per year as side products in the existing manufacturing processes of propylene oxide, epichlorohydrin, and butylene oxide, and are presently incinerated at a cost. Thus, developing *RrDHL*-based biocatalytic processes to recover value from these chloroalkanes represents an important environmental and process opportunity (**4**). Unfortunately, as a practical biocatalyst, this *RrDHL* enzyme suffers from low activity and low stability under the industrial process conditions.

To overcome these drawbacks and develop commercially viable biocatalytic processes, we sought to use directed evolution to engineer *RrDHL* mutants with increased activity and stability. Directed evolution mimics the process of natural evolution in the test tube, involving the generation of molecular diversity by random mutagenesis and gene recombination followed by selection/screening on the basis of desired functional changes (**10**). In contrast to rational design, this approach does not require prior extensive structural and mechanistic information on the biological molecules. Directed evolution has been successfully applied to alter enzyme functions, including thermostability, substrate specificity, catalytic activity, enantioselectivity, and pH profile (**11**). From these studies, it is clear that a key requirement for a successful directed evolution experiment is the development of an efficient screening or selection method for the enzyme function of interest.

Here we describe a sensitive, 96-well plate-based screening method for directed evolution of highly active and stable *RrDHL* mutants towards substrate 1,2,3-trichloropropane (TCP). The conventional assay for dehalogenase activity is based on colorimetric detection of halide release using ferric nitrate and mercuric thiocyanate (**9**), which is an end-point assay and not amenable to high throughput screening. To increase the throughput of library screening, we developed a quantitative, colorimetric assay for *RrDHL* that uses a pH indicator to monitor the rate of proton release, according to the work reported by Janes et al. (**12**). We chose bromothymol blue (BTB) as a pH indicator and *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) as a buffer because they have very similar pK_as that ensure the color change is proportional to the number of protons released. The screening procedures are illustrated in **Fig. 1**. The gene encoding *RrDHL* is over-expressed in *E. coli* at high level using plasmid pET24a(+) (Novagen, Madison, WI). A library of *RrDHL* variants is created using error-prone PCR and grown in 96-well plates. The activity of each variant is measured using a 96-well plate reader by following the absorbance change at 620 nm. To screen for *RrDHL* variants with increased thermostabilities, two replica 96-well assay plates are made for each growth plate, and the ratio between the activity with heat treatment (residual activity) and the activity without heat treatment (initial activity) is used as a thermostability index. Only variants with both higher thermostability index and similar to or higher initial activity than the parental enzyme are selected for the next round of evolution. To screen for *RrDHL* variants with increased activity, only one replica 96-well assay plate is needed. Variants with higher specific activity than the parental enzyme are selected for the next round of evolution.

Using this screening method coupled with repeated cycles of random mutagenesis by error-prone PCR, we successfully obtained an evolved *RrDHL* mutant exhibiting 140-fold longer half-life of thermal inactivation at 53°C and

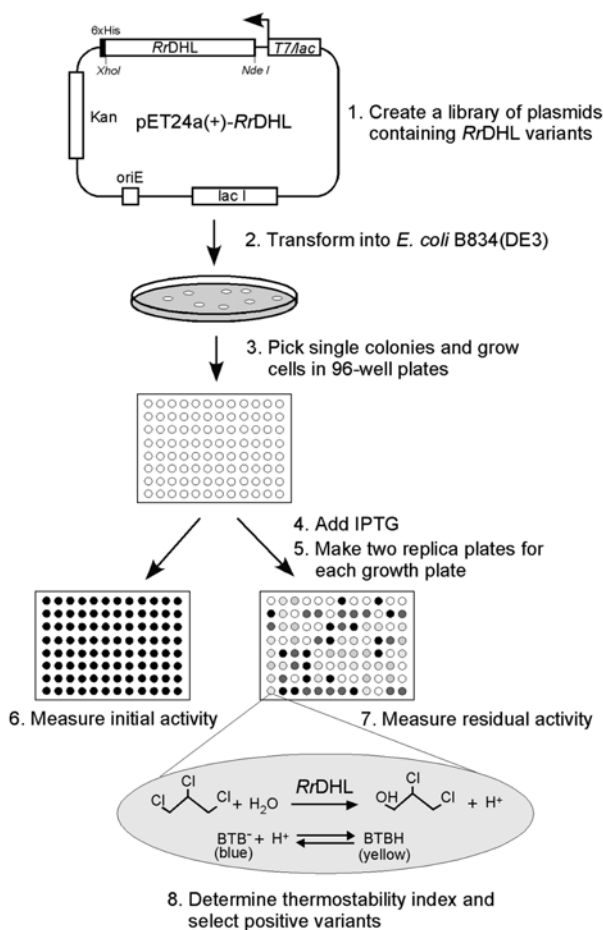


Fig. 1. Schematic representation of the screening method for directed evolution of hydrolytic haloalkane dehalogenase variants with increased thermostability. For directed evolution of dehalogenase variants with increased activity, a similar screening method is used in which no heat treatment is required.

10-fold higher specific activity than that of the wild-type enzyme (**13**). This screening method should be applicable to other enzymes, such as amidases, esterases, lipases, proteases, and kinases, which produce protons in the reactions.

2. Materials

1. Plasmid pET24a(+) and *E. coli* strain B834(DE3) (Novagen, Madison, WI). B834(DE3) competent cells are prepared using the standard calcium chloride method.

2. *E. coli* HB101 high transformation efficiency competent cells (Promega, Madison, WI).
3. 1 M IPTG (isopropyl- β -D-thiogalactopyranoside) in water: sterilize by filtering through a 0.22- μ m filter (Sigma, St. Louis, MO).
4. BTB (bromothymol blue) and BES [*N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid] (Sigma).
5. TCP (1,2,3-trichloropropane) (Aldrich, Milwaukee, WI).
6. 0.1 N standardized HCl (Sigma).
7. 5 mM MnCl₂ (Sigma).
8. Restriction endonucleases: *Nde*I and *Xho*I and appropriate 10X reaction buffers (New England BioLabs, Beverly, MA).
9. T4 DNA ligase and its 10X reaction buffer (Roche Diagnostics, Indianapolis, IN).
10. Oligonucleotide primers: P5N (5'-GAT ATA CAT ATG TCA GAA ATC GGT ACA GG-3', underline sequence is *Nde*I restriction site) and P3X (5'-GGT GCT CGA GTG CGG GGA GCC AGC G-3', underline sequence is *Xho*I restriction site).
11. *Taq* DNA polymerase and its 10X reaction buffer (Promega).
12. *Pfu* DNA polymerase and its 10X reaction buffer (Stratagene, La Jolla, CA).
13. 10X error-prone PCR reaction buffer: 100 mM Tris-HCl, pH 8.3 at 25°C, 500 mM KCl, 70 mM MgCl₂, 0.1% (wt/vol) gelatin.
14. 10X error-prone PCR dNTP mix: 2 mM dATP, 2 mM dGTP, 10 mM dCTP, and 10 mM dTTP (Roche Diagnostics, Indianapolis, IN).
15. 10X standard PCR dNTP mix: 2 mM of each dNTP (Roche Diagnostics).
16. 50 mg/mL kanamycin solution (1000X).
17. Luria-Bertani (LB) medium: 10 g bacto-tryptone, 5 g bacto-yeast extract, and 10 g sodium chloride, pH 7.0. Sterilize by autoclave.
18. LB agar plates: LB medium with 15 g/L agar. Sterilize by autoclave.
19. T4 DNA ligase and its reaction buffer (Roche Diagnostics).
20. Agarose gel electrophoresis supplies and equipment.
21. EZ load precision molecular mass ruler (Bio-Rad, Hercules, CA).
22. MJ PTC-200 thermocycler (MJ Research Inc., Watertown, MA).
23. SpectraMax 96-well plate reader (Molecular Devices, Sunnyvale, CA).
24. Cary-100 UV-Vis spectrophotometer (Varian Inc., Walnut, CA).
25. Incubators and incubator-shakers.
26. Water baths.
27. DNeasy system for genomic DNA isolation, QIAprep spin plasmid miniprep kit, QIAquick PCR purification kit, QIAEX II gel extraction kit and Ni-NTA Spin kit (QIAGEN, Valencia, CA).

3. Methods

The methods described below outline 1) the cloning and expression of *RrDHL* (see **Note 1**), 2) creation of a library of *RrDHL* variants, 3) a screening procedure for *RrDHL* variants with increased thermostability, and 4) a screening procedure for *RrDHL* variants with increased specific activity.

3.1. Cloning and Expression of RrDHL

1. Combine 10 μg plasmid pET24a(+), 10X NEB buffer 2, 30 U *Nde*I and 30 U *Xho*I in a total volume of 100 μL . Incubate at 37°C for 2 h.
2. Load the reaction mixture on 1% agarose gel and run electrophoresis for approx 40 min at 100 V.
3. Extract and recover the *Nde*I and *Xho*I digested vector DNA using QIAEX II gel extraction kit. Estimate DNA concentration using gel electrophoresis with EZ load precision molecular mass ruler (DNA mass standards).
4. Isolate genomic DNA from *Rhodococcus rhodochrous* TDTM-003 using DNeasy system (Qiagen) or other reliable method.
5. Combine 10 ng genomic DNA, 10X cloned *Pfu* buffer, 0.5 μM each primer (P5N and P3X), 10X standard PCR dNTP mix (2 mM each dNTP), and 2.5 U Turbo *Pfu* polymerase in a total volume of 100 μL .
6. Run the PCR reaction using the following program: 96°C for 2 min; 25 cycles of 94°C for 1 min; 50°C for 1 min; and 72°C for 1 min; followed by 72°C for 7 min.
7. Purify the PCR product according to the manufacturer's protocol in the QIAQuick PCR purification kit, and digest the PCR product with *Nde*I and *Xho*I followed by product purification. Estimate DNA concentration using gel electrophoresis with EZ load precision molecular mass ruler.
8. Combine ~100 ng of *Nde*I and *Xho*I digested plasmid pET24a(+), 30 ng of *Nde*I and *Xho*I digested PCR product, 10X T4 DNA ligase buffer, and 0.5 μL T4 DNA ligase (1 U/ μL) in a total volume of 10 μL . Incubate at 16°C for 12–16 h.
9. Transform 2–5 μL of ligation mixture into B834(DE3) competent cells using standard heat shock method, and plate out on LB agar plates containing 50 $\mu\text{g}/\text{mL}$ kanamycin. Incubate the plates at 37°C for 14–16 h.
10. Pick 2–3 colonies and confirm the successful cloning of the *RrDHL* gene by colony PCR or restriction enzyme digestion analysis. The resultant desired plasmid is denoted pET24a(+)-*RrDHL*. As described below in detail, expression of the *RrDHL* enzyme is induced by IPTG and the enzyme activity is determined using a pH-indicator-based assay.

3.2. Random Mutagenesis and Library Creation

1. Combine 10–100 ng of the plasmid DNA containing the *RrDHL* gene (pET24a(+)-*RrDHL*), 0.3–1.0 μM each primer (P5N and P3X), 10X error prone PCR buffer, 10X error-prone PCR dNTP mix, and 5 U *Taq* in a total volume of 100 μL .
2. Run the PCR reaction using the following program: 96°C for 2 min; 13 cycles of 94°C for 1 min; 50°C for 1 min; and 72°C for 1 min; followed by 72°C for 7 min.
3. Purify the PCR product according to the manufacturer's protocol in the QIAQuick PCR purification kit, and estimate the DNA concentration using gel electrophoresis with EZ load precision molecular mass ruler (~0.01 $\mu\text{g}/\mu\text{L}$).
4. Digest the PCR product with *Nde*I and *Xho*I, and purify the product using QIAquick PCR purification kit. Estimate the DNA concentration of the purified

product using gel electrophoresis with EZ load precision molecular mass ruler.

5. Combine ~100 ng of *Nde*I and *Xho*I digested plasmid pET24a(+), 30 ng of *Nde*I and *Xho*I digested PCR product, 10X T4 DNA ligase buffer, and 0.5 μ L T4 DNA ligase (1 U/ μ L) in a total volume of 10 μ L. Incubate at 16°C for 12–16 h.
6. Transform *E. coli* high efficiency HB101 competent cells with 2–5 μ L of ligation mixture according to the protocol recommended by the manufacturer (Promega), and plate out a small aliquot of the transformed cells on LB agar plates containing 50 μ g/mL kanamycin to estimate the library size while growing the rest of the transformed cells in 3 mL LB medium containing 50 μ g/mL kanamycin (see **Note 2**).
7. Isolate the plasmids in the transformed cells using QIAprep spin plasmid miniprep kit and estimate the DNA concentration using a UV-Vis spectrophotometer.
8. Transform plasmid DNA into B834(DE3) competent cells using standard heat shock method, and plate out on LB agar plates containing 50 μ g/mL kanamycin. Incubate the plates at 37°C for 14–16 h.
9. Pick single colonies into 96-well plates containing 100 μ L of LB medium and 50 μ g/mL kanamycin per well using sterilized toothpicks or an automated colony picker such as QPix (Genetix, Hampshire, UK). For each 96-well plate, use 3 wells for controls (colonies containing the plasmid with the parental *RrDHL* gene).
10. Incubate the 96-well plates at 30°C for 5 h with slow shaking (150–200 rpm) (see **Note 3**).
11. Add 100 μ L of LB medium containing 50 μ g/mL kanamycin and 2 mM IPTG to induce protein expression, and continue to incubate overnight (see **Note 4**).

3.3. Thermostability Screen

1. Prepare two replica 96-well assay plates for each growth plate by taking 20 μ L aliquots of cell culture from each well.
2. Incubate one of the two 96-well assay plates at a target temperature (it is set at 55°C in the first round screening) for 2 min in an oven (see **Note 5**). Take the plate out and allow it to cool to room temperature. Add 200 μ L of assay solution containing 1 mM BES, 50 μ M BTB and ~10 mM TCP, pH 7.8 (see **Notes 6 and 7**). Monitor the absorbance change for 8 min at 620 nm using a SpectraMax 96-well plate reader. Calculate the enzyme activity (= initial activity) (see **Note 8**).
3. Incubate the second of the two 96-well assay plates at 55°C for 10 min in an oven. Follow the same steps as described above and determine the enzyme activity (= residual activity).
4. Calculate the ratio of residual activity to initial activity (defined as the thermostability index) for each variant. The positive variants should be those whose thermostability indexes are higher than the parental enzyme and whose initial activities are similar to or higher than the parental enzyme.

3.4. Activity Screen

1. Prepare a replica 96-well assay plate for each growth plate by taking 20 μL aliquot of cell culture from each well.
2. Add 200 μL of assay solution containing 1 mM BES, 50 μM BTB, and ~ 10 mM TCP, pH 7.8 (see **Notes 6** and **7**). Monitor the absorbance change for 8 min at 620 nm using a SpectraMax 96-well plate reader. Calculate the enzyme activity (see **Note 8**).
3. Select variants with higher activity than the parental enzyme for further characterization (see **Note 9**). These variants are purified from *E. coli* using Ni-NTA Spin kit (Qiagen) according to the manufacturer's instructions. The protein expression levels of these variants are evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The enzyme activity is measured in a 96-well plate reader using the pH-indicator-based dehalogenase activity assay described above. The positive variants should be those whose protein expression levels are similar to the parental enzyme, but whose activities are higher than the parental enzyme.

4. Notes

1. The gene encoding *RrDHL* was previously cloned into a pTrcHis expression vector (Invitrogen, San Diego, CA) in a fusion protein construct (**14**). This fusion protein construct contains an 11-amino acid poly-histidine tail at the *N*-terminus, a 293-amino acid *RrDHL* enzyme, and a 13-amino acid EXFLAG peptide tail at the C-terminus. Based on the crystal structure of the *RrDHL* enzyme, it seems that the *N*-terminal peptide tail may block entrance of the substrate into the enzyme active site. Thus, to eliminate potential adverse effect of this peptide tail on enzyme activity, a new construct containing the *RrDHL* enzyme and a poly-histidine tail at the C-terminus was created by cloning the *RrDHL* gene from the genomic DNA of *Rhodococcus rhodochrous* TDTM-003 into pET24a(+) vector. The expression of the *RrDHL* enzyme was induced by IPTG in *E. coli* and the protein expression level was found to be about 15% of total cellular proteins. The specific activity of the new construct toward TCP was 50% higher than the original construct.
2. The transformation efficiency of B834(DE3) or other BL21-derived strains is only 10^3 – 10^4 transformants per μg ligated DNA ($\sim 10^6$ transformants per μg plasmid DNA) using either the electroporation method or the calcium chloride method. Because of this low transformation efficiency, it is quite laborious and time-consuming to create a library of more than 10^4 variants. To overcome this problem, *E. coli* strains with very high transformation efficiency such as HB101 ($>10^8/\mu\text{g}$) and XL1-blue ($>10^9/\mu\text{g}$) may be used to create a library of plasmids containing *RrDHL* variants. This library of plasmids is then transformed to B834(DE3) to yield a large library of variants.

3. To increase the reliability and sensitivity of the screening method, it is important to ensure that cells grow very uniformly in the 96-well plates. Two small tricks are very helpful. First, to minimize water evaporation of the medium, a wet paper towel is added under each 96-well plate in the plate holder. Second, to avoid the formation of cell clumps in the wells and provide enough aeration for normal cell growth, a glass bead (3-mm diameter, Kimble Glass Inc., Vineland, NJ) is added into each well acting as a stirring bar. In addition, it was found through experiments that more soluble active enzymes per well were produced at 30°C than at 37°C. Also, the evaporation problem at 30°C was less severe than that at 37°C. Thus, 30°C was used for optimal cell growth.
4. For optimal protein expression, the cells are induced at the mid-log growth phase. After IPTG induction, the maximal protein expression level may be reached within a few hours (5–6 h). The choice of overnight incubation is only for the sake of convenience.
5. The 96-well assay plate is contained in an aluminum box made to closely fit the plate, ensuring uniform heating. The specific incubation temperature is chosen such that the thermostability index of the parental enzyme is 20–30% after a 10 min incubation. This temperature needs to be adjusted for subsequent rounds of screening when the thermostability of the enzyme has been increased. *See Chapters 11 and 12* for more on screening for thermostability.
6. It is extremely important to choose an appropriate pH indicator and buffer pair for accurate measurement of dehalogenase activity. The main selection criterion is that the pK_a of the buffer and the pK_a of the pH indicator should be as close to equal as possible. BTB is chosen as the desired pH indicator mainly because it works most sensitively between pH 6.0 and 7.6. The color of BTB will gradually change from blue to yellow with increasing amount of protons generated by the enzyme. BES is chosen because its pK_a (7.1) is very close to that of BTB (7.0). The BTB concentration is determined such that the initial absorbance at 620 nm is ~1.2. The BES concentration (1 mM) is determined such that it is low enough to maximize sensitivity, while high enough to ensure accurate measurements and small pH changes throughout the assay. The small pH changes are important because kinetic parameters may change with changing pH (*12*).
7. The maximal solubility of TCP in water is about 10 mM. To prepare the assay solution, TCP is added in excess and mixed with other components using a stirring bar for 1 h. Droplets of TCP can be seen at the bottom of the bottle containing the assay solution. Since the K_M of the dehalogenase enzyme is about 1 mM, the reaction rate should reach its maximum at saturated TCP.
8. The rate of absorbance change is correlated to enzyme activity in the unit of μmol of product formation per min by a conversion factor. This conversion factor is determined by titrating the assay solution with HCl. For RrDHL, an OD₆₂₀ change of -1.212 corresponded to 1 μmol of protons produced.
9. It is possible that the increased enzyme activity comes from increased protein expression level caused by codon changes. Thus, to eliminate false positives, the specific activity of the selected variants should be determined using purified

enzymes. Because of the poly-histidine tag, a dozen variants can be purified in 0.5 h using a Ni-NTA Spin kit (Qiagen) and subjected to enzyme activity analysis and protein expression analysis.

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Detection of Aromatic α -Hydroxyketones with Tetrazolium Salts

Michael Breuer and Bernhard Hauer

1. Introduction

In principle there are two ways for the biocatalytic synthesis of α -hydroxyketones. Oxidoreductases may be used to convert diketones or diols into the respective α -hydroxyketones. This kind of reaction is exploited, e.g., in the industrial synthesis of the α -glucosidase inhibitor miglitol ([2*R*,3*R*,4*R*,5*S*]-1-[2-hydroxyethyl]-2-[hydroxymethyl]-3,4,5-piperidintriol) (**1**). Alternatively, thiamine diphosphate dependent transketolases are capable of synthesizing α -hydroxyketones by catalyzing the transfer of activated glycolaldehyde (**2**). In natural biosyntheses these enzymes are usually found in sugar metabolism. Another thiamine diphosphate-dependent enzyme is used for the industrial production of *R*-phenylacetylcarbinol ([1*R*]-1-hydroxy-1-phenyl-propan-2-one). *R*-phenylacetylcarbinol (*R*-PAC) is the first chiral intermediate in the production process of pseudoephedrine and ephedrine. Since 1921, it has been known that yeast (*Saccharomyces cerevisiae*) are able to catalyze the formation of *R*-PAC (**3,4**). The further synthetic steps from *R*-PAC to pseudoephedrine are carried out by classical chemical synthesis (**5**). The actual enzyme in yeast catalyzing the synthesis of *R*-PAC is pyruvate decarboxylase (PDC; EC 4.1.1.1). In vivo this enzyme converts pyruvate to acetaldehyde. 2- α -Hydroxyethyl-thiamine diphosphate (“activated acetaldehyde”) is an intermediate of the catalytic cycle of PDC. Its α -carbanion reacts with several aldehydes in a nucleophilic attack to form the respective acyloins (**6**). In this manner, benzaldehyde and pyruvic acid form *R*-PAC and CO₂. Pyruvate decarboxylase has been isolated from a number of different sources (**7**). In contrast to the enzymes from different yeast and fungi, pyruvate decarboxylase from the bac-

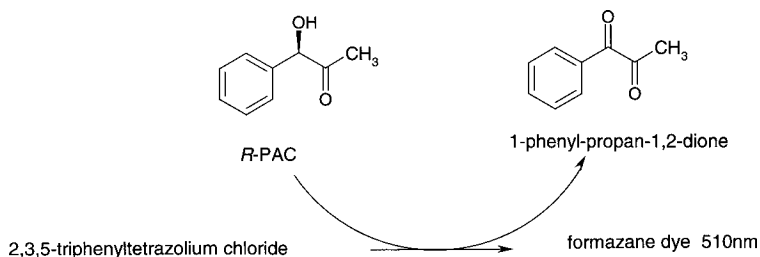
terium *Zymomonas mobilis* catalyzes the formation of substantial amounts of *R*-PAC from acetaldehyde and benzaldehyde (**Scheme 1**) (8,9).



PDC from *Z. mobilis* was optimized by directed evolution to improve the stability of the catalyst.

As a tool for this endeavor, a high-throughput assay was developed. A number of methods for detecting *R*-PAC and similar acyloins are described in the literature (10). Those based on chromatographic procedures (11) are too slow to be employed in a high-throughput screening process. A colorimetric method using the Voges-Proskauer reaction was described and later modified (12). This method is not specific, as it also detects acetoin (3-hydroxy-2-butanone). Acetoin is a byproduct of PDC formed by the addition of two molecules acetaldehyde. Interference by acetoin should be avoided in an assay for *R*-PAC.

The redox potential of *R*-PAC is sufficient to reduce tetrazolium compounds (13). Tetrazolium red (2,3,5-triphenyltetrazolium chloride) was found to be an especially convenient reagent for the detection of aromatic acyloins (14). Reaction of *R*-PAC with tetrazolium red yields a red formazane dye that can be easily determined spectrophotometrically (**Scheme 2**). 2,3,5-triphenyltetrazolium chloride is rather selective for aromatic acyloins (see **Note 1**).



Additionally, it was also found that tetrazolium red can also be used to detect 2-hydroxypropiophenone, an isomer of *R*-PAC (15). This compound, however, is not formed by pyruvate decarboxylase, but by benzoylformate decarboxylase (EC 4.1.1.7) (see **Note 2**).

2. Materials

2.1. Biological and Chemical Materials

All chemicals were purchased from Sigma Chemicals, Deisenhofen, Germany. Authentic *R*-phenylacetylcarbinol was a gift from BASF PharmaChemikalien GmbH&Co KG, Minden, Germany.

1. Appropriate *Escherichia coli* strain (e.g., *E. coli* SG13009).
2. IPTG (Isopropyl- β -D-thiogalactoside) inducible expression plasmid containing pyruvate decarboxylase gene.
3. LB* (Luria broth) and LB agar plates* (**16**).
4. Appropriate antibiotics.
5. ddH₂O.*
6. Substrate solution: 30 mM acetaldehyde, 40 mM benzaldehyde, 20 mM MgSO₄, 2 mM thiamine diphosphate, 50 mM 2-[*N*-Morpholino]ethane-sulfonic acid, pH 7.0.
7. Tetrazolium red solution: 0.2%(w/v) 2,3,5-triphenyltetrazolium chloride in methanol.
8. 3 M NaOH.

2.2. Equipment

1. Toothpicks.*
2. Automated colony picker (e.g., Qpix2, Genetix Ltd., Hampshire, UK).
3. 96-well microtiter plates* (Greiner Labortechnik GmbH, Frickenhausen, Germany).
4. Rotary shaking incubator (5.6g, 37°C).
5. Replica plater* (Sigma Chemicals, Deisenhofen, Germany).
6. Microplate centrifuge (Rotanta 96 RSC, Hettich-Zentrifugen GmbH&Co. KG, Tuttlingen, Germany).
7. Microplate shaker (monoshake, H+P Labortechnik AG, Oberschleissheim, Germany).
8. Microplate reader (e.g., Spectra Max 190, Molecular Devices, Sunnyvale, CA).
9. Appropriate software (e.g., Softmax Pro Molecular Devices).
10. MS Excel software.

3. Methods

3.1. Production of Pyruvate Decarboxylase

Escherichia coli SG13009 harboring a plasmid *pPDC*-His₆ with pyruvate decarboxylase from *Zymomonas mobilis* were prepared as described elsewhere (**11**). Variants of pyruvate decarboxylase may be generated with appropriate techniques (see companion volume “Directed Enzyme Library Creation.”

*Sterilize before use.

1. Competent *Escherichia coli* are transformed with a library of plasmid-borne PDC variants plated on selective agar medium, and grown approximately 12 h at 37°C.
2. Colonies from freshly plated LB agar plates are picked with toothpicks to inoculate 96-well plates containing 150 μ L LB supplemented with appropriate antibiotics. Each 96-well plate is inoculated with *E. coli* carrying reference PDC. This reference is the gene that served as starting material in the respective cycle of enzyme evolution. These wells serve as standards. Libraries larger than 500 clones are preferably picked using an automated picking robot.
3. Cells are grown at 37°C and 5.6g for approx 12 h.
4. These cultures are used to inoculate fresh 96-well plates. At this point the libraries are also replica-plated.
5. Three hours after inoculation, protein expression is induced by adding IPTG (1 mM final conc.).
6. After growing the cultures for additional 16 h, cells are harvested by centrifugation (5 min, 3850g).

3.2. Enzyme Incubation

1. Cell pellets are suspended in 200 μ L substrate solution.
2. The 96-well plates are agitated for 10 s to promote cell lysis. Cell lysis is caused by benzaldehyde. Additional lysing agents, e.g., bugbuster (Novagen, Madison, WI), do not increase the efficiency of cell lysis.
3. After incubating the mixture for 45 min at 25°C insoluble matter is removed by centrifugation (5 min, 3850g).
4. 100 μ L supernatant is transferred to fresh 96-well plates and is assayed for R-PAC.

3.3. R-PAC Detection

After the addition of 20 μ L tetrazolium red solution and 10 μ L NaOH (3 M) the tetrazolium compound is reduced by R-PAC to the respective formazan dye. This reaction is followed at 510 nm spectrophotometrically (see Notes 3–5).

3.4. Data Analysis

Data are either evaluated using the software of the photometer or after transfer to MS Excel. The rate of formazan formation and final OD_{510 nm} values depend on R-PAC concentration. R-PAC concentrations up to 0.5 mM correlate well with absorption at 510 nm measured 2 min after the addition of tetrazolium solution and NaOH (see Fig. 1A). Larger amounts of formazan increase OD_{510 nm} beyond the linear range of the spectrophotometer. In these cases, the initial rate of formazan formation is useful to estimate the content of R-PAC in the sample (see Fig. 1B).

Especially when large numbers of samples are analyzed, plate-to-plate differences may occur. Therefore samples should only be compared to the standards of the same 96-well plate.

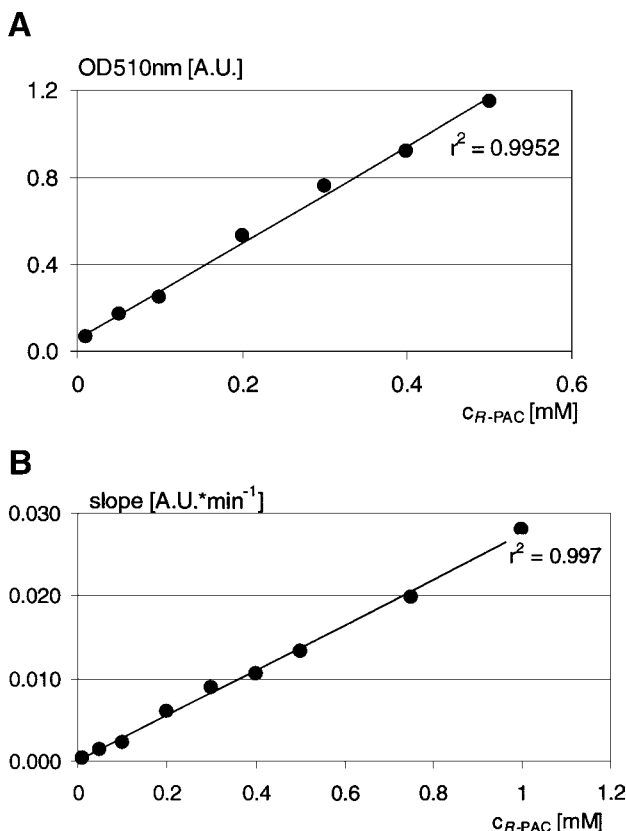


Fig. 1. Correlation between *R*-phenylacetylcarbinol concentration and the formation of the red formazane dye. **(A)** OD_{510 nm} measured 2 min after the addition 2,3,5-triphenyltetrazolium chloride to different amounts of *R*-PAC (r^2 : 99.5%). **(B)** Correlation between the absorption increase measured at 510 nm and *R*-PAC concentration (r^2 : 99.7%).

4. Notes

1. In contrast to other colorimetric assays for acyloins, 2,3,5-triphenyltetrazolium chloride appears to be fairly specific for the detection of aromatic acyloins.
2. To examine the selectivity of the test system, we checked for false positive reaction of tetrazolium red in the presence substrates (benzaldehyde and acetaldehyde) or byproducts (acetoin) of the reaction. It is known that pyruvate decarboxylase also catalyses the synthesis of acetoin from two acetaldehyde molecules. To estimate any interference by this by-product, the response of the assay to 3 mM acetoin was analyzed.

1	-	-	+		0.048
2	-	+	+		0.523
3	+	+	+		0.547
4	-	+	-		0.052
5	+	+	-		0.101
6	+	-	-		0.153
7	-	-	-		0.044
8	+	-	+		0.159
	acetoin	substrates	PDC		OD _{510nm} after 120 sec

Fig. 2. Detection of *R*-PAC with tetrazolium red. The formation of the red color can only be observed in the presence of active enzyme and its substrates (samples 2 and 3). In sample 2, 0.2 mM *R*-PAC is formed. No formazan dye is observed when either substrates, catalyst, or both are omitted from the reaction mixture (samples 1, 4, and 7). Acetoin (3 mM) gives rise to much less color (samples 5, 6, and 8) and does not influence *R*-PAC-detection (sample 3).

- Figure 2** shows the result of this experiment. Significant formation of the red formazan is only observed when both active enzyme and the respective substrates are present in the reaction mixture (samples 2 and 3). The positive control (sample 2) gives an intense red color (OD_{510 nm}: 0.523). This is equivalent to 0.2 mM *R*-PAC. Both acetaldehyde and benzaldehyde alone do not react with tetrazolium red (sample 4). When assaying 3 mM acetoin only a very faint reddish hue (OD_{510 nm}: 0.15, sample 6) was observed.
- A comparison of samples 2 and 6 shows that tetrazolium red is more than 60 times more selective for *R*-PAC than for acetoin. When *R*-PAC (0.2 mM) is analyzed in the presence of 3 mM acetoin (sample 3) the absorption is increased by only 2.9%. Acetoin, the by-product of the biotransformation does not interfere considerably with the color formation due to *R*-PAC. A 15-fold excess of acetoin results only in a small increase of the assay response. Under standard assay conditions the concentration of acetoin formed by pyruvate decarboxylase is less than 15% of *R*-PAC. Thus, when screening for PDC activity the additional formation of formazan by acetoin is negligible.
- Assaying *R*-PAC formation with this assay can give a good estimate of the enzyme performance. However, not all relevant characteristics of pyruvate decarboxylase can be determined in a single assay done in high-throughput. We therefore recommend checking those variants identified by the tetrazolium red assay thoroughly and in a more sophisticated manner. We recommend growing cells in shake flasks for optimal enzyme production, and analyzing specific carboligase activity as well as enantioselectivity of *R*-PAC formation.

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Selection of Heat-Stable *Clostridium cellulovorans* Cellulases After In Vitro Recombination

Koichiro Murashima and Roy H. Doi

1. Introduction

Cellulases degrade cellulose, which is the most abundant biological polymer on the earth (**1**). Although the chemical composition of cellulose is very simple, consisting of only glucose residues connected by β -1,4-glycosidic bonds, no single enzyme is able to degrade crystalline cellulose. To degrade crystalline cellulose to glucose, at least three enzymes have to cooperate: endoglucanase (EC 3.2.1.4), exoglucanase (cellobiohydrolase, EC 3.2.1.91), and β -glucosidase (EC 3.2.1.21) (**1**).

From the standpoint of industrial application of cellulases, there have been many efforts to transform cellulosic biomass into fermentable sugar, which could be converted to ethanol by fermentation (**2**). This process would allow the production of fuel from cellulosic biomass. Recently, endoglucanases have been applied in the textile and detergent industries. In the textile industry, endoglucanases are used for removing fuzz from the surface of cellulosic fibers, and enhancing the softness and the brightness of cotton fabrics (**3**). In the detergent industry, endoglucanases are used to facilitate the removal of soil by swelling the cotton fabric (**4**). Directed evolution of cellulase should be a powerful tool to improve properties such as heat stability, and to obtain cellulases suitable for industrial applications.

Clostridium cellulovorans produces two types of cellulases: cellulosomes (a cellulase enzyme complex) and non-cellulosomal cellulases (**5**). Cellulosomes contain a variety of cellulolytic subunits attached to the non-enzymatic scaffolding component termed CbpA (**6**). So far, seven cellulosomal and two non-cellulosomal cellulase genes have been isolated from *C. cellulovorans* (**7**).

Among these cellulase genes, several genes are homologous to each other, for example *engK* – *engM*, *engH* – *engL*, and *engB* – *engD* – *engE* (7). These gene sets are suitable for construction of DNA shuffled gene libraries for directed evolution.

Commonly, cellulase activity is measured by a liquid assay, in which reducing sugars liberated from cellulosic substrates by cellulases are measured (8). The substrates for the liquid assay are Avicel for crystalline cellulose degradation activity, and carboxymethyl cellulose (CMC) for endoglucanase activity. Although the liquid assay is accurate and well-established, this method requires a complicated procedure, including boiling and dilution of the reaction mixture (8). Therefore, the liquid assay is not suitable for high-throughput screening of cellulases from DNA shuffled gene libraries.

The method described here detects endoglucanase activity by a plate assay. This method is based on the interaction between Congo Red and CMC (9). Since Congo Red can be removed from CMC degraded by endoglucanases, the endoglucanase activity can be detected as clearing zones on CMC-agar plates. The procedure of this plate assay is simple and suitable for high-throughput screening of endoglucanases. As a successful example, we describe screening of heat stable endoglucanases derived from the DNA-shuffled library between the *engB* and the *engD* of *C. cellulovorans* (10). A combination of the plate assay and a heat treatment facilitated screening and selection of heat stable endoglucanases from the DNA-shuffled library. The method described here could be used for screening not only heat stable endoglucanases, but also alkaline- or acid-resistant and salt-tolerant endoglucanases.

2. Materials

2.1. Biological and Chemical Materials

All chemicals were purchased from Sigma unless otherwise indicated.

1. Appropriate *E. coli* strain(s) (such as *E. coli* TOP 10, Invitrogen).
2. Plasmid (such as pBAD./Thio, Invitrogen) containing endoglucanase gene (such as *engD* [10] or *engB-CBD* [10]) under control of an appropriate promoter.
3. DNA shuffled library of endoglucanase genes inserted into appropriate expression vector (such as pBAD/Thio).
4. LB (Luria Broth) and LB agar plates.*
5. Appropriate antibiotic(s) (for example ampicillin for pBAD/Thio vector).
6. Appropriate inducer(s)* (for example arabinose for pBAD/Thio vector).
7. CMC-agar:* 0.3% w/v carboxymethyl cellulose sodium salt (CMC-Na, medium viscosity), 0.7% agarose, and 0.002% arabinose in 25 mM sodium acetate buffer, pH 6.0.*

*Sterilize before use.

8. 0.25% w/v Congo Red.
9. 1 M NaCl.
10. CelLytic B-II bacterial cell lysis/extraction reagent.
11. 500 mM sodium acetate buffer, pH 6.0.
12. CMC-Congo Red-agar : 0.1% w/v CMC-Na, 0.004% Congo Red, 0.6% agarose in 10 mM sodium acetate buffer, pH 6.0.
13. Petri dish(s) (90-mm diameter and 145-mm diameter).
14. Appropriate *Taq* polymerase (such as Ampli *Taq*, PE Biosystems).
15. Appropriate PCR primers.
16. 10X dNTPs for error-prone PCR: 2.0 mM dATP, 2.0 mM dGTP, 10 mM dTTP, 10 mM dCTP.
17. 10X buffer for error-prone PCR: 100 mM Tris-HCl buffer, pH 8.0, 83 mM MgCl₂, 1.5 mM MnCl₂, 500 mM KCl, 0.1% gelatin.
18. PCR purification kit (Qiagen).
19. QIAEX II gel extraction kit (Qiagen).

2.2. Equipment

1. Appropriate shaker for culture of *E. coli*.
2. Appropriate incubator.
3. Thermocycler (such as Gene AMP PCR system 9700, PE Biosystem).
4. Appropriate centrifuge.
5. Agarose gel running system.

3. Methods

3.1. Construction of the Endoglucanases Library

To screen for active mutants on selection plates, it is necessary to express selected enzymes as soluble proteins (**11**). Since fusion to thioredoxin is known to help solubilize expressed proteins (**12**), we chose the pBAD/Thio vector as an expression vector, which adds thioredoxin to the *N*-terminus of the protein of interest (*see* **Note 1**). The expression of genes inserted into this vector is controlled by the AraB promotor, induced by arabinose.

The library of endoglucanases was constructed by *in vitro* recombination of the genes for two endoglucanase EngB and EngD from *C. cellulovorans*, based on the fact that the catalytic domains of both cellulases are highly homologous (**13**). Although EngD was expressed as a soluble protein in *E. coli*, EngB was expressed as an insoluble protein in inclusion bodies. Fusion with the cellulose binding domain (CBD) of EngD has been shown to solubilize EngB expressed by *E. coli* (**10**). Therefore, the *engB* gene fused with the CBD of *engD* (*engB*-CBD) was used as one of the parent genes, and it was shuffled with the *engD* gene. The shuffled DNA fragments were inserted into pBAD/Thio vector to construct the library.

3.1.1. Preparation of DNA Fragments for DNA Recombination

The DNA fragments of *engD* and *engB-CBD* were amplified by PCR from the vectors pBAD-*engD* (10), which contained the *engD* gene, and pBAD-*engB-CBD* (10), which contained the *engB-CBD* gene. To access more distant sequences, random mutagenesis was performed by error-prone PCR before DNA recombination. For the *engB-CBD* gene, primers engB-CAD-N (5'-AAGACAGGTATTCGTGACAT-3') and engD-CBD-C (5'-TTTACTGTG CATTCACTACCA-3') were used with pBAD-*engB-CBD* as the template. For the *engD* gene, primers engD-CAD-N (5'-TTTACAGGTGTACGTGACGT) and engD-CBD-C were used with pBAD-*engD* as the template. One hundred nanograms of template vector and 100 pmol of each primer were mixed with 100 μ L of reaction mixture for error-prone PCR (10 mM Tris-HCl buffer, pH 8.0, 8.3 mM MgCl₂, 0.15 mM MnCl₂, 50 mM KCl, 0.01% gelatin, 0.2 mM dATP, 0.2 mM dGTP, 1 mM dTTP, 1 mM dCTP, and 5U *AmpliTaq*). A PCR program of 95°C for 5 min and 40 cycles of 94°C for 30 s and 55°C for 10 s, and 72°C for 5 min was performed. After removal of the primers by use of a PCR purification kit, the band around 1.4 kbp was purified from the 1.0% agarose gel using the QIAEX II gel extraction kit and the purified fragments were used for DNA recombination.

3.1.2. In Vitro Recombination of *engD* and *engB-CBD* Genes

The in vitro recombination between *engB-CBD* gene and *engD* gene was carried out by the staggered extension process (StEP) (14). The DNA fragments were directly cloned to the pBAD/Thio-TOPO vector by the TA cloning technique. This library vector was transformed into *E. coli* TOP10 cells, plated on LB plates containing 50 μ g/mL of ampicillin, and then grown overnight at 37°C.

3.2. Plate Assay Screen for Active Endoglucanases

Thermostabilization by protein engineering often causes a loss of enzymatic activity (15). Therefore, before selection of heat stable endoglucanases from the library, the mutants were screened on LB agar plates over-laid with agar containing soluble cellulose (CMC) to identify those with almost the same endoglucanase activity as the parents.

1. Grow the *E. coli* TOP10 cells, which were transformed with the library vectors, on LB plates (diameter 90 mm) containing 50 μ g/mL of ampicillin overnight at 37°C.
2. Melt CMC-agar by boiling. Then, overlay 5 mL of the CMC-agar, cooled to around 50°C after boiling, onto the LB plates. Leave the plates at room temperature until the CMC-agar solidifies (5–10 min).
3. Incubate the plates at 37°C for 2 h (see Note 2).

4. Add 0.25% Congo Red on the plate to stain the CMC-agar. Leave the plate for 20 min.
5. Remove the Congo Red by decantation.
6. Add 1 M NaCl on the plate to destain. Repeat twice.
7. Transfer the clones which show almost the same size clearing zone as the parental clones to new LB-plate(s), and store them as active mutants (see **Note 3–5**).

3.3. Screening Heat-Stable Mutants by Combination of CMC-Plate Assay and Heat Treatment

Subsequently, the heat stable mutants are screened with the CMC-Congo Red-agar from the active mutants selected as described above. Since the parental endoglucanases EngB and EngD lose their endoglucanase activities completely after incubation at 55°C for 30 min, those mutants which retained their activity after incubation at 55°C for 30 min were selected as heat-stable mutants.

3.3.1. Sample Preparation and Heat Treatment

1. Culture the active mutant clones in 1 mL of LB with 100 µg/mL of ampicillin and 0.002% arabinose. Grow overnight at 30°C with shaking at 220 rpm.
2. Collect the *E. coli* cells by centrifugation.
3. Extract soluble proteins from the *E. coli* cells with 150 µL of CellLytic B-II bacterial cell lysis/extraction reagent according to the product manual (see **Note 6**).
4. Centrifuge the solution and collect the supernatant.
5. Dilute 2.5 µL of the supernatant into 250 µL of 10 mM sodium-acetate buffer, pH 6.0 (see **Note 2**).
6. Incubate 50 µL of diluted supernatant at 55°C for 30 min (see **Note 7 and 8**).

3.3.2. Endoglucanase Plate Assay of Heat Treated Sample

1. Add 50 mL of CMC-Congo Red-agar into empty petri dishes (diameter 145 mm). After solidification of CMC-Congo Red-agar, cut wells with the broader side of yellow chip, and remove the gel by forceps.
2. Put 40 µL of the heat treated sample in the holes in the CMC-Congo Red-agar plate.
3. Incubate at 37°C for 2 h (see **Note 2**).
4. Added 1 M NaCl to the CMC-Congo Red-agar plate, and leave at room temperature for 10 min to destain. Repeat.
5. Select the mutants, whose soluble proteins show clearing zones around the holes in the CMC-Congo Red-agar plate, as thermostable mutants (see **Note 5**).

4. Notes

1. Other expression vectors can be used with appropriate inducer as long as the parental endoglucanase is expressed in an active and soluble form.
2. The incubation period and/or the dilution should be adjusted according to the amount of expression and activity of the parental endoglucanases.

3. In the case of EngB and EngD, these parental endoglucanases showed clearing zones of about 10-mm diameter.
4. Roughly, the endoglucanase activity assayed by the plate assay is in good agreement with that by the standard liquid assay. For example, EngH, another endoglucanase of *C. cellulovorans*, had much lower endoglucanase activity by the standard assay (4.131 U/ μ mol [16]) than EngB (652 U/ μ mol [10]) and EngD (1458 U/ μ mol [10]). The *E. coli* cells expressing EngH showed smaller clearing zones on CMC-agar plates (around 1-mm diameter) even after 6 h incubation than those expressing EngB and EngD after 2 h incubation. Also, ExgS, the exoglucanase of *C. cellulovorans*, has much lower endoglucanase activity by standard assay (0.287 U/ μ mol [16]) and did not show any clearing zone even after 6 h incubation.
5. If the clearing zone is too obscure to detect, add 1 M acetic acid on the plate after destaining. The treatment with acetic acid turns the color of Congo Red darker, and gives clearer contrast.
6. Other extraction methods, such as sonication or lysozyme treatment, could be used instead of CellLytic B-II bacterial cell lysis/extraction reagent.
7. The conditions for heat treatment should be changed according to the properties of the parental enzymes. By heat treatment, the parental endoglucanases should be inactivated completely.
8. In place of the heat treatment, alkaline or acid conditions that inactivate the parental endoglucanases completely could be used to screen for alkaline- or acid-resistant endoglucanases.

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Screening and Selection Strategies for Disulfide Isomerase Activity

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1. Introduction

Disulfide isomerases (EC 5.3.4.1) were first discovered almost 40 years ago by Christian Anfinsen, and have since been shown to occur in numerous organisms from bacteria to man (*1*). These enzymes play a key role in disulfide bond formation, an essential step in the oxidative folding of secreted proteins. The effort to determine the *in vivo* function and substrate specificity of disulfide isomerases has been hampered by the fact that, *in vitro*, the same isomerase enzyme can catalyze protein thiol oxidation, disulfide bond reduction, or disulfide bond rearrangement depending upon the redox conditions (*2*). Despite decades of studies, the detailed catalytic mechanism of disulfide bond isomerization is still not completely understood.

The practical significance of disulfide isomerases stems from their role in facilitating the folding of complex proteins expressed in heterologous hosts. For many multidisulfide polypeptides, the yield of correctly folded biologically active protein is limited by disulfide bond isomerization. In bacteria, the co-expression of disulfide isomerases has been shown to markedly improve the folding of bovine pancreatic trypsin inhibitor (BPTI, 3 disulfides) (*3*), urokinase (12 disulfides) (*4*), human nerve growth factor (3 disulfides) (*5*), and even that of human tissue plasminogen activator (tPA), a multi-domain protein with 17 disulfides (*4,6,7*).

However, in many instances, even under conditions of disulfide isomerase overexpression, only a small fraction of the heterologous protein produced by the cell is correctly folded. For example, while co-expression of the bacterial disulfide isomerase DsbC increases the yield of active tPA by more than 40-fold relative to control cells, the correctly folded protein corresponds to only

about 5% of the total protein produced by the cell. In the first example of the engineering of isomerases for heterologous protein production, mutations in the active site of DsbC have been shown to increase the yield of correctly folded vtPA (4).

Unfortunately, the directed evolution of disulfide isomerases to enhance their activity towards specific protein substrates is quite complicated. In the cell, these enzymes interact with folding intermediates which are, by definition, transient species. Moreover, they do not function alone but instead act in concert with other cellular thiol:disulfide isomerases/oxidoreductases. For these reasons, it is impossible to engineer disulfide isomerases using in vitro prepared protein substrates, and an in vivo assay must be used instead.

Our laboratory has developed a series of high throughput screening assays suitable for the isolation of improved disulfide isomerases from libraries of protein mutants. Although the methodologies described in this chapter deal with the isolation of mutant enzymes that enhance the yield of active human tissue plasminogen activator in *E. coli*, analogous strategies can be readily adapted to other protein substrates or for expression in different organisms.

Human tissue-type plasminogen activator (tPA) is a pharmaceutically important thrombolytic agent that requires the formation of 17 specific disulfide bonds from a total of 35 cysteine residues (8); it is completely inactive when produced in wild-type *E. coli*. Because tPA is a protease, it is possible to assay for correctly folded tPA enzymatically. Although the co-expression of DsbC can greatly improve the folding of full-length tPA, as discussed above, the overall levels of active tPA are still quite low. Therefore, a variant of tPA (vtPA), in which three of its five functional subdomains (finger, epidermal growth factor, and kringle 1) are deleted, is often used as a model protein substrate; vtPA requires the formation of nine disulfide bonds from 18 cysteine residues, and can be actively produced at very low levels in wild-type *E. coli* (9). Because of its complex multi-domain structure, number (and non-linear pattern) of disulfide bonds, the existence of a commercially available enzymatic assay, and its commercial value, tPA (or vtPA) is an excellent model substrate for the engineering of *E. coli* for the production of multidisulfide proteins.

Three strategies for library screening applications are outlined: 1) a low throughput microtiter-well assay; 2) a phage-linked assay, called PLainFold (Phage-Linked Assisted in vivo Folding), whereby the catalytic activity of overexpressed periplasmic disulfide isomerases is exploited to allow for the folding of a protein substrate displayed on filamentous bacteriophage, which in turn can be enriched via binding to a specific ligand; and 3) a genetic selection for disulfide isomerization activity. Method (1) is a low throughput assay suitable for the screening of a few thousand clones. The phage-linked assay

(2) is useful for enriching clones with higher catalytic activity and offers the advantage of very-high throughput. Finally the genetic selection (3) allows for the differentiation between active and inactive mutants, but is not sufficiently quantitative to assist in the isolation of improved catalysts.

2. Materials

2.1. Microtiter-Well Plate (v)tPA Screening

1. An appropriate strain of *E. coli* that is optimized for the periplasmic production of recombinant multidisulfide substrate proteins. The protease-deficient SF110 (*F-ΔlacX74 galE galK thi rpsL ΔphoA degP41 ΔompT*), or an isogenic strain in which the *dsbC* locus has been knocked out via P1 transduction, is the strain commonly used by the authors (10).
2. A plasmid that encodes for the periplasmic expression of the desired multidisulfide protein. For example, pTrc-stII-vtPA, a derivative of pTrc99a (Amersham Pharmacia) in which the vtPA coding sequence is fused to the *stII* leader peptide downstream of the ribosome binding site of the expression cassette, is the plasmid commonly used by the authors for library screening (7). pTrc99a is IPTG-inducible, confers ampicillin resistance and has a ColE1 origin of replication. For the genetic selection described in **Subheading 3.3.**, in which only one plasmid is used, i.e., no foldase variants or other factors are being co-overexpressed, pBAD33-stII-vtPA (which produces lower levels of vtPA than the pTrc99a derivative) is the construct used by the authors; it was constructed by replacing the gene for full-length tPA in pBAD-stII-tPA (6) with that of vtPA.
3. Depending upon the experimental context, a compatible plasmid system is usually necessary for the expression of foldase variants that are co-expressed to improve the folding of the multidisulfide protein of interest. A modified version of pBAD33 (11), a low-copy plasmid with an arabinose-inducible expression cassette that confers chloramphenicol resistance and has a pACYC origin of replication along with an M13 bacteriophage origin, is the compatible plasmid commonly used by the authors (see **Note 1**).
4. LB (Luria-Bertani) growth media.
5. Ampicillin 100 mg/mL (filter sterilize and store in 1 mL aliquots at -20°C) and chloramphenicol 25 mg/mL (in ethanol; store at -20°C). Working concentrations in liquid media or solid medium are 100 $\mu\text{g/mL}$ and 25 $\mu\text{g/mL}$, respectively.
6. L-Arabinose 20% w/v (filter sterilize and store at room temperature) and IPTG (isopropyl- β -D-thiogalactopyranoside) 100 mM (filter sterilize and store in 1 mL aliquots at -20°C).
7. Sterile 96-well cell culture plates with flat bottoms and low evaporation lids (Costar).
8. Microtiter-well plate shaker.
9. 5–50 μL and 50–200 μL multi-channel pipetmen.
10. Bugbuster Lysis reagent (Novagen).

11. 10X Tris buffer: 0.5 M Tris-HCl, pH 7.4, 0.1% Tween 80. Dissolve 5.72 g of Tris-HCl and 1.66 g of Tris base in 100 mL of ddH₂O, then add 100 μ L Tween 80.
12. 1X Tris buffer: 50 mM Tris-HCl, pH 7.4, 0.01% Tween 80. Dilute 10X Tris buffer 1:10 in ddH₂O.
13. Plasminogen (0.5 mg/mL) (human glu-type, American Diagnostica): Dissolve plasminogen in 1X Tris buffer to 0.5 mg/mL final concentration. Make 1 mL aliquots and store at -20°C .
14. Spectrozyme PL (1 mM) (American Diagnostica): Dissolve Spectrozyme PL in 1X Tris buffer to 1 mM final concentration. Make 5 mL aliquots and store at -20°C .
15. 1X TAR (tPA assay reagent): 0.04 mg/mL Plasminogen, 0.4 mM Spectrozyme PL, 50 mM Tris pH, 7.4 and 0.01% Tween 80. Combine 1 mL of 0.5 mg/mL Plasminogen, 5 mL of 1 mM Spectrozyme PL, and 6.5 mL of 1X Tris buffer.
16. 1:4X TAR: 0.01 mg/mL Plasminogen, 0.1 mM Spectrozyme PL, 50 mM Tris-HCl, pH 7.4 and 0.01% Tween 80. Dilute 1X TAR 1:4 in 1X Tris buffer.

2.2. PLainFold (Phage-Linked Assisted In Vivo Folding)

1. *E. coli* strain TG1 (*supE hsd Δ 5 thi Δ (lac-proAB) F' [traD36 proAB⁺ lacI^q lacZ Δ M15]*) (Stratagene), or other strain that is commonly used in phage display selections. In order to decrease the amount of background signal from properly folded substrate-gIIIp fusions (from endogenous DsbC production), the *dsbC* locus of TG1 has been inactivated by P1 transduction of a *dsbC* allele that has been disrupted via the insertion of a kanamycin resistance cassette. The resulting strain has been designated TG1RL.
2. An appropriate plasmid with the coding sequence for vtPA inserted between a bacterial leader peptide and the mature coding sequence of gIIIp. This plasmid should be IPTG-inducible, be repressible via the addition of glucose, and confer ampicillin resistance. pAT100-vtPA is the construct commonly used by the authors; it contains fragments from pUC19 (12), pHEN2 (13), and pAK100 (14) (see Note 2).
3. A compatible phagemid system that encodes for the expression of periplasmic disulfide isomerase (in this case, the *E. coli dsbC* gene) variants that are co-expressed to improve the folding of the substrate(vtPA)-gIIIp fusion. A modified version of pBAD33 (11), a low-copy plasmid with an arabinose-inducible expression cassette that confers chloramphenicol resistance and has a pACYC origin of replication along with an M13 bacteriophage origin, is the compatible plasmid commonly used by the authors (see Note 1). Expression of DsbC, or variants, should be inducible via the addition of arabinose and repressed by the presence of glucose.
4. TB (Terrific broth) (Difco). TBG is TB + 2% glucose (w/v) + 0.6% glycerol (v/v). Solid TBG medium is TBG plus 15 g/L Bacto-Agar.
5. TYG: 8 g/L NaCl + 10 g/L Tryptone + 5 g/L Yeast Extract + 2% glucose (w/v). Solid TYG medium is TYG plus 15 g/L Bacto-Agar.
6. Ampicillin (Amp) 100 mg/mL, Chloramphenicol (Cm) 25 mg/mL, Kanamycin (Km) 50 mg/mL (filter sterilize and store in 1 mL aliquots at -20°C), Tetracycline (Tet) 5 mg/mL (in ethanol; store at -20°C). Working concentrations in liquid media and solid medium are 100 μ g/mL (Amp), 25 μ g/mL (Cm), 50 μ g/

mL (Km), and 30 $\mu\text{g/mL}$ (Tet), respectively. In this manuscript, antibiotic supplementation is indicated in superscript; for example, the nomenclature TBG^{Km/Amp} refers to TBG media or medium supplemented with 50 $\mu\text{g/mL}$ Kanamycin and 100 $\mu\text{g/mL}$ Ampicillin.

7. L-Arabinose 20% (w/v) and IPTG 100 mM.
8. Helper Phage R408 (Stratagene).
9. PBS (Phosphate-Buffered Saline): Dissolve 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na₂HPO₄, and 0.24 g of KH₂PO₄ in 800 mL of distilled H₂O. Adjust the pH to 7.4 with HCl. Add H₂O to 1 L. Autoclave and store at room temperature.
10. PEG/NaCl: 20% (w/v) Polyethylene glycol 6000, 2.5 M NaCl. Autoclave and store at room temperature.
11. BSA (Bovine Serum Albumin) 5 mg/mL. Filter sterilize and store at 4°C.
12. PBST: PBS + 0.05% Tween 20. Filter sterilize and store at room temperature.
13. 50 mM NH₄OAc, pH 4.95. Filter sterilize and store at room temperature.
14. 0.1 M glycine/HCl, pH 2.2. Filter sterilize and store at room temperature.
15. 1 M Tris-HCl, pH 7.5. Autoclave and store at room temperature.
16. DMSO (dimethylsulfoxide).
17. *Erythrina caffra* trypsin inhibitor (ETI) (American Diagnostica).
18. EZ-LinkTM NHS-LC-Biotin (Pierce).
19. Centricon[®] YM-3 microconcentrator (Millipore).
20. Azide.
21. Dynabeads[®] M-280 (DynaL Biotech).
22. Magnetic Particle Concentrator (DynaL Biotech).

2.3. Genetic Selection

1. *E. coli* strain MCZ4 (*DH5 α Δ pabA::Tn5Kn*), or another PABA-auxotrophic strain, transformed with an appropriate vector that encodes for the periplasmic expression of vtPA. This strain can be obtained from the authors. pBAD33-stII-vtPA is the plasmid used by the authors in the context of this genetic selection (see **Subheading 2.1.**).
2. M9 minimal media. M9 minimal medium is supplemented with 15 g/L Bacto-Agar.
3. Kanamycin 25 mg/mL and ampicillin 100 mg/mL. Working concentrations are 50 $\mu\text{g/mL}$ and 100 $\mu\text{g/mL}$, respectively.
4. L-Arabinose: 20% (w/v).
5. Citrate buffer: 0.1 M sodium citrate, pH 5.5. Autoclave and store at room temperature.
6. MNNG: *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Sigma) 1 mg/mL in citrate buffer.
7. Phosphate buffer: 0.1 M sodium phosphate, pH 7.0. Autoclave and store at room temperature.
8. *N*- α -benzoyl-L-arginine-*p*-aminobenzoic acid (*N*- α -benzoyl-L-arginine-PABA).

3. Methods

The methods enumerated below comprise a set of approaches that can be utilized to select or screen for enhanced periplasmic disulfide isomerase activities in different contexts (see **Note 3**). All of these methods harness the enzy-

matic activity and/or selective binding of correctly folded, and therefore active, vtPA, in which the *in vivo* yield is dependent on the catalytic activity (or upregulation) of disulfide isomerases.

3.1. Microtiter-Well Plate (v)tPA Screening

The microtiter-well plate screen relies on a quantitative chromogenic assay and in-well chemical lysis of cell cultures to release vtPA into solution (*see Fig. 1*).

The quantitative chromogenic assay is an indirect assay in which the measured activity is the release of a free chromophore (*p*-nitroanilide) that absorbs light at 405 nm; this chromophore is released by the action of plasmin. The actual assay solution consists of plasminogen and the chromogenic substrate Spectrozyme PL. Correctly folded (v)tPA converts the zymogen plasminogen into plasmin, which can then act on the chromogenic substrate (present in excess) that is then cleaved to release the active chromophore. By measuring the change in absorbance at 405 nm, the amount of active (v)tPA present in a protein lysate solution can be indirectly ascertained (*see Fig. 2*).

The detailed procedure involves the following steps: 1) Individual colonies from a plated library are inoculated into separate microtiter-wells; 2) expression of the disulfide isomerase (*see Note 4*) and vtPA are induced, and then, 3) at the appropriate time, the cultures are harvested and lysed chemically. Finally, 4) the lysates are incubated with a less concentrated form (diluted 1:4 from the normal working concentration, *see Note 5*) of the chromogenic assay reagent (used to reduce costs) and the ΔA_{405} timecourse profile of each plate is monitored. Samples with ΔA_{405} greater than wild-type controls indicate isomerase variants with improved catalytic activity.

1. Patch individual colonies from a plate onto a fresh petri dish (as appropriate) and also inoculate into separate wells of a microtiter-well plate (containing 200 μ L of LB supplemented with the appropriate antibiotics) with the same sterile toothpick or pipet tip (*see Notes 6 and 7*). Incubate the microtiter-well plate(s) overnight at 37°C with shaking on a microtiter-well plate shaker (*see Note 8*).
2. After an appropriate amount of time (8–18 h), i.e., when the entire population has reached stationary phase, subculture each plate 1:25 into fresh plates containing 200 μ L of LB supplemented with the appropriate antibiotics at 37°C (*see Note 9*). Because of the timing of subsequent steps of this protocol, it is recommended that at least 10-min intervals be used between plates.
3. When the majority of the clonal populations in each well have reached an OD₆₀₀ of approx 0.4–0.6 (*see Note 10*), induce the expression of disulfide isomerase, typically using arabinose to a final concentration of 0.2% w/v and record the OD₆₀₀ of the plate. After 30 min, induce the expression of vtPA via the addition of the appropriate agent, typically IPTG to a final concentration of 1 mM.

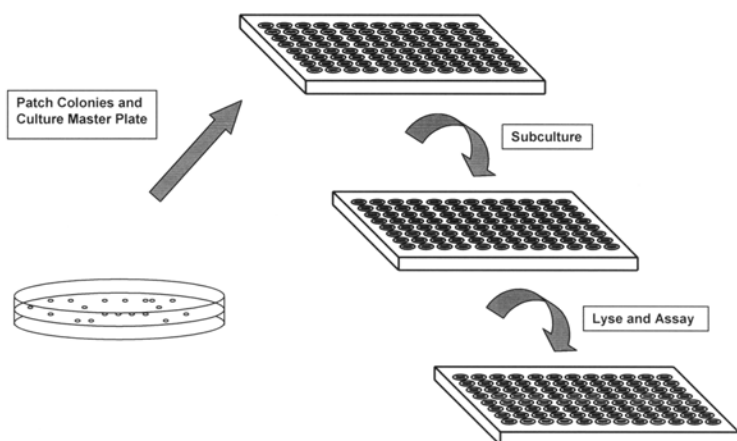


Fig. 1. Schematic diagram of microtiter-well plate assay.

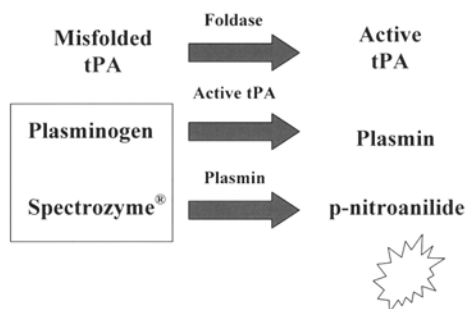


Fig. 2. Illustration of mechanism of (v)tPA indirect chromogenic assay.

4. After 3 h of shaking at 37°C, remove the plate from the incubator and record the OD₆₀₀. Serially transfer 30 µL of each culture into a fresh plate containing 40 µL of Bugbuster lysis reagent in each well. Allow lysis to take place for 1–2 h with shaking at room temperature (*see Note 11*).
5. Add 200 µL of 1:4X TAR, equilibrated at room temperature, to each well and record the A₀ value at 405 nm. Incubate the plate at 37°C (without shaking) and take measurements at different times, as appropriate (typically every hour).

3.2. PLAINFold (Phage-Linked Assisted In Vivo Folding)

The system to be detailed here is a novel method of phage display in which the substrate molecule of interest (in this case vtPA) is N-terminally displayed on the minor coat protein gIIIp of the filamentous bacteriophage M13. gIIIp

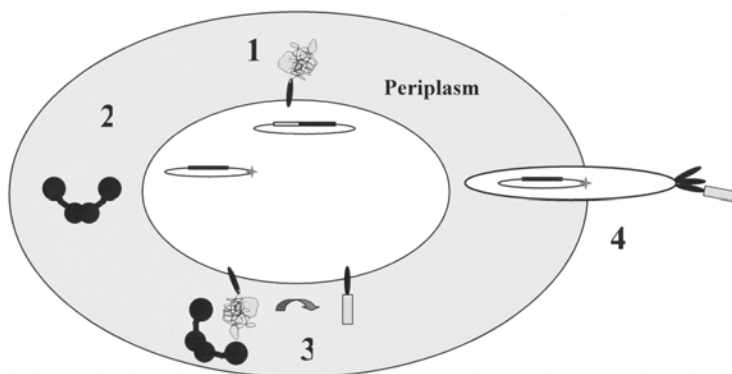


Fig. 3. Illustration of the major schematic concepts of phage display platform. (1) The vtPA-gIIIp fusion is expressed from a dummy plasmid. The N-terminal portion of gIIIp, including N-terminal fusions, is folded in the periplasm, while the C-terminal portion of gIIIp is embedded in the membrane for attachment to the exiting phage particle. (2) DsbC (shown as a dimmer), or another isomerase/foldase, is overexpressed and secreted to the periplasm. The sequence of this foldases is encoded for on a phagemid that contains a phage (M13) origin of replication. (3) Active foldases can help convert misfolded vtPA-gIIIp fusions into the native conformation. (4) Recombinant phage are produced in which the folding state of the vtPA-gIIIp fusion (which is displayed on an average of one copy per phage particle) is physically coupled to the coding sequence for the foldase responsible for this folding state.

fusions are exported to, and fold within, the bacterial periplasmic space. Disulfide isomerase mutants (*see Note 2*) are also expressed in the periplasmic space, where they can interact with, and assist the folding of, the substrate-gIIIp fusion protein. Eventually, the gIIIp fusion is incorporated into phage particles (*see Fig. 3*).

The gene encoding the disulfide isomerase is present in a phagemid containing an M13 origin of replication and thus is packaged within the phage that displays the gIIIp fusion on its surface. In this manner, the substrate-gIIIp fusion becomes physically linked to the DNA encoding the disulfide isomerase. Since each bacterial cell produces hundreds of phage particles, the fraction of phages displaying correctly folded substrate-gIIIp fusions is proportional to the catalytic activity of the expressed disulfide isomerase. When recombinant phages are panned against a ligand of the displayed substrate protein, only those phage particles displaying correctly folded substrate-gIIIp fusions are bound. After washing away unbound phage and then selectively eluting only the bound phage, phage variants that have correctly folded substrate-gIIIp fusions become enriched.

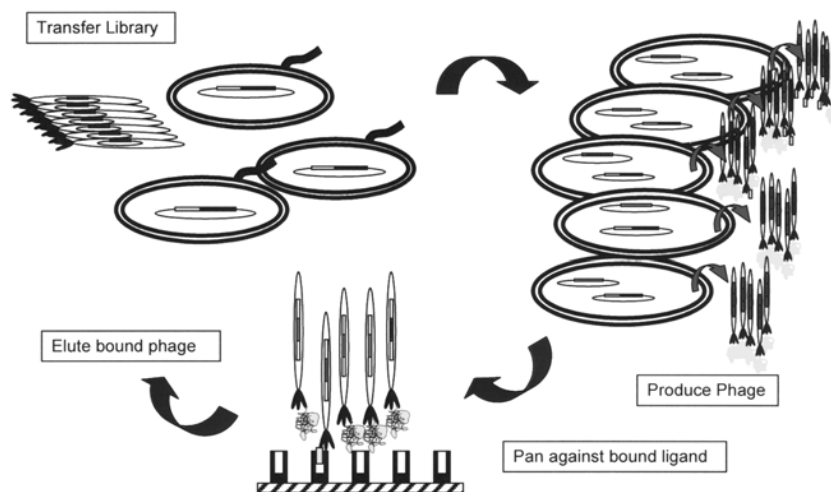


Fig. 4. Schematic diagram of cycle of phage display selection.

Following several rounds of panning, the library population is enriched for foldase variants that promote the folding of the substrate of interest (*see Fig. 4*). These libraries can then be transferred into an appropriate strain in which only the substrate of interest (and not the gIIIp fusion) is produced, and screened for improved folding of the substrate protein expressed by itself, and not in the context of the gIIIp fusion.

For the directed evolution of DsbC variants, we utilized a derivative of the common *E. coli* strain TG1, often used in phage display protocols, lacking a functional *dsbC* gene. The introduction of a *dsbC* null allele marked with an antibiotic resistance gene is performed via P1 transduction. The vtPA-gIIIp fusion is secreted via the pelB signal peptide and is encoded by a plasmid lacking a phage origin of replication. For example, we used pAT100-vtPA (*see Note 2*), which allows for the IPTG-inducible production of the vtPA-gIIIp fusion, is repressible by glucose, and confers ampicillin resistance. A library of dsbC foldase/isomerase mutants (expressed with the native dsbC signal peptide) is encoded for on a derivative of the phagemid pBAD33, modified via the inclusion of the optimized ribosome binding site from pTrc99a upstream of the expression cassette start codon (*13*); this phagemid confers chloramphenicol resistance and allows for the arabinose-inducible production of isomerase variants. Infection with the helper phage R408 is necessary for recombinant phage production.

The steps detailed below describe 1) initial library capture and propagation (i.e., from a library cloning strain), 2) the transfer of the appropriate phage

library into the phage display selection strain, 3) the production of recombinant phage particles, 4) panning of phage against binding partner and subsequent elution, and 5) initial synthesis of ETI-beads. **Steps 2–4 (Subheadings 3.2.2.–3.2.4.)** comprise a single round of the selection process; each round takes two days to complete.

3.2.1. Initial Transfer of Cloned Disulfide Isomerase Library into Phage and First Selection Round

Although library construction protocols vary (see relevant chapters in the accompanying volume), most protocols involve the electroporation of the ligation mixture into commercially available ultra-electrocompetent cells; XL-1 Blue (Stratagene) is commonly used for these purposes. The actual strain is not important, but it must be an F' derivative for the successful infection of helper phage and subsequent capture of the phagemid-encoded isomerase library for further manipulation. In this first step, helper phage are used to capture the phagemid isomerase library and transfer it into the PLainFold selection strain (TG1RL/pAT100-vtPA).

1. Subculture the transformed isomerase library into 400 mL of TBG^{Tet/Cm} pre-warmed to 37°C by adding an aliquot of cells equivalent to a final OD₆₀₀ of 0.05 to the fresh media (*see Note 12*).
2. After the culture has reached an OD₆₀₀ of 0.4–0.6 with shaking at 37°C, 250 rpm, transfer 20 mL of culture into a 50-mL Falcon tube and infect with helper phage R408 at a multiplicity of infection of 20 (*see Notes 13 and 14*). Mix the solution with gentle swirling and incubate without agitation for 30 min at 37°C.
3. Harvest the cells by centrifugation at 3300g, 30°C for 15 min. Discard the supernatant.
4. Resuspend the cells in 100 mL of TBG^{Tet/Cm} pre-warmed to 37°C and grow overnight (with shaking) at 37°C.
5. Harvest the phage prep essentially as described in **steps 8–11 of Subheading 3.2.3.**, but harvest twice as much culture as described and scale-up as necessary, i.e., use 16 mL of PEG/NaCl for the precipitation and resuspend the phage pellet in 4 mL of PBS + 15% glycerol.
6. Titrate the amount of phage, i.e., the size of the input phage library, as described in **steps 12–15 of Subheading 3.2.3.**

The initial production of recombinant phage proceeds essentially as described in **Subheading 3.2.2.–3.2.3.** (*see Note 15*). In **step 2 of Subheading 3.2.2.**, the appropriate volume of library phagestock is added (in lieu of phage eluate) to transfer the isomerase library into the screening strain. After the recombinant phage have been produced and titrated, the first cycle of panning concludes the initial selection round. In the initial panning stage, it is important that the number of input phage be approx 1000× larger than the diversity (i.e., number of

transformants) in the original library. After the phage titer has been determined (in **step 15** of **Subheading 3.2.3.**), dilute the recombinant phagestock to the appropriate concentration (i.e., such as approx 100 μL of phage are used in **step 4** of **Subheading 3.2.4.**) and proceed with the panning as described in **Subheading 3.2.4.**

3.2.2. Library Transfer

This step begins each round of the phage display selection process, in which phage libraries are used to infect fresh TG1RL/pAT100-vtPA. In the first round of panning, the phage-captured isomerase library is transferred into the selection strain. In subsequent rounds of panning, the phage population eluted from the immobilized ligand in the panning step is used to re-infect fresh cultures of the selection strain. This process transfers the phagemid library into the selection strain for the further production of recombinant phage.

1. Subculture TG1RL/pAT100-vtPA, from an overnight culture or from a frozen stock, into 30 mL of TBG^{Km/Amp} pre-warmed to 37°C and grow to an OD₆₀₀ of 0.4–0.6 (*see Note 16*).
2. Take 10 mL of this culture, transfer it to a 50-mL Falcon tube, and infect the cells with the 240 μL phage eluate from the final step of the panning procedure (*see Subheading 3.2.4.*). Take another 1 mL of the mid-log phase TG1RL/pAT100-vtPA culture and add it to the neutralized beads and residual eluate in the 1.5-mL eppendorf tube used in the panning process; pool this mixture with the infected 10 mL of cells in the Falcon tube. Swirl gently to mix, let sit at 37°C for 30 min, and shake at 250 rpm, 37°C for 1 h.
3. Take 50 μL of the cell mixture and make a 10-fold dilution series, plating 50 μL of the 1:10, 1:100, and 1:1000 dilutions of the infected cells on TYG^{Km/Amp/Cm}. Incubate the plates overnight at 30°C, and calculate the number of phage colony-forming units (cfu) that came out from the previous round of panning.
4. Harvest the remaining 10.95 mL of infected cells by centrifugation at 3300g, 4°C for 10 min.
5. Pour off the supernatant from the pelleted cells, and resuspend the cells (by pipetting using a filter tip) in the residual media plus 500 μL of fresh TB (for a total of ca. 750 μL). Plate aliquots of approx 250 μL on three large TYG^{Km/Amp/Cm} plates. Incubate at 30°C overnight.

3.2.3. Production of Monovalent Recombinant Phage

This series of steps results in the production of a population of phage particles displaying primarily a single copy of the vtPA-gIIIp fusion on their coat. By allowing the production of phage particles to proceed in the presence of overexpressed disulfide isomerase variants, the folding state of the vtPA-gIIIp moiety is determined by the activity of the particular foldase variant that is encoded by the phagemid.

1. Resuspend the cells from each large plate of the transferred library (TG1RL/pAT100-vtPA) in 2 mL of TB + 15% glycerol via gentle scraping with a sterile scraper made from a glass Pasteur pipet. Pool suspensions from each plate in a 50-mL Falcon tube and vortex briefly to produce a homogenous mixture. Make two 1-mL aliquots of each library in cryotubes and snap freeze at -80°C as library backups.
2. Measure the OD_{600} of the mixture by an appropriate dilution of the collected cells; 1:100 (made via two consecutive 1:10 dilutions) or 1:200 dilutions should suffice.
3. Subculture the library in 100 mL of $\text{TBG}^{\text{Km/Amp/Cm}}$ pre-warmed to 37°C in a 500-mL baffled shake flask via the inoculation of an amount of cells (based on results from **Step 2** above) that is equivalent to a final OD_{600} of 0.08.
4. After the library has reached an OD_{600} of 0.4–0.6 at 37°C , pipet 10 mL of culture into a 50-mL Falcon tube and infect the cells with helper phage R408 at a multiplicity of infection (MOI) of 20; let the cells sit at 37°C without agitation for 30 min (*see Note 13*).
5. Harvest the infected cells by centrifugation (of the Falcon tube) at $3300g$, 37°C for 10 min. Pour off the supernatant, re-spin briefly for 1–2 min, and aspirate off any residual media. This step is necessary to remove all glucose from the cells to prevent the repression of *dsbC* and *vtPA-gIIIp* induction.
6. Resuspend the cells in 25 mL of $\text{TB}^{\text{Km/Amp/Cm}}$ + 0.2% arabinose, from a total of 50 mL of this media pre-warmed to 37°C in a 250-mL baffled shake flask. Vortex briefly, then pour the solution into the remaining 25 mL of media and grow at 37°C , 250 rpm for 30 min.
7. Add IPTG to a final concentration of 0.1 mM and grow at 30°C overnight (14–16 h) with shaking at 250 rpm.
8. Harvest 37.5 mL of the overnight phage culture (in a JA-20 tube) at $15,000g$, 4°C for 15 min.
9. Pour the supernatant into a 50-mL Falcon tube containing 8 mL of PEG/NaCl and incubate on ice for 1.5 h.
10. Centrifuge the Falcon tube at $3300g$, 4°C for 30 min. Pour off the supernatant, re-spin for 1–2 min, and aspirate off residual PEG/NaCl using a filter-tip (*see Note 17*).
11. Resuspend the phage pellet in 2 mL of PBS + 15% glycerol by vortexing. Spin down debris via centrifugation at $14,000g$, 4°C for 10 min. After reserving 20 μL of this phage stock for subsequent titration and panning (*see Note 18*), divide the phage stock into two aliquots of approx 1 mL and freeze at -80°C .
12. Begin a 10-fold dilution series of the phage stock by adding 20 μL of phage stock to 180 μL of PBS. After reserving 100 μL of this 1:10 dilution for the panning process, continue the dilution series until a 10^{-4} dilution has been made.
13. Infect 1 mL of fresh TG1 cells, subcultured in 10 mL of TB (in a 50-mL Falcon tube) to an OD_{600} of 0.4–0.6 at 37°C , with 10 μL of the 10^{-4} phage stock dilution in a small, sterile polypropylene culture tube. Mix gently by flicking the tube, and let the tube sit at 37°C without shaking for 30 min. Shake at 250 rpm, 37°C for 1 h.

14. Make a 10-fold dilution series of the infected cells. Plate 50 μL of infected cells diluted 1:100 and 1:1000 on TYG^{Cm} and grow at 30°C overnight.
15. Count the number of colonies on each plate and calculate the phage titer.

3.2.4. Phage Panning

In this final portion of a round of phage display screening, the population of recombinant phage is panned against an immobilized ligand that selectively binds to the correctly folded substrate protein, which is displayed on phage (as a fusion to gIIIp). To pan for correctly-folded vtPA, *Erythrina caffra* trypsin inhibitor (ETI) is covalently linked to a solid support, in this case streptavidin-coated paramagnetic beads. After panning, bound phage, which presumably display correctly folded vtPA-gIIIp fusions, are selectively eluted and used as the starting point for further rounds of selection.

1. Prepare the ETI-beads (*see Subheading 3.2.5.* for initial coupling and preparation of ETI-beads) for the panning process by resuspending the bead slurry via gentle pipetting.
2. Transfer 25 μL of the bead mixture to a 1.5-mL eppendorf tube and use a magnetic particle concentrator to collect the beads on one side of the tube. Aspirate off the supernatant, remove the tube, and wash the beads by adding 25 μL PBS to the beads. Repeat this wash procedure twice, resulting in 25 μL of washed and resuspended beads.
3. Add 80 μL of PBS and 20 μL of 5 mg/mL BSA to each tube with beads, and mix gently by flicking the tube.
4. Add 100 μL of the phage stock that was diluted 1:10 from **Step 12** of **Subheading 3.2.3.** to the tube. Mix by flicking the tube, and incubate end-over-end for two hours at room temperature (*see Note 19*).
5. Collect the beads on the side of the tube by using the magnet. Aspirate off the supernatant and discard.
6. Wash twice with 500 μL of PBST, and resuspend the beads in 200 μL of PBST. Transfer this mixture to a fresh 1.5-mL eppendorf tube, and perform 10 washes with 200 μL of 50 mM NH₄OAc, pH 4.95. Perform nine more washes with 200 μL of PBST, and perform a final wash with 200 μL of PBS, leaving as little residual PBS as possible.
7. Elute the bound phage via resuspension of the beads in 200 μL of 0.1 M glycine/HCl, pH 2.2. Incubate end-over-end at room temperature for 10 min.
8. Collect the beads on the side of the tube using the magnet, and transfer the 200 μL of supernatant to a fresh eppendorf tube containing 40 μL of 1 M Tris-HCl, pH 7.5. Resuspend the remaining beads in 10 μL of 1 M Tris-HCl, pH 7.5 (*see Note 20*).

3.2.5. Preparation of ETI-Beads

1. Dilute ETI to 2 mg/mL in PBS.
2. Add 75 μL of EZ-LinkTM NHS-LC-Biotin, diluted to 3 mg/mL in DMSO, to 1 mL of 2 mg/mL ETI/PBS. Incubate the mixture on ice for two hours. Add 1 mL of cold PBS.

3. Centrifuge the mixture in a Centricon® YM-3 microconcentrator to remove unreacted biotin. Dilute the sample in 1 mL of cold PBS and re-centrifuge. Repeat this step two more times and adjust the final volume of the solution to 1 mL with cold PBS/0.02% azide.
4. Couple the biotinylated-ETI to streptavidin-coated paramagnetic beads, Dynabeads® M-280. Follow manufacturer recommendations.

3.3. Genetic Selection for Isolation of Mutant Strains with Increased tPA-Folding

We have designed and implemented a novel genetic selection based on complementation of *p*-aminobenzoic acid (PABA) auxotrophy, which allows for the identification of strains with enhanced disulfide isomerase activity. A null mutation in any of the *pabA*, *pabB*, or *pabC* genes renders *E. coli* unable to grow on minimal media unless at least 5 ng/mL of free PABA is supplemented. However, cleavage of a PABA-adduct, *N*- α -benzoyl-L-arginine-PABA, by (v)tPA, results in the release of a sufficient amount of this nutrient to allow for PABA-deficient mutants to grow and form colonies in the absence of PABA supplementation (**15**) (see **Note 21**).

The PABA-auxotrophic strain MCZ4 (*DH5 α Δ pabA::Tn5Kn*), transformed with the plasmid pBAD33-stII-vtPA (see **Subheading 2.1.**), is the strain that has been used as the starting point for this genetic selection.

1. Subculture a fresh overnight culture of MCZ4/pBAD33-stII-vtPA (or another PABA-auxotrophic strain of *E. coli* transformed with pBAD33-stII-vtPA) in 10 mL of media supplemented with the appropriate antibiotics (kanamycin and ampicillin in this case).
2. After the strain has reached an OD₆₀₀ of approx 0.3, harvest 5 mL of culture via centrifugation at 3300g, room temperature for 10 min.
3. Wash the pellet twice with cold citrate buffer (0.1 M sodium citrate, pH 5.5). Resuspend the pellet in 1.9 mL of cold citrate buffer and store on ice.
4. Add 100 μ L of freshly prepared MNNG solution (1 mg/mL in citrate buffer) and incubate the solution at 37°C for 30 min.
5. Harvest the cells via centrifugation at 3300g, room temperature for 10 min.
6. Wash the pellet twice with 5 mL of phosphate buffer (0.1 M phosphate, pH 7.0). Resuspend the pellet in 2 mL of phosphate buffer.
7. Dilute the cell suspension 1:100 and plate 200 μ L of this suspension on M9 minimal medium supplemented with 8 μ M of *N*- α -benzoyl-L-arginine-PABA and 0.2% arabinose.
8. Because we are interested in examining mutations that increase the active production of vtPA, and not mutations that bypass the cellular requirement for free PABA, mutant strains must be scored to eliminate false positives. Patch an appropriate number of colonies from the plated, mutagenized population on both M9 minimal medium and M9 minimal medium supplemented with *N*- α -benzoyl-

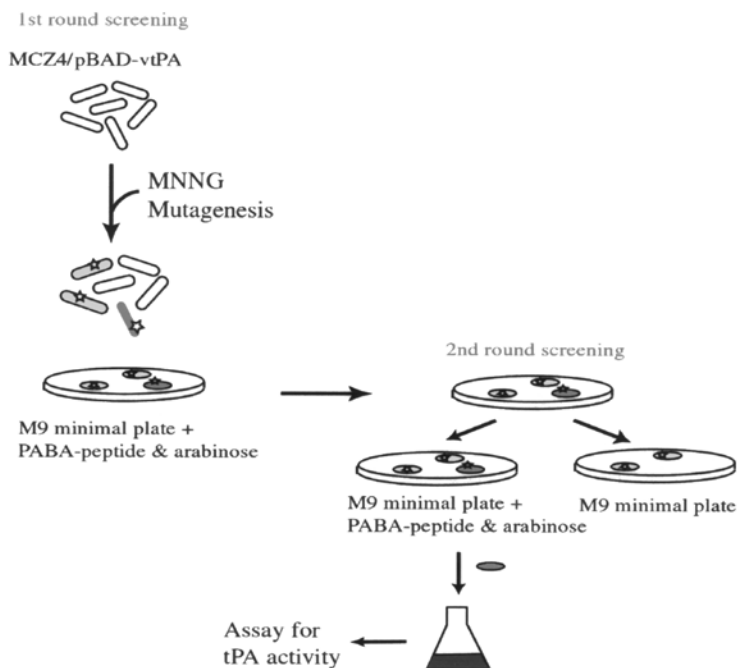


Fig. 5. Illustration of genetic selection methodology.

L-arginine-PABA and arabinose (as above). False positives will grow on both plates; true positive mutations will not be viable on unsupplemented M9 medium (see Fig. 5).

After mutant strains have been isolated, the microtiter-well assay described in **Subheading 3.1.** can be used to quantitatively assess the increase in the production of active vtPA (see **Note 22**).

4. Notes

1. pBAD33 does not contain a ribosome binding site upstream of its expression cassette. In order to allow for efficient protein synthesis, the multiple cloning site of pTrc99a, including optimized ribosome binding site, was cloned into the MCS of pBAD33.
2. pAT100 was constructed in two steps. First, the *pelB-gIIIp* region from pHEN2 (13) was transferred as a *Hind*III/*Eco*RI fragment into similarly-digested pUC19 (12). The resulting plasmid was then digested with *Af*III and *Sfi*I and ligated with a *lacI-pelB* fragment from similarly digested pAK100 (14). pAT100-vtPA was created by PCR amplification and cloning of the vtPA gene from pTrc-stII-vtPA into the *pelB-gIIIp* intergenic region.

3. The phage display selection and genetic selection described in **Subheadings 3.2.** and **3.3.** are applicable to periplasmically expressed proteins. Although the microtiter-well plate assay described in **Subheading 3.1.** should also work for the directed evolution of cytoplasmic foldases, the efficiency of the chemical lysis method in releasing the contents of the cytoplasm can be somewhat variable.
4. In order for DsbC to function as an isomerase, its active-site cysteines must be maintained in the reduced form (**16,17**); this function is performed in vivo by the membrane protein DsbD. As of yet, active heterologous disulfide isomerases have not been successfully expressed in the periplasm of *E. coli*. As such, these screening and selection strategies will most likely only be useful for the evolution of bacterial disulfide isomerases.
5. This microtiter-well screening assay was adapted from a large-scale shake flask assay involving many of the same steps (**4,7**). The main difference is that, instead of chemically lysing the samples, the cells were lysed via French press. Following this step, the total soluble protein concentration of each sample was determined and normalized. Finally, 5 μg of total soluble protein from each sample were assayed with the 1X TAR solution for vtPA activity. While the use of 1X TAR is feasible for a limited number of samples, it becomes prohibitively expensive for larger numbers of samples. It was determined that the use of 1:4X TAR is adequate for a first screening step; large-scale assays are usually performed to confirm data from the microtiter-well assay.
6. The use of patched clones may be detrimental to the success of the experiment arising from plasmid instability issues. For this reason, it is recommended that only individual colonies be used for the inoculation of master plates.
7. The outer “square” of wells in the microtiter-well plates have a greater surface area for heat transfer, a characteristic that can affect the results of the chromogenic assay (which proceeds more quickly at higher temperatures) and thus skew results. For this reason, the authors typically only use the inner 60 wells of a 96-well plate for analysis.
8. The authors have also used an experimental setup in which cultures are allowed to grow without shaking of the microtiter-well plates. However, because the transfer of oxygen is impeded, growth rates are much slower and the amount of time required to run an experimental cycle is greatly increased. Furthermore, at very high rates of shaking, spillage from well to well can occur. Appropriate rates of shaking should be pre-determined, and the rate of shaking should be kept constant for all plates in a round of assays.
9. Two-hundred microliters of media per well is used to prevent possible well-to-well contamination. A subculture ratio of 1:25, i.e., 8.3 μL into 200 μL of fresh media, was chosen to stay within the acceptable error range of the multi-channel pipetteman used by the authors (volumes of less than 5 μL are generally not very reliable in most multi-channel pipetmen).
10. Different plate readers will have different correlations to standard spectrophotometers based upon the path-length and the volume of culture in each well. The user will need to correlate growth curves between the two devices in order to

determine what OD value on the plate reader corresponds to an OD₆₀₀ of 0.4–0.6 for a 1-cm pathlength. At 37°C with moderate shaking, it typically takes 1.5–2.5 h for the cell population to reach mid-log phase growth.

11. The amount of lysis reagent used is in excess of the amount recommended by the manufacturer. Typically, one hour is more than enough time for complete lysis.
12. If the library has been plated, scrape and pool the entire library together as described in **step 1** of **Subheading 3.2.3**. If larger liquid libraries are used instead, it may be necessary to scale up this and other steps to ensure that the entire library is captured.
13. 1 mL of cells at an OD₆₀₀ of 1.0 grown in LB media is equivalent to approx 8×10^8 cells. Therefore, 20 mL of cells at an OD₆₀₀ of 0.5 should be infected by 1.6×10^{11} pfu of R408 to reach an MOI of 20 ($0.5 \text{ OD units} \times 20 \text{ mL cells} \times 8 \times 10^8 \text{ cells/mL/OD} \times 20 = 1.6 \times 10^{11} \text{ pfu}$).
14. Continue growing the 380 mL of remaining culture for 2–3 h at 37°C, 250 rpm. Harvest the culture, aspirate off any residual media, and then freeze the cell pellet for later use. The pellet can be used as the starting point for a large-scale plasmid preparation of the library, which can then easily be transferred into other background strains.
15. The initial transfer of the library into the selection strain (TG1RL/pAT100-vtPA) is critical; in order to prevent the loss of diversity from the original library, it is important that the number of multiply-infected cells be minimized. In order to accomplish this, the ratio of cells:phage in the infection step should be at least 10:1. In order to ensure that the entire library is sampled, the volume of cells infected should be adjusted such that the number of cfu that are produced is at least 10-fold larger than the original number of library members. For example, the authors typically infect 20 mL of the freshly subcultured selection strain with the input phage library at a ratio of 10:1. Because this is twice the volume of cells typically manipulated (as described in **steps 2–5** of **Subheading 3.2.2.**) in the library transfer step, subsequent values are scaled up as necessary, i.e., harvested cells are resuspended in a total of 1.5 mL of TB and plated on six large TYG^{Km/Amp/Cm} plates in 250- μ L aliquots. Depending on the library size, these values may have to be manipulated to ensure that adequate coverage of the library is maintained.
16. In order to reduce the variability in the amount of time required for subcultured strains to reach mid-log phase growth, it is recommended that mid-log phase aliquots of commonly manipulated strains, i.e., TG1RL/pAT100-vtPA and TG1, be frozen and stored. By subculturing the inoculum of the frozen preparations into a constant amount of fresh (pre-warmed) media, the subculture times can be confidently predicted to reduce the amount of waiting time.
17. During the centrifugation step, you should begin the subculture of the TG1 freezer stock for the subsequent titration of phage stock. The exact timing of growth of TG1 for titration steps will be dependent upon the inoculum you prepare.
18. It is important to over-sample the input phage population by 100–1000 times for each round of panning. However, because the exact amount of over-sampling is not important (only that a relatively consistent level is used), the user is free to

make an educated guess and continue with the panning instead of waiting an entire day for the exact phage titer to be calculated. For the authors, the use of 100 μL of a 1:10 dilution (made from 20 μL of the original phage stock) resulted in the use of 1×10^9 – 1×10^{10} cfu for each panning stage, resulting in a 700- to 2500-fold oversampling rate.

19. After approx 90 min of incubation of the phage with ETI-beads, you should begin the subculture of the TG1RL/pAT100-vtPA freezer stock for subsequent re-infection with eluted phage. Again, the exact timing of this step will depend upon the final OD_{600} and amount of inoculum you use; it is crucial that you give yourself enough time to complete the panning protocol before the subcultured cells reach the target OD_{600} .
20. After an appropriate number of rounds of the PLainFold selection, the library of enriched variants should be transferred into an appropriate non-suppressor screening strain in which only the substrate (vtPA), and not the substrate-gIIIp fusion, is expressed, i.e., SF110/pTrc-stII-vtPA. This pool of putative positive clones can then be screened as described in **Subheading 3.1**. For F^- screening strains like SF110, this will require the transfer of the phage-captured library into an F' strain, e.g., XL-1 Blue, used to isolate plasmid DNA that can then be electroporated into the screening strain of interest.
21. This genetic selection may also be adapted for the isolation of improved disulfide isomerase libraries.
22. In this case, the production of vtPA would be induced directly (with arabinose, in the context of pBAD-stII-vtPA) at mid-log phase as described in **Subheading 3.1**; no secondary inductions are required. Otherwise, the microtiter-well assay would be performed exactly as described in **Subheading 3.1**.

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An Overview of High-Throughput Screening Systems for Enantioselective Enzymatic Transformations

Manfred T. Reetz

1. Introduction

The directed evolution of enantioselective enzymes (**1,2**) for use in organic synthesis constitutes an attractive alternative to traditional forms of asymmetric catalysis based on chiral transition metal complexes or catalytic antibodies. It involves the proper combination of molecular biological methods for random gene mutagenesis and gene expression coupled with appropriate high-throughput screening systems that allow the rapid determination of the enantiomeric purity of a chiral product. Typically, thousands of samples arising from the catalytic action of the evolved enzyme variants on a given substrate of interest need to be assayed within a reasonable time span, ideally within one day.

The enantioselectivity of a wild-type enzyme in a given transformation is traditionally determined by the so-called *ee*-value (*see* **Note 1**) of the product or, in the case of kinetic resolution of a racemate, by the selectivity factor *E* (*see* **Note 2**). Normally gas chromatography (GC) or high performance liquid chromatography (HPLC) based on chiral columns or NMR spectroscopy of diastereomeric derivatives is employed, but the conventional forms of these analytical tools can only handle a few dozen samples per day. Therefore, high-throughput *ee*-assays need to be developed, as in the likewise new field of combinatorial asymmetric transition metal catalysis (**3**). In principle, the assays developed in the latter area can also be adapted to suit the needs of directed evolution of enantioselective enzymes, although to date this has not been put into practice.

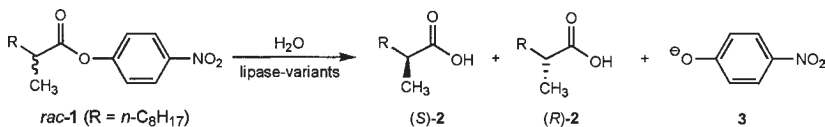
Many of the *ee*-screening systems are complementary, and no single assay is truly universal. Selection, as opposed to screening, has not been developed

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to date in the directed evolution of enantioselective enzymes, although a screening system based on differential cell growth has been described (4). Colony-based *ee*-assays have not been developed so far. This chapter summarizes the state of the art concerning high-throughput *ee*-screening systems, focusing on the principles as well as on practical aspects (5,6). Individual protocols for select *ee*-assays are presented in Chapter 27.

2. Assays Based on UV/Vis Spectroscopy

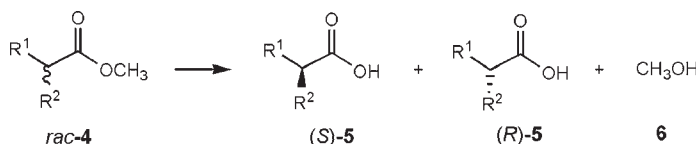
The first high-throughput *ee*-assay used in the directed evolution of enantioselective enzymes was based on UV/Vis spectroscopy (7). It is a rather crude system restricted to the hydrolytic kinetic resolution of chiral *p*-nitrophenol esters catalyzed by lipases or esterases. The system is based on a principle that forms the basis of several other *ee*-assays developed later. In order to evaluate thousands of lipase-variants from *Pseudomonas aeruginosa* as potential biocatalysts in the hydrolytic kinetic resolution of chiral esters, the *p*-nitrophenol ester (*S*)-1/(*R*)-1 was prepared as a model substrate. Hydrolysis in buffered medium generates *p*-nitrophenolate (3) which shows a strong UV/Vis absorption at 405 nm. Thus, reactions can be carried out on microtiter plates, a simple plate reader measuring absorption as a function of time (typically during the first 8 min). However, since the racemate delivers only information concerning the overall rate, the (*S*)- and (*R*)-substrates were prepared and studied separately pairwise on 96-well microtiter plates. If the slopes of the absorption/time curves differ considerably, a hit is indicated, i.e., an enantioselective lipase-variant has been identified, which is then studied in detail in a lab-scale reaction using traditional chiral gas chromatography (GC). Using epPCR (7), saturation mutagenesis (8), and DNA shuffling (9), a total of 40,000 lipase-variants were generated and screened in the model reaction. Several enantioselective lipases variants were obtained, the best one showing an *E*-value of >51 (9). The wild-type lipase displays an *E*-value of only 1.1.



The disadvantage of this assay has to do with the fact that a built-in chromophore is required (*p*-nitrophenol), yet *p*-nitrophenol esters will never be used in real (industrial) applications. Moreover, since the (*S*)- and (*R*)-substrate are tested separately pairwise, the enzyme does not compete for the two substrates, rendering the assay rather crude.

A different UV/Vis-based approach to screening enantioselective hydrolases makes use of a colorimetric assay (10) that is more general than the original

Quick-E-Test (**11**). Since hydrolysis of esters leads to a change in acidity, as in the hydrolytic lipase- or esterase-catalyzed kinetic resolution, quantification is possible by the use of an appropriate pH indicator (Kazlauskas test (**10a**)). A linear correlation between the amount of acid generated and the degree of protonation of the indicator pertains if a buffer (e.g., *N,N*-bis[2-hydroxyethyl]-2-[aminoethanesulfonic acid]) and a pH indicator (e.g., *p*-nitrophenol) having the same pK_a value are used. The advantage of this system relates to the fact that *p*-nitrophenol esters are not necessary, i.e., “normal” substrates such as methyl esters **4** can be used.



In this assay, as in related systems based on other indicators (**10b,12**), the original idea of using the (*S*)- and (*R*)-substrates pairwise separately on microtiter plates (**7**) needs to be applied. Then, hits are easily identified using a standard plate reader or even visual inspection. Thus, dozens (or more) of microtiter plates (96- or 384-format) can be processed per day.

The Kazlauskas test for enantioselective hydrolases is cheap and practical. As noted by the author, true *E*-values in the kinetic resolution of chiral esters are not provided because the (*S*)- and (*R*)-substrates are tested separately (**10a**). However, the relative initial rates provide an estimate of enantioselectivity, and the hits can then be studied conventionally using the racemate in conjunction with standard analytical tools, such as chiral GC or HPLC. Sometimes serious discrepancies arise, however (**10b**). A related colorimetric assay makes use of a more sensitive indicator (bromothymol blue) (**12**). Finally it must be kept in mind that *ee*-assays showing a precision of only ± 10 –20% in the *ee*-value, as in these screens and in some others, are well suited to identify hits in the early phases of a directed evolution project. However, higher precision (better than $\pm 5\%$) is required in the later stages, e.g., when going from *ee* = 90 to higher enantioselectivities (**5b**).

Several *ee*-assays have been developed which make use of enzyme-coupled processes. If the actual product of an enzymatic reaction under study can be transformed by another enzyme into a secondary product that gives rise to a spectroscopic signal, an enzyme-coupled assay is possible. This was first demonstrated using fluorescence as the spectroscopic detection method, high-throughput also being possible (**13**). Specifically, chiral esters containing a fluorogenic moiety were subjected to enzyme-catalyzed hydrolysis, the initial product (alcohol) then being degraded enzymatically with formation of a product detectable by fluorescence. In the case of enantioselective hydrolases this

idea coupled with the concept of employing the (*S*)- and (*R*)-substrates separately pairwise led to the establishment of a useful high-throughput *ee*-assay for hydrolases (**13**). The only disadvantage of this otherwise elegant approach relates to the necessity of incorporating a fluorogenic moiety in the starting material. This means that in the case of directed evolution, the optimized enzyme variants catalyze the transformation of a specific substrate that is not likely to be industrially relevant.

In a different and more general approach, the hydrolase-catalyzed kinetic resolution of chiral acetates was studied using a high-throughput *ee*-assay also based on an enzyme-coupled test, the presence of a fluorogenic moiety not being necessary (**14**). The assay is based on the idea that the acetic acid formed in the hydrolysis of a chiral acetate can be transformed stoichiometrically into NADH via a series of coupled enzymatic reactions using commercially available enzyme kits (see **Fig. 1**). The NADH is then easily detected UV-spectroscopically in the wells of microtiter plates using a standard plate reader. It was estimated that about 13,000 samples can be evaluated per day. Using various commercially available hydrolases the kinetic resolution of (*S,R*)-1-methoxy-2-propylacetate was studied. The agreement between the apparent selectivity factor E_{app} and the actual value E_{true} determined by GC turned out to be excellent at low enantioselectivity ($E = 1.4\text{--}13$), but less so at higher enantioselectivity (20% variation at $E = 80$) (**14**).

A different enzymatic method for determining the *ee*-value of thousands of samples containing chiral alcohols has been developed, called EMD_{ee} (**15**). Although it has not yet been used to assay libraries of mutant enzymes, such adaptation should be possible. The method is based on the idea that an appropriate enzyme can be used to selectively process one enantiomer of a product from a catalytic reaction. The well-known catalytic addition of diethylzinc **8** to benzaldehyde **7** was chosen as test-bed for demonstrating EMD_{ee}. However, it is easy to imagine a hydrogenase-catalyzed ketone reduction. The reaction product, 1-phenylpropanol **9**, can be oxidized to ethyl phenyl ketone **10** using the alcohol dehydrogenase from *Thermoanaerobium sp.*, this process being completely (*S*)-selective (see **Fig. 2**). It was possible to measure the rate of this enzymatic oxidation by monitoring the formation of NADPH by UV spectroscopy at 340 nm.

Decisive for the success of the assay is the finding that the rate of oxidation constitutes a direct measure of the *ee* value. High-throughput was demonstrated by analyzing 100 samples in a 384-well format by using a UV plate reader. Each sample contained 1 μmol of 1-phenylpropanol **9** in a volume of 100 μL . The accuracy of the *ee*-value amounts $\pm 10\%$, as checked by independent GC determinations. About 100 samples can be processed in 30 min, which means 4800 *ee*-determinations per day. Of course, for each new alcohol to be assayed,

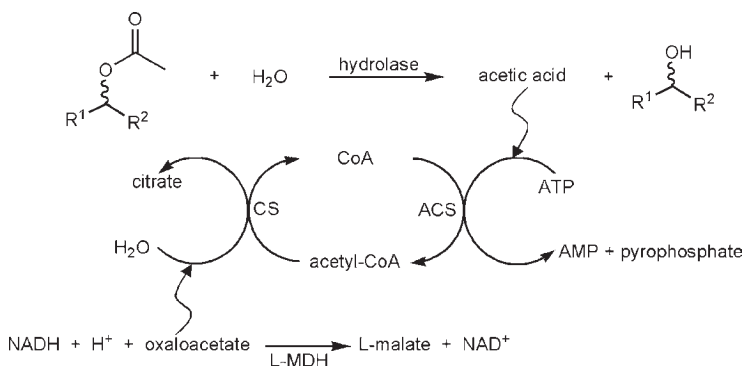


Fig. 1. The hydrolase-catalyzed reaction releases acetic acid, which is converted by acetyl-CoA synthetase (ACS) to acetyl-CoA in the presence of (ATP) and coenzyme A (CoA) (**14**). Citrate synthase (CS) catalyzes the reaction between acetyl-CoA and oxaloacetate to give citrate. The oxaloacetate required for this reaction is formed from L-malate and NAD^+ in the presence of L-malate dehydrogenase (L-MDH). Initial rates of acetic acid formation can thus be determined by the increase in adsorption at 340 nm due to the increase in NADH concentration. Use of optically pure (*R*)- or (*S*)-acetates allows the determination of the apparent enantioselectivity E_{app} (**14**).

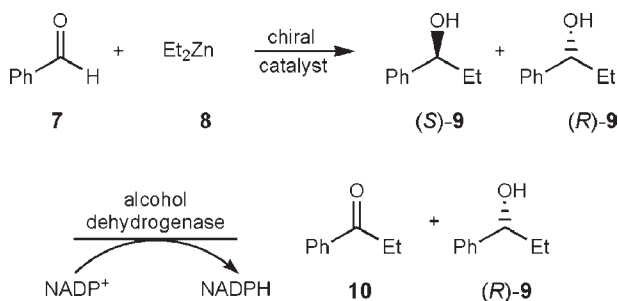


Fig. 2. EMDee in the case of 1-phenylpropanol produced by asymmetric addition of diethylzinc to benzaldehyde (**15**).

the alcohol dehydrogenase needs to be selective. If this is not the case, a different (selective) alcohol dehydrogenase has to be found in order for the assay to function properly.

Yet another UV/Vis based high-throughput screening of enantioselective catalysts is possible by enzyme immunoassays (**16**), a technology that is routinely applied in biology and medicine. As in the case of some of the other screening systems, this new assay was not developed specifically for enzyme-catalyzed processes. In fact, it was illustrated by analyzing (*R*)/(*S*)-mixtures of

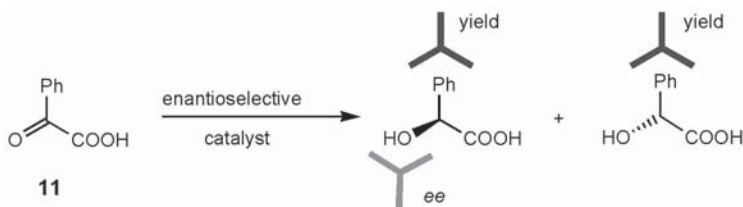


Fig. 3. Scheme illustrating high-throughput screening of enantioselective catalysts by competitive enzyme immunoassays (**16**). The antibody marked blue recognizes both enantiomers, whereas the antibody marked red is (*S*)-specific, making the determination of yield and *ee* possible.

mandelic acid generated by enantioselective Ru-catalyzed hydrogenation of benzoyl formic acid **11** (see Fig. 3). By employing an antibody that binds both enantiomers it is possible to measure the concentration of the reaction product, thereby allowing the yield to be calculated. The use of an (*S*)-specific antibody then makes determination of the *ee* possible (see Fig. 3).

Of course, the success of this assay depends upon the availability of specific antibodies, and indeed these can be raised to almost any compound of interest. Moreover, simple automated equipment comprising a plate washer and plate absorbance reader is all that is necessary. About 1000 *ee* determinations are possible per day, precision amounting to $\pm 9\%$ (**16**).

3. Assays Based on Fluorescence

Fluorescence-based assays have the advantage of high sensitivity (**13**). However, if the fluorescence active probe is attached to the substrate undergoing enzyme catalyzed enantioselective transformation, the process of directed evolution will lead to the creation of an enzyme specific for this (complicated) substrate, not likely to be used in industrial applications. A different approach is to use a molecular sensor which fluoresces upon formation of a certain product, as in acylation reactions (**17**). It should be possible to adapt this technique in the lipase-catalyzed kinetic resolution of chiral alcohols.

Another potentially useful fluorescence-based *ee*-assay makes use of DNA microarrays (**18**). This type of technology had previously been employed to determine relative gene expression levels on a genome-wide basis as measured by the ratio of fluorescent reporters (**19**). In the case of the *ee*-assay, chiral amino acids were used as model compounds. Mixtures of a racemic amino acid are first subjected to acylation at the amino function with formation of *N*-Boc protected derivatives. The samples are then covalently attached to amine-functionalized glass slides in a spatially arrayed manner (see Fig. 4). In a second step, the uncoupled surface amino functions are acylated exhaustively. The third

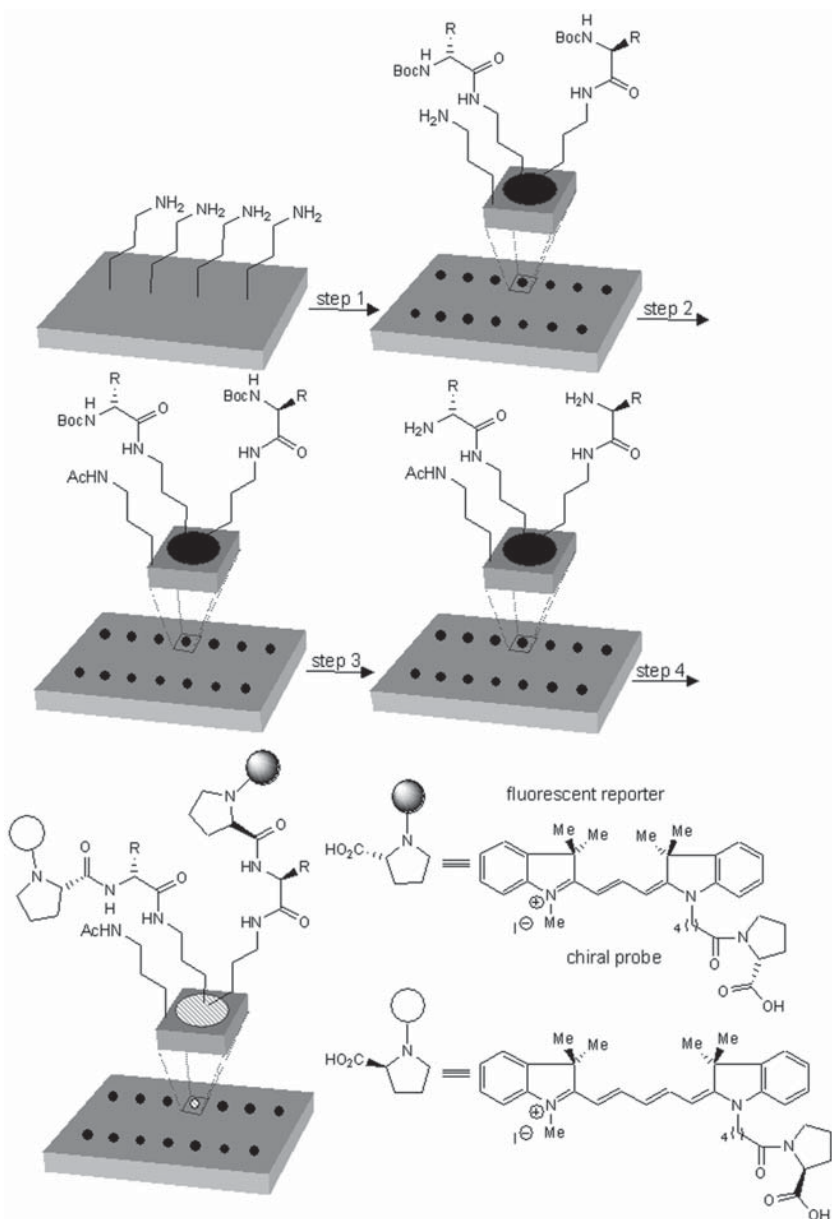


Fig. 4. Reaction microarrays in high-throughput *ee*-determination (**18**). Reagents and conditions: step 1) BocHNCH(R)CO₂H, PyAOP, *i*Pr₂NEt, DMF; step 2) Ac₂O, pyridine; step 3) 10% CF₃CO₂H and 10% Et₃SiH in CH₂Cl₂, then 3% Et₃N in CH₂Cl₂; step 4) pentafluorophenyl diphenylphosphinate, *i*Pr₂NEt, 1:1 mixture of the two fluorescent proline derivatives, DMF, -20°C.

step involves complete deprotection to afford the free amino function of the amino acid. Finally, in a fourth step two *pseudo*-enantiomeric fluorescent probes are attached to the free amino groups on the surface of the array. An appreciable degree of kinetic resolution in the process of amide coupling is a requirement for the success of the *ee*-assay (Horeau's principle) (20). In the present case, the *ee* values are accessible by measuring the ratio of the relevant fluorescent intensities. About 8000 *ee* determinations are possible per day, precision amounting to $\pm 10\%$ of the actual value. Although it was not explicitly demonstrated that this *ee*-assay can be used to evaluate enzymes (e.g., proteases), this should in fact be possible. The question whether other types of substrates (and enzymes) are amenable to this type of screening also needs to be addressed.

4. Assays Based on Mass Spectrometry (MS)

Since enantiomers have identical mass spectra, the relative amounts of the (*R*)- and (*S*)-forms present in a given sample (i.e., the *ee*-value) cannot be measured by conventional MS-techniques. However, in one approach this information does in fact become available if two conditions are met (21): 1) A mass-tagged chiral derivatization agent is applied to the mixture, and 2) in the process of derivatization a significant degree of kinetic resolution occurs (Horeau's principle [20]). The relative amounts of mass-tagged diastereomers can then be measured by MS simply by integrating the appropriate peaks, the uncertainty in the *ee*-value amounting to $\pm 10\%$ (21). High-throughput application (e.g., in enzyme catalysis) has not been demonstrated, but this should pose no problems.

A different MS-based approach does not require any derivatization reaction (22) and has been applied successfully in the directed evolution of enantioselective enzymes. It makes use of deuterium-labeled *pseudo*-enantiomers or *pseudo-meso*-compounds. The method is practical and can be applied in studies involving kinetic resolution of racemates or desymmetrization of prochiral compounds bearing reactive enantiotopic groups (see Fig. 5).

Since the products of these transformations are always *pseudo*-enantiomers differing in absolute configuration and in mass, integration of the MS-peaks and data processing affords the *ee*- or *E*-values. Any type of ionization can be employed, but electrospray ionization (ESI) is used most commonly (22). In some cases an internal standard is advisable if the determination of %-conversion is necessary. The uncertainty in the *ee*-value amounts to only $\pm 2\%$. In the original version about 1000 *ee*-values could be measured per day (22a), but this has recently been increased to about 10,000 samples per day as a result of a second-generation system based on an eight-channel multiplexed sprayer system (22b). The method has been used to assess lipase-variants from *Bacillus subtilis*, produced by the methods of directed evolution (Reetz, M. T.,

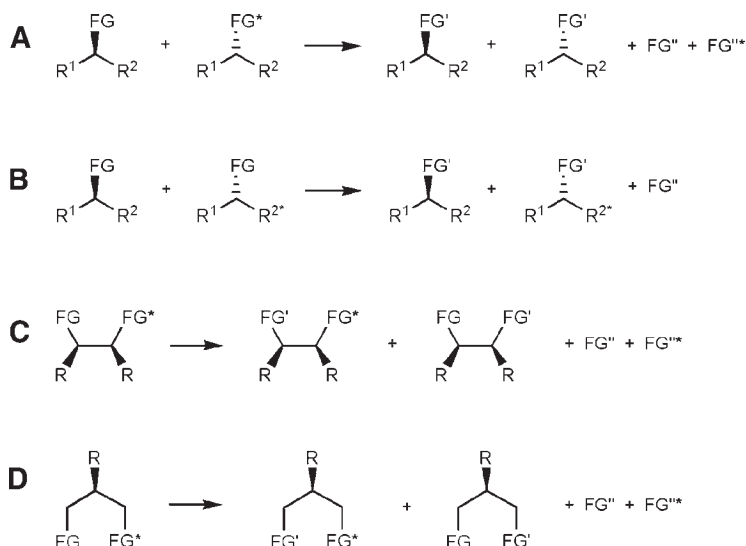
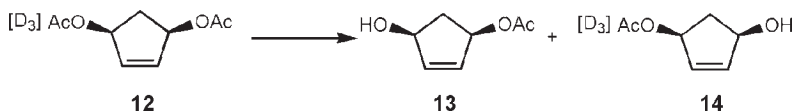


Fig. 5. (A) Asymmetric transformation of a mixture of *pseudo*-enantiomers involving cleavage of the functional groups FG and labeled FG* (**22**). (B) Asymmetric transformation of a mixture of *pseudo*-enantiomers involving either cleavage or bond formation at the functional group FG; isotopic labeling at R² is indicated by the asterisk. (C) Asymmetric transformation of a *pseudo-meso* substrate involving cleavage of the functional groups FG and labeled FG*. (D) Asymmetric transformation of a *pseudo*-prochiral substrate involving cleavage of the functional groups FG and labeled FG*.

Jaeger, K.-E., et al., unpublished work). They are employed as catalysts in the desymmetrization of *meso*-1,4-diacetoxy-cyclopentene. The goal is to obtain enantioselective variants of this lipase which are expressed in *E. coli*. Accordingly, the D₃-labeled *pseudo-meso*-compound **12** is used as the substrate, the two products of the asymmetric transformation being non-labeled and the D₃-labeled *pseudo*-enantiomers **13** and **14**, easily distinguished by ESI-MS. The method has also been used in kinetic resolution, as in epoxide hydrolase catalyzed reactions of racemic epoxides (**22b**).

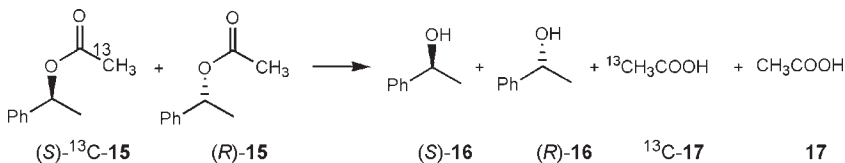
It should be noted that in the case of kinetic resolution, in which by nature the *ee*-value varies with the degree of conversion, a single measurement may not suffice, especially if the individual mutant enzymes in a library show different rates, or if the amount of protein varies significantly from well to well.



5. Assays Based on NMR Spectroscopy

Although NMR measurements are usually considered to be slow processes, recent advances in the design of flow-through cells have allowed the method to be applied in combinatorial chemistry (23). Thereafter, these technological advancements were applied to the development of two different NMR-based high-throughput *ee*-assays (24). In one version classical derivatization using a chiral reagent or NMR shift agent is parallelized, about 1400 *ee*-measurements being possible per day with a precision of $\pm 5\%$. In the second embodiment, illustrated here in detail, a principle related to that of the MS-system described in **Subheading 3**, is applied, i.e., chiral or *meso*-substrates are labeled so as to produce *pseudo*-enantiomers or *pseudo-meso*-compounds which are then used in the actual screen. Consequently, application is restricted to kinetic resolution of racemates and desymmetrization of prochiral compounds bearing reactive enantiotopic groups as illustrated previously in **Fig. 5**.

The most practical form of this assay utilizes ^1H NMR spectroscopy, ^{13}C -labeling being used to distinguish between the (*R*)- and (*S*)-forms of a chiral compound under study. Practically any carbon atom in the compound of interest can be labeled, but methyl groups in which the ^1H signals are not split by ^1H , ^1H coupling are preferred because the relevant peaks to be integrated are the singlet arising from the CH_3 -group of one enantiomer and the doublet of the $^{13}\text{CH}_3$ -group of the other. A typical example that illustrates the method concerns the lipase- or esterase-catalyzed hydrolytic kinetic resolution of *rac*-1-phenyl ethyl acetate, derived from *rac*-1-phenyl ethanol. However, the acetate of any chiral alcohol or the acetamide of any chiral amine can be used. Labeling can be carried out at any position of a compound, as in (*S*)- ^{13}C -15. The synthesis is straightforward, since it simply involves acylation of the (*S*)-alcohol using commercially available ^{13}C -labeled acetyl chloride. Then a 1:1 mixture of labeled and non-labeled compounds (*S*)- ^{13}C -15 and (*R*)-15 is prepared to simulate a racemate. It is used in the actual catalytic hydrolytic kinetic resolution, which affords a mixture of true enantiomers (*S*)-16 and (*R*)-16 as well as labeled and non-labeled acetic acid ^{13}C -17 and 17, respectively, together with unreacted starting esters. At 50% conversion (or at any other point of the reaction) the ratio of (*S*)- ^{13}C -15 to (*R*)-15 reveals the enantiomeric purity of the non-reacted ester, while the ratio of ^{13}C -17 to 17 correlates with the relative amounts of (*S*)-16 and (*R*)-16, respectively.



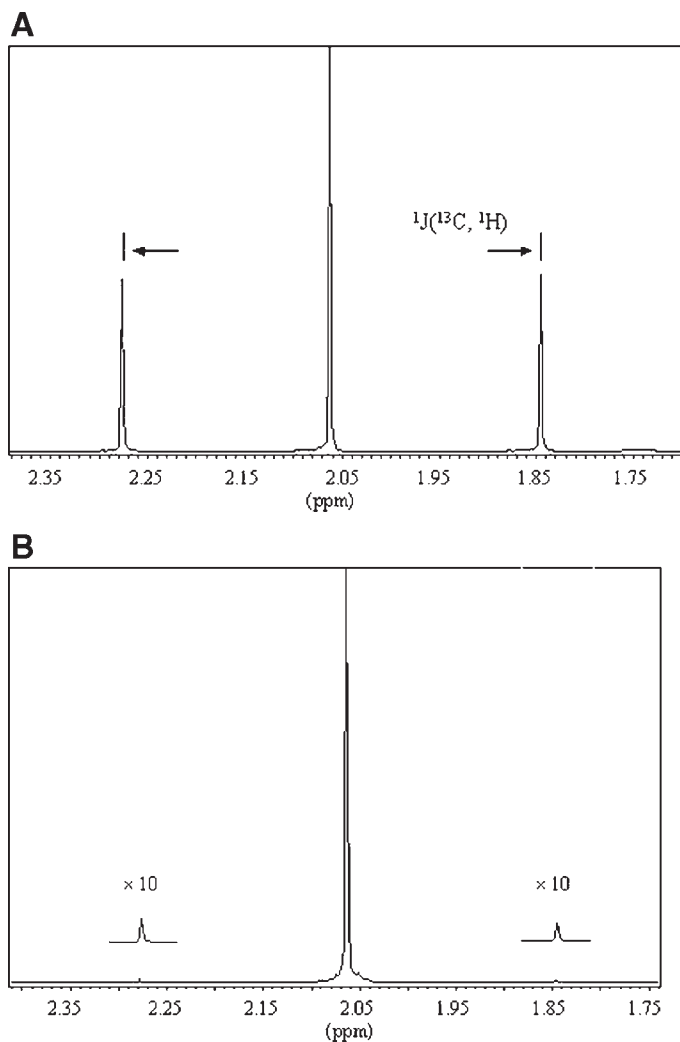


Fig. 6. Expanded region of the ^1H NMR spectra of (A) racemic mixture of (S) - ^{13}C -**15**/ (R) -**15** and (B) (R) -**15** alone (**24**).

Figure 6A shows an excerpt of the ^1H NMR spectrum of a “racemic” mixture of (S) - ^{13}C -**15** and (R) -**15** featuring the expected doublet of the ^{13}C -labeled methyl group and the singlet of the non-labeled methyl group. **Figure 6B** displays the singlet of the non-labeled methyl group of (R) -**15**, including the ^{13}C -satellites due to the presence of natural ^{13}C in the sample (**24**).

Thus, the two *pseudo*-enantiomers are nicely distinguishable by ^1H NMR spectroscopy, the exact ratio of the two being accessible by simple integration of the respective peaks. This ultimately provides the *ee*-value. The quantitative analysis can be accomplished automatically by suitable software such as AMIXTM (available from Bruker Biospin GmbH, Rheinstetten, Germany). The presence of naturally occurring ^{13}C in the non-labeled (*R*)-substrate is automatically considered in the data processing step. As demonstrated in control experiments, the agreement with the corresponding *ee*-values obtained by independent GC analysis is excellent ($\pm 2\%$), the correlation coefficient amounting to $R^2 = 0.9998$ (24).

Since the *ee*-value in an actual kinetic resolution depends on the degree of conversion, the selectivity factor *E* needs to be ascertained, accessible if the conversion can be measured. In the present system, this is possible by automatic integration of the corresponding methine signals of the unreacted substrate ester at 5.9 ppm and the product alcohol at 4.9 ppm. Then the *E*-value can be estimated according to the method of Sih (25). In other cases an internal standard may be more appropriate.

In summary, the two embodiments of the NMR-based high-throughput *ee*-assay constitute the currently most general and precise way to determine the enantiopurity of large numbers of samples (24). A second generation version of these two embodiments based on *Chemical Shift Imaging* (CSI) (26) using bundles of capillaries is in the process of being optimized (Reetz, M. T., Tielmann, P., Eipper, A., Ross, A., unpublished work), which will enable up to 10,000 *ee*-determinations to be performed per day. A completely different NMR approach relies on the production of diastereomers formed in the reaction of isotopically-labeled chiral starting material (27). However, it is less general.

6. Assays Based on Capillary Array Electrophoresis or Gas Chromatography

Conventional chromatography using chiral stationary phases can only handle a few dozen *ee*-determinations per day. However, capillary array electrophoresis (CAE), as used in the Human Genome Project, can be adapted to handle up to 20,000 *ee* determinations per day, as in the case of chiral amines (28) or alcohols (Reetz, M. T., Belder, D., Ludwig, M., unpublished results). Following an enzymatic reaction producing such products, derivatization by a fluorescence-active reagent is necessary. For thousands of samples this needs to be done robotically. Owing to the high sensitivity of fluorescence detection, precision is high, $\pm 3\%$. Thus, this is an excellent *ee*-assay.

In some cases GC, which is considerably cheaper, can be modified so that in optimal cases about 700 exact *ee*- and *E*-determinations are possible per day (29). Such medium-throughput may suffice in certain applications. The case

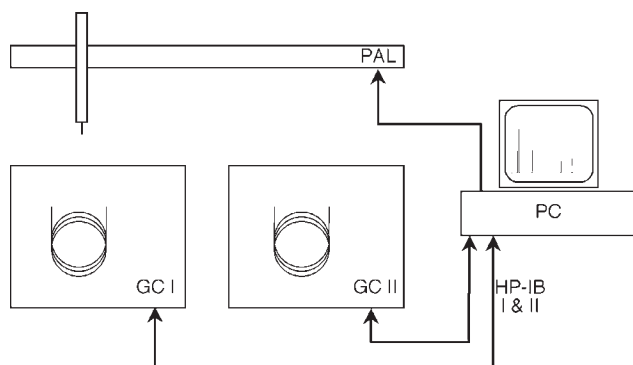
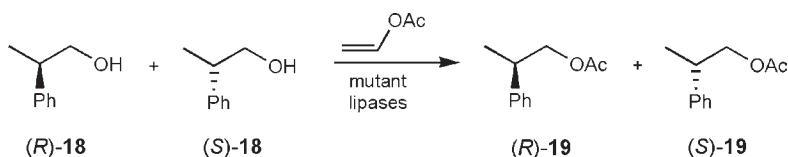


Fig. 7. Schematic representation of a medium-throughput GC *ee*-screening system comprising two GC instruments (29).

study concerns the lipase-catalyzed kinetic resolution of the chiral alcohol (*R*)- and (*S*)-**18** with formation of the acylated forms (*R*)- and (*S*)-**19**. Thousands of mutants of the lipase from *Pseudomonas aeruginosa* were created by error-prone PCR for use as catalysts in the model reaction (29).



The initial approach concerns the use of two columns in a single GC oven (29). However, this turned out to have a number of disadvantages and should be avoided. The successful construction consists of two GC instruments (e.g., GC instruments and data bus (HP-IB) are commercially available from Hewlett-Packard, Waldbronn, Germany), one prep-and-load sample manager (PAL) (commercially available from CTC, Schlieren, Switzerland) and a PC (see Fig. 7). The instruments are connected to the PC via a standardized data bus (HP-IB) (commercially available from Hewlett-Packard) to control pressure, temperature, and handle other data such as that of the detector. A wash station as well as a drawer system with a maximum of eight microtiter plates are included. The sample manager is attached to the unit in such a way as to reach both injection ports. Since the sample manager can inject samples from 96- or 384-well microtiter plates, over 3000 samples can be handled without manual intervention. The software (Chemstation®) (commercially available from Hewlett-Packard, Waldbronn, Germany) enables additional programs

(macros) to be applied before and after each analytical run. Such a macro controls the sample manager, each position on the microtiter plate being labeled via the sequence table. Another macro ensures analysis following each sample run in a specified manner, i.e., the peaks of the chiral compound **18** are analyzed quantitatively. The analytical data are transferred to an Excel™ spreadsheet via DDE (Dynamic Data Exchange) (commercially available from Microsoft, Unterschleissheim, Germany) in table form or in microtiter-format, allowing for a rapid overview. Finally, the setup includes H₂-guards which monitor the hydrogen concentration in the ovens; at concentrations exceeding 1% (potentially explosive at >4% H₂) the systems responds and automatically switches to nitrogen as the carrier gas (**29**).

Using a stationary phase based on a β -cyclodextrin derivative (2,3-di-*O*-ethyl-6-*O*-*tert*-butyldimethylsilyl- β -CD) complete separation of (*R*)- and (*S*)-**18** (but not of (*R*)/(*S*)-**19**) is possible within 3.9 min. Since the configuration illustrated in **Fig. 7** comprises two simultaneously operating GC units, about 700 exact *ee*-determinations of (*R*)/(*S*)-**18** are possible per day. Moreover, the corresponding values for the conversion and the selectivity factor *E* (or *s*) are likewise automatically provided in microtiter-format, which means that the *ee* of (*R*)/(*S*)-**19** is also accessible. Of course, every new substrate has to be optimized anew using commercially available chiral stationary phases (**29**). A related system has been developed for HPLC analysis (Reetz, M. T., Belder, D., et al., unpublished).

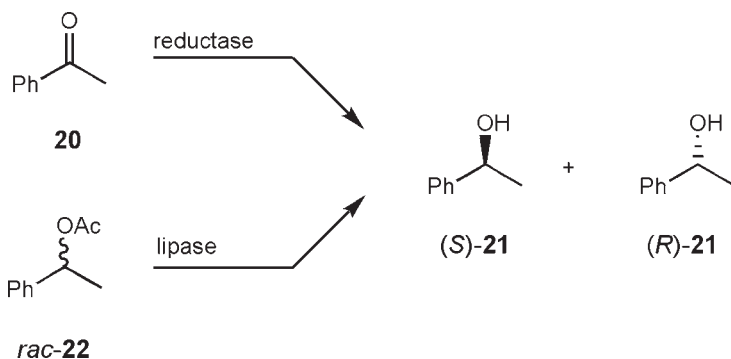
7. Assays Based on Circular Dichroism (CD)

An alternative to chiral GC or HPLC which require chiral columns, is the use of normal columns to simply separate the starting materials from the enantiomeric products, enantiomeric excess (*ee*) of the mixture of enantiomers then being determined by circular dichroism (CD) spectroscopy. Indeed, this principle was first established in 1980 and developed further in later research (**30**). Recently, it was shown that the method can be applied in the screening of combinatorially-prepared enantioselective transition metal catalysts (**31**). However, it should be amenable to enzyme catalyzed processes as well. The method uses sensitive detectors for HPLC to determine in a parallel manner both the circular dichroism ($\Delta\epsilon$) and the UV-absorption (ϵ) of a sample at a fixed wavelength in a flow-through system. The CD-signal depends only on the enantiomeric composition of the chiral products, whereas the absorption relates to their concentration. Thus, only short HPLC-columns are necessary. Upon normalizing the CD-value with respect to absorption, the so-called anisotropy factor *g* is obtained:

$$g = \frac{\Delta\epsilon}{\epsilon}$$

It is therefore possible to determine the *ee*-value of a mixture without recourse to complicated calibration. The fact that the method is theoretically valid only if the *g*-factor is independent of concentration and if it is linear with respect to *ee* has been emphasized repeatedly. However, it needs to be pointed out that these conditions may not hold if the chiral compounds form dimers or aggregates, because such enantiomeric or diastereomeric species would give rise to their own particular CD effects (32).

This precautionary measure was considered in the development of a CD-based *ee*-assay for chiral alcohols (32). In work concerning the directed evolution of enantioselective enzymes, there was the need to develop fast and efficient ways to determine the enantiomeric purity of these compounds, which can be produced enzymatically either by reduction of the prochiral ketone (e.g., **20**) using reductases or by kinetic resolution of *rac*-acetates (e.g., **22**) by lipases. In both systems the CD approach is theoretically possible. In the former case an LC-column would have to separate the **20** from the product (*S*)/(*R*)-**21**, whereas in the latter case (*S*)/(*R*)-**21** would have to be separated from (*S*)/(*R*)-**22**.



Since acetophenone **20** has a considerably higher extinction coefficient than 1-phenylethanol **21** at a similar wavelength (near 260 nm), separation of starting material from product is absolutely necessary, and was accomplished using a relatively short HPLC-column based on a reversed-phase system (32). In experiments using enantiomerically pure product **21**, the maximum value of the CD-signal was determined. Mixtures of **21** having different enantiomer ratios (and therefore *ee*-values) were prepared and analyzed precisely by chiral GC in control experiments. The same samples were studied by CD, resulting in the compilation of *g*-values. Upon plotting the *g*- against the *ee*-values, a linear dependency was in fact observed with a correlation factor of $r = 0.99995$, translating into the following simple equation for enantioselectivity (32):

$$ee = 3176.4 g - 8.0$$

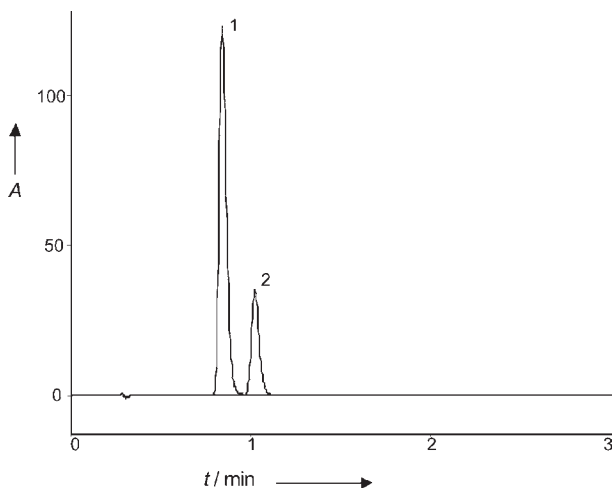


Fig. 8. HPLC-chromatogram of a mixture of **20** (peak 1) and (*S*)/(*R*)-**21** (peak 2) (**5a**, **29**).

The possible question of dependency of the *g*-factors on concentration was then studied. A mixture of (*S*)- and (*R*)-**21** corresponding to an enantiomeric excess of *ee* = 20% was prepared at a concentration of 10 $\mu\text{L/mL}$ in acetonitrile, which was then successively diluted. It was shown that no dependency of *g* on concentration (standard deviation = 2.6%) exists. Thus, possible aggregation arising from hydrogen bonding between two or more molecules of the product (*S*)- and (*R*)-**21** in this medium, which could lead to artifacts, is not involved, making the system amenable to CD-analysis and therefore to high-throughput analysis.

Separation of **20** from (*S*)/(*R*)-**21** was accomplished using reversed phase silica as the column material and methanol/water (47/53) as the eluant with complete optimization (**32**). In view of the results concerning the dependency of the *g*-factor on concentration, aggregation can be excluded in this protic medium. **Figure 8** shows the corresponding HPLC-chromatogram, in which the mixture is fully separated in less than 1.5 min. Thus, using the JASCO-CD-1595 instrument in conjunction with a robotic autosampler, it is possible to perform about 700–900 exact *ee* determinations per day (**32**).

8. Assays Based on IR-Thermography

Photovoltaic IR cameras equipped with focal plane array detectors provide a two-dimensional thermal image as a spatial map of the temperature and emis-

sivity distribution of all objects in the picture (33). It is customary to use different colors to visualize different photon intensities of the detected infrared radiation, i.e., red areas indicate “hot spots” and blue areas denote “cold spots”.

Recently, emissivity corrected IR-thermography of large libraries of heterogeneous catalysts was developed, a technique that requires only very small amounts of catalysts (<200 μg) (34). The main object of this study was to visualize temperature differences solely owing to the catalytic activity of the catalysts, which was achieved by applying a linear correction to the detector response and subtracting the IR image of the library just before the start of the reaction, i.e., as background (offset) from the images during catalytic experiments. This means that local emissivity differences are no longer visible, and the heat evolution from the catalytic reactions on a microtiter plate can then be reliably detected.

Thereafter, application in the area of enantioselective homogeneous transition metal catalysis and enzyme catalysis was reported (35). A commercially available Eppendorf-Thermomixer was modified such that the top was replaced by an aluminum plate into which holes were drilled for cylindrical glass reaction vessels about 8 mm in diameter and 35 mm in height. The whole microtiter plate can be shaken so as to ensure agitation of the reaction contents in each well. The method was illustrated by experiments involving kinetic resolution of chiral substrates, i.e., the (*R*)- and (*S*)-compounds were reacted separately pairwise.

Time-resolved detection of an enantioselective enzyme-catalyzed kinetic resolution was demonstrated (35). The enzyme (lipase from *Candida antarctica*) was added to the wells of the microtiter plate in immobilized form, i.e., the reaction was catalyzed by a heterogeneous catalyst. Using (*S*)- and (*R*)-1-phenylethanol **21** as the substrates separately and vinyl acetate as the acylating agent, it was demonstrated that the reaction is highly (*R*)-selective, i.e., hot spots appeared above the wells of the microtiter plate containing (*R*)-**21** (see Fig. 9). The result is in perfect agreement with the literature data according to which the *ee*-value of the acylated form at 50% conversion is >99% in favor of (*R*)-**22**.

These and further developments (36) show that IR-thermography is a useful tool in the high-throughput identification of highly active and enantioselective enzymes (or other catalysts) in exothermic processes. The method allows one to distinguish such “hits” from other members of a library of catalysts much less active or less enantioselective. However, quantitative correlation with enantioselectivity is not possible so far (36). The disadvantage of the method hinges on the fact that small differences in enantioselectivity, as usually observed in sequential rounds of enzyme mutagenesis, cannot be picked up by IR-thermographic assays. The fact that meaningful comparisons on microtiter can only be made if the same amount of enzyme is present in each well also needs to be kept in mind when attempting to apply this technology.

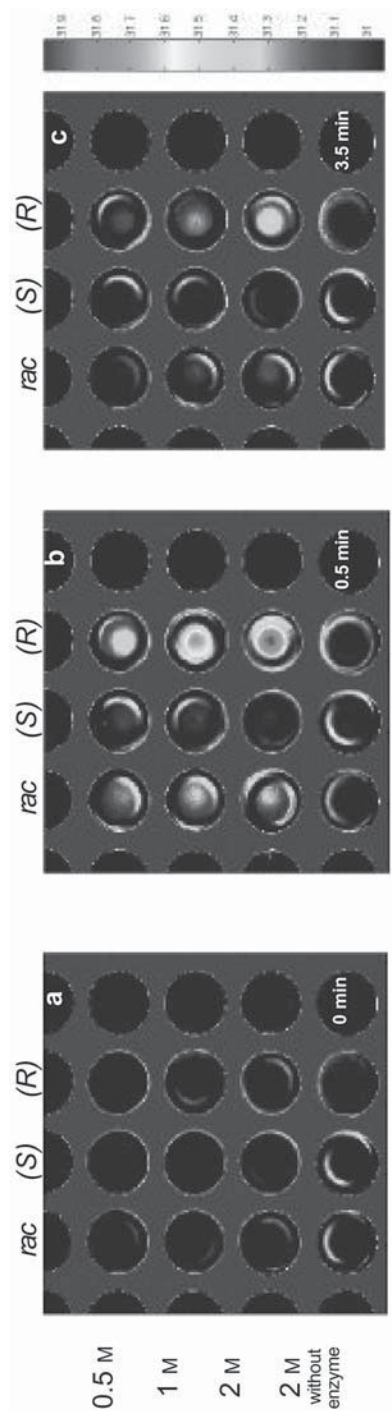
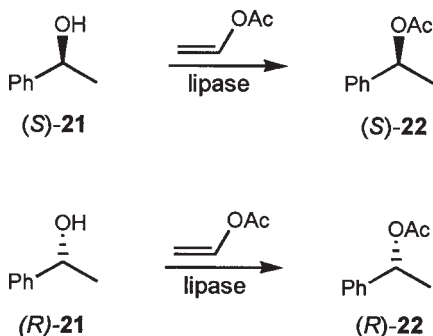


Fig. 9. Time-resolved IR thermographic imaging of the lipase-catalyzed enantioselective acylation of **21** after (A) 0.5, (B) 0.5 and (C) 3.5 min (**35**). The control experiment without enzyme is given in the bottom row in each case. The bar on the far right is the temperature/color key of the temperature window used [$^{\circ}\text{C}$].



9. Conclusions

A number of rapid *ee*-assays are currently available, throughput ranging between 700 and 20,000 samples per day. The choice as to which assay to use depends upon the particular analytical problem, which means that no general advice can be given. Some of the tests were developed for use in asymmetric transition metal catalysis, which means that some time needs to be invested for adaptation in enantioselective enzyme catalysis. For example, following the enzyme-catalyzed transformation in the wells of microtiter plates in aqueous medium, robotic extraction with organic solvents prior to *ee*-analysis is generally necessary. These operations are usually routine.

Perhaps the two most practical, general and easy to use *ee*-assays currently available are the ones based on mass spectrometry (22) and NMR spectroscopy (24). Between 1400 and 10,000 samples can be processed per day. The accuracy in the *ee*-determination ranges between $\pm 2\%$ and $\pm 5\%$, considerably better than that reported for most other assays. As already delineated, this is important in the late stages of any project concerning the directed evolution of enantioselective enzymes, i.e., typically when going from an *ee* of 90% to higher values. A special form of chiral GC as an *ee*-assay allows for up to 700 samples to be analyzed per day, accuracy amounting to $\pm 2\%$ (29). For some applications this medium-throughput *ee*-assay suffices. The chapter which follows describes protocols of these three *ee*-screening systems, assays that are currently being used in the author's own laboratory.

10. Notes

1. The enantiomeric excess (*ee*) is defined as the percent excess of one enantiomer over the racemate:

$$\%ee = \frac{|R - S|}{R + S} \cdot 100 = |\%R - \%S|$$

2. The selectivity factor E in a kinetic resolution, sometimes also called the enantiomeric ratio, reflects the relative rate of reaction of one enantiomer with respect to the other and is approximated by the formula of Sih (25):

$$E = \frac{\ln \left(1 - C \left[1 + ee_p \right] \right)}{\ln \left(1 - C \left[1 - ee_p \right] \right)}$$

where C = conversion; ee_p = enantiomeric excess of the product.

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Select Protocols of High-Throughput *ee*-Screening Systems for Assaying Enantioselective Enzymes

Manfred T. Reetz

1. Introduction

As delineated in **Chapter 26**, the directed evolution of enantioselective enzymes for use in synthetic organic chemistry requires the availability of high-throughput screening systems for determining the enantiomeric excess (*ee*) of thousands of samples (**1**). In this chapter the protocols of some of the most practical *ee*-screens are presented.

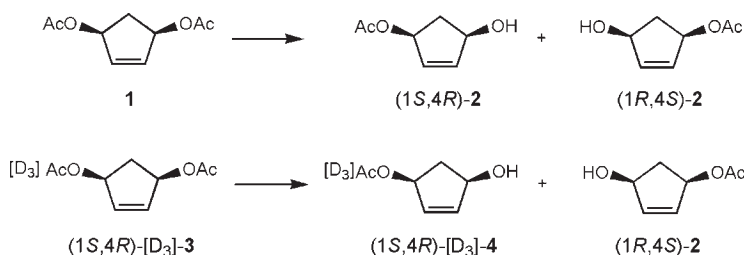
2. Assays Based on Mass Spectrometry (MS) (**1,2**)

A typical example for the application of the MS-based *ee*-assay concerns the directed evolution of an enantioselective lipase from *Bacillus subtilis* for use as a catalyst in the hydrolytic desymmetrization of *meso*-1,4-diacetoxycyclopentene **1**, the two enantiomeric products being (1*S*,4*R*)-**2** and (1*R*,4*S*)-**2**. The wild-type lipase shows only a slight preference for (1*S*,4*R*)-**2** with *ee* = 38% (Reetz, M. T., Jaeger, K. E., et al., unpublished). Since the two enantiomers have identical mass spectra, they cannot be distinguished by MS. Therefore, in the MS-based high-throughput screen (**2**) (see **Chapter 26**), the *pseudo-meso*-compound (1*S*,4*R*)-[D₃]-**3** needs to be prepared and used in the assay, because now the two products of hydrolytic desymmetrization, (1*S*,4*R*)-[D₃]-**4** and (1*R*,4*S*)-**4** are *pseudo*-enantiomers differing in absolute configuration and mass. Integration of the respective MS peaks provides the *ee*-value.

2.1. Details of Automation Required for High-Throughput *ee*-Screening

Having plated the bacterial colonies on agar plates, they are collected and placed individually in the deep-wells of microtiter plates (96-format) contain-

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ing Luria-Bertani (LB) medium with the aid of an appropriate robot (e.g., Colony Picker Q-Pix commercially available from Genetix). Up to 10,000 colonies can be handled per day. The LB-medium is added using an 8-channel dispenser (e.g., Dispenser Multidrop DW from Labsystems).

The preparation of the reaction solutions and the process of carrying out the lipase-catalyzed reactions in the wells of 96-format microtiter plates are also fully automated. The preparation of the reaction solutions in the deep wells (2.2 mL) of microtiter plates (96-format) is automated using a pipet robot (e.g., Genesis supplied by Tecan GmbH, Maennedorf, Switzerland). Accordingly, pipet scripts are programmed (Gemini Software supplied by Tecan) for robotically filling the wells with buffer and substrate solutions (*see* **Note 1**). In order to activate all of the modules of the robot, the Facts software (supplied by Tecan GmbH) is used. The pipet robot consists of a workstation with spaces for 12 microtiter plates, a robot arm for transport and a carousel for storing the reaction plates as well as a 96-fold pipet module (*see* **Fig. 1**).

Following lipase-catalyzed desymmetrization reactions of the substrate (e.g., **3**) in the wells of 96-format microtiter plates, an extraction step is necessary prior to ESI-MS analysis (*see* **Note 2**). This process is controlled by the Facts software. Accordingly, four modules are controlled simultaneously: The robot arm, the carousel for storing the microtiter plates, the 96-pipet system, as well as the 8-fold pipet head. Iteration occurs within 12 min.

The control of the HPLC-pump as well as the auto-sampler and the MS is ensured by the Masslynx 3.5 Software (Micromass). Following optimization of the measurement conditions, a list of process measurements is set up (sample list) and the desired HPLC and MS steps are called upon. Following a measurement the ESI-source is automatically brought to room temperature (shut down). Using a 96-microtiter plate, 576 samples can be processed per measurement. The chromatograms are integrated by the Quanlynx and Openlynx software package (Micromass) and transformed into an Excel table. The use of a macro allows the calculation of the absolute intensities and therefore the *ee* as well as the conversion. The *E*-values in the case of kinetic resolution are automatically calculated by the formula derived by Sih (**3**).

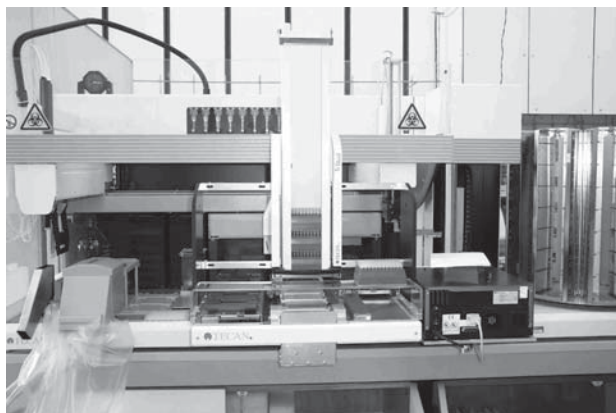


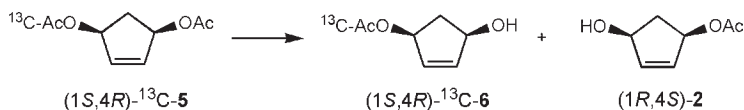
Fig. 1. Pipet robot *Genesis* (Tecan GmbH, Maennedorf, Switzerland) with integrated carousel (right) (2).

2.2. Data Processing

Data processing is possible by the use of the Openlynx Browser (Micromass). The overall process occurs continuously and makes possible the analysis of up to 10,000 samples per day, provided the eight-channel multiplexed sprayer system (e.g., from Micromass) is used (2b). It is also possible to use 384-format microtiter plates.

3. Assay Based on NMR Spectroscopy (4)

In order to illustrate the use of the NMR-based high-throughput *ee*-screen (4), the lipase-catalyzed hydrolytic desymmetrization of the *meso*-compound **1** is again considered (see Subheading 2). In this case the *pseudo-meso*-compound (1*S*,4*R*)-¹³C-**5** needs to be prepared and used in the enzymatic reaction, because the two products of desymmetrization are *pseudo*-enantiomers, (1*S*,4*R*)-¹³C-**6** and (1*R*,4*S*)-**2**, easily distinguished by NMR spectroscopy (Chapter 26).



3.1. Detailed Description of the High-Throughput NMR-Assay

After carrying out enzymatic reactions in the wells of microtiter plates (96-format) in water (in the present case lipase-catalyzed hydrolytic reaction of (1*S*,4*R*)-¹³C-**5**, a standard automatic extraction step follows (see Note 3).

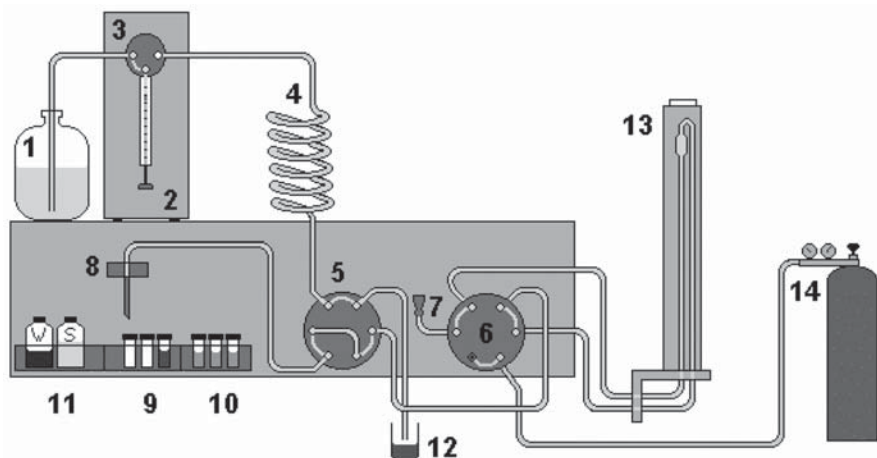


Fig. 2. Schematic representation of the BEST™ system (Bruker Biospin GmbH); (4). (1) Bottle with transporting liquid; (2) Dilutor 402 single syringe (5 mL) with 1100- μ L tube; (3) Dilutor 402 3-way valve; (4) Sample loop (250–500 μ L); (5) 6-way valve (standard version) loading sample; (6) 6-way valve (standard version) injecting sample; (7) Injection port; (8) XYZ needle; (9) Rack for sample vials; (10) Rack for recovering vials; (11) Rack for washing fluids and waste bottle (3 glass bottles); (12) External waste bottle; (13) Flow probe with inner lock container; (14) Inert gas pressure bottle for drying process.

Depending upon the particular substrate to be assayed and upon the type of solvent used, it may be necessary to remove the solvent. However, this is often not necessary. In the case of enzymatic reactions in organic medium, solvent extraction is not required. For NMR analysis, such solvents as CDCl_3 , D_6 -DMSO, or D_2O are used. A minimum of about 6 μmol of substrate/product per mL of solvent is needed. In the present case, chloroform is chosen as the solvent. Although the flow-through cell system does not need too much solvent (about 1 L in 24 h), the solvents can be mixed with the undeuterated form in 1:9 ratios in order to reduce costs (4). In order to speed up the NMR measurements, not the whole spectrum of the compound mixture is recorded, but only specific parts of it, e.g., methyl groups.

Various commercially available flow-through NMR cells can be used, e.g., the BEST™-NMR system (Bruker Biospin GmbH) as described here or the VAST™ NMR system (Varian). The schematic description is shown in Fig. 2. In addition to the flow-through cell and the NMR spectrometer (300 MHz), the system requires an autosampler, e.g., a Gilson 215 Autosampler (4).

The lipase-catalyzed (*Bacillus subtilis*) hydrolytic desymmetrization of the *pseudo-meso*-compound (1*S*,4*R*)-¹³C-**5** in water is carried out in the wells of microtiter plates (e.g., 96-format), followed by extraction (pipet robot) using 300 μ L of CDCl₃. For storage, the resulting organic layers are placed in the wells of microtiter plates (e.g., 96-format). The samples are then transferred to the autosampler of the BEST™-NMR system and analyzed using the high-speed mode as described below. The samples are taken by the movable needle and transferred into the first valve system. At the same time washing solution (CDCl₃) is introduced by the Dilutor 402 into the six port selection valve. Injection occurs via the injection port, whereby the washing solution is first fed into the second six port selection valve followed by the sample to be measured. The washing solution is then transferred rapidly via tubings into the flow-through cell by a hydraulic impulse. Immediately thereafter the sample follows which is separated from the washing solution by a small air gap. The washing solution is pumped through the flow-through cell, and once the sample has entered it, pumping is stopped and the NMR measurement is automatically initiated (maximum of four scans). During this time the washing solution is stored behind the cell (**4**).

Since the same solvent is used for all samples, NMR locking and shimming is principally necessary only once at the beginning of the process. However, since the shim may not be constant, locking and shimming should be repeated after about every 10th sample. Following the NMR measurement, the sample and the washing solution are flushed out of the system by automatic pumping in the reverse direction. During the NMR measurement of one sample the next one is prepared by the autosampler (e.g., Gilson 215 Autosampler). About 1400 samples can be handled per day. The analysis of the spectral data occurs with the aid of appropriate software, e.g., Software AMIX™ (Bruker Biospin GmbH). For this purpose the region of the spectrum to be integrated needs to be defined.

The precision in the *ee*-values amounts to $\pm 2\%$ as checked by independent GC analysis. In the case of another version of the high-throughput *ee*-assay based on traditional derivatization using chiral reagents such as Mosher's acid chloride (**Chapter 26**), the same equipment and software can be used. Again, about 1400 samples can be handled per day, precision in the *ee*-value in these cases being $\pm 5\%$ (**4**). Thus, these two NMR-based *ee*-screening systems are practical, precise, and rather general (see **Note 4**).

3.2. Data Processing

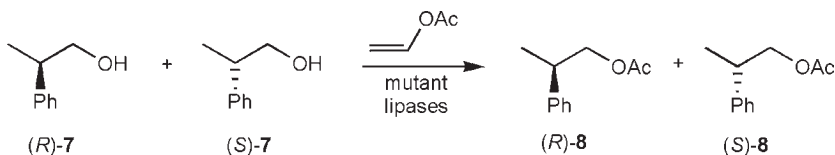
The individual data concerning the NMR peaks integrated can then be transferred onto Excel™ spreadsheets. With the help of a macro the *ee*- or *E*-values are readily tabulated (**4**).

MTP 1	1	2	3	4	5	6	7	8	9	10	11	12														
A	15	4	15	3	33	15	0	-	0	-	27	11	28	12	26	11	8	-2	30	12	0	-	0	-	c	ee
	1.6		1.4		2.1		-		-		2.0		2.1		2.1		0.7		1.9		-		-		E	
B	0	-	15	5	16	4	0	-	0	-	21	8	26	10	0	-	18	4	12	2	21	5	16	0	c	ee
	-		1.8		1.5		-		-		1.9		1.9		-		1.6		1.3		1.6		1.5		E	
C	12	3	0	-	0	-	20	7	0	-	28	13	20	5	0	-	22	8	0	-	0	-	20	6	c	ee
	1.5		-		-		1.8		-		2.2		1.5		-		2.0		-		-		1.7		E	
D	29	13	27	11	8	-4	19	7	25	10	30	13	16	4	28	12	0	-	29	13	6	-2	26	11	c	ee
	2.2		2.0		0.4		2.0		2.1		2.1		1.5		2.2		-		2.2		0.6		2.1		E	
E	12	2	29	13	0	-	35	18	22	8	0	-	19	5	27	13	0	-	6	-2	24	10	17	5	c	ee
	1.5		2.2		-		2.4		1.9		-		1.6		2.4		-		0.5		2.2		1.8		E	
F	0	-	23	9	27	12	12	1	22	9	23	9	15	4	13	2	23	9	18	6	23	8	12	2	c	ee
	-		2.1		2.2		1.1		2.1		2.0		1.7		1.4		2.0		1.9		1.9		1.4		E	
G	27	10	0	-	26	11	9	0	0	-	0	-	29	13	12	1	22	7	0	-	0	-	5	-2	c	ee
	2.0		-		2.2		1.1		-		-		2.2		1.2		1.8		-		-		0.5		E	
H	37	21	0	-	17	5	18	6	31	15	22	8	0	-	10	0	0	-	0	-	13	3	19	6	c	ee
	2.5		-		1.7		1.9		2.3		1.9		-		1.1		-		-		1.5		1.8		E	

Fig. 3. Excel™ spreadsheet of GC data in microtiter-format showing values for %conversion (c), %ee and selectivity factor (*E*) for mutant lipases catalyzing the hydrolytic kinetic resolution of alcohol **7** (**5**).

4. Assay Based on Gas Chromatography (**5**)

The special adaptation of gas chromatography for relatively rapid *ee*-determination is described in **Chapter 26** (**5**). Its use is illustrated here, specifically in the directed evolution of lipase variants from *Pseudomonas aeruginosa* employed in the kinetic resolution of (*R*)- and (*S*)-2-phenyl-1-propanol **7** with formation of the acetates (*R*)- and (*S*)-**8** (**5**).



As delineated in **Chapter 26**, the optimized GC unit is comprised of two instruments, a single sampler, and a common data processing system. The samples are automatically injected (see **Note 5**). The data are then visualized in microtiter plate format using Microsoft Excel, showing for each mutant %conversion, %*ee*, and the selectivity factor *E* for the lipase-catalyzed reaction of substrate **7** (**5**) (see **Fig. 3**).

5. Notes

1. This will vary, depending upon the particular substrate and enzyme. In the present case, typical for lipase-catalyzed desymmetrization, the reaction solutions are composed of 125 μL phosphate buffer (10 mM, pH 7.5), 50 μL supernatant and 25 μL substrate ([1*S*,4*R*]-D₃-**3**) solution (0.1 M in dimethylsulfoxide). The reactions are allowed to take place in the wells of 96-format microtiter plates at room temperature in an incubator while shaking for 24 h.
2. In the present case, ethyl acetate was used in the extraction step (200 μL reaction solution + 200 μL solvent). The samples need to be diluted prior to ESI-MS measurements using standard solution: methanol/10 mM NaOAc, 4:1 plus undeuterated *meso*-1,4-diacetoxy-cyclopentene **1** as an internal standard necessary for the determination of conversion.
3. In this particular case, the same extraction procedure is used as in the case of the MS-based system (**Subheading 2; Note 2**), except that no dilution is undertaken.
4. A second generation version of this NMR-based *ee*-assay is in the process of being optimized based on Chemical Shift Imaging (CSI) (**6**) using bundles of capillaries (Reetz, M. T., Tielmann, P., Eipper, A., in cooperation with Ross, A. at Hoffmann-La Roche, unpublished results). This enables up to 10,000 *ee*-determinations per day.
5. Following reaction in the wells of a 96-format microtiter plate (glass!) in an incubator for 24 h, centrifugation is performed. Using a pipet robot, about 15 μL of the reaction solutions are placed in another glass microtiter plate together with 170 μL of toluene prior to automatic GC measurements.

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Directed Evolution of the Substrate Specificities of a Site-Specific Recombinase and an Aminoacyl-tRNA Synthetase Using Fluorescence-Activated Cell Sorting (FACS)

Stephen W. Santoro and Peter G. Schultz

1. Introduction

A variety of *in vivo* selection and screening methods have been developed for the directed evolution of protein function. Typically, *in vivo* selection strategies involve the identification of new binding or catalytic functions based on their ability to confer a selective growth advantage on the host cell (usually *Escherichia coli*). *In vivo* screening approaches differ from selections in that screening involves the detection of a desired activity on the basis of its ability to produce an identifiable signal in an activity assay.

For the evolution of enzyme substrate specificity, selection and screening approaches each offer advantages and limitations. Altering the specificity of an enzyme to selectively utilize a new substrate usually requires a “double-sieve” strategy, such that activity with the new substrate causes cell survival, while activity with the old substrate causes cell death. Since it is not always easy to link an enzymatic activity to cell survival *and* death, this requirement limits the generality of such approaches. In contrast, screening approaches require only that an enzymatic activity be linkable to a signal that can be assayed. Screening systems are readily adaptable for use as double-sieves: positive and negative screening identifies enzyme variants active in the presence and absence of a substrate, respectively. Moreover, screening stringency can often be varied more readily than selection stringency. Thus, *in vivo* screening approaches offer the advantage of versatility for evolving the substrate specificity of an enzyme.

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On the other hand, selection approaches offer the advantage that the time required to carry out a cycle of selection does not typically scale with the size of the starting library. In contrast, the time required to carry out a cycle of screening increases with the size of the library being screened, which can make screening very large libraries impractical. High-throughput methods can be used to reduce the time requirements for screening large libraries. One such method, fluorescence activated cell sorting (FACS), can be used to rapidly screen individual bacterial cells containing protein variants (1,2). Screening can be carried out at a rate of about 10^8 cells per hour, sufficient to cover the size of the largest protein libraries that can currently be constructed in *E. coli*. The primary requirement for using FACS to evolve a desired enzymatic activity is that it be possible to link the activity to the production of a fluorescence signal.

Here, the use of FACS in the directed evolution of substrate specificity is presented for two different enzymes: Cre, a site-specific recombinase from bacteriophage P1 (3), and MjYRS, the tyrosyl-tRNA synthetase from *Methanococcus Jannaschii* (4). For both enzymes, a switch in substrate specificity (as opposed to a broadening of specificity) requires a double-sieve strategy. Positive selection pressure favors enzyme variants that recognize the new substrate, while negative pressure favors variants that cannot recognize the original substrate. For recombinase evolution, a method involving a combination of positive and negative screening is presented. For aminoacyl-tRNA synthetase evolution, a method involving positive selection and negative screening is presented.

2. Materials

2.1. Bacterial Strains, Genetic Constructs, and Oligonucleotide Primers

2.1.1. Site-Specific Recombinase Evolution

1. *E. coli* DH10B-DE3 strain (see Note 1), prepared using a λ -DE3 lysogenization kit (Novagen) with DH10B cells (Life technologies).
2. Plasmid pS (see Fig. 1A), designed and constructed as previously described (3) to allow the fluorimetric and cytometric assessment of site-specific recombinase activity in *E. coli* (see Notes 2 and 3).
3. Oligonucleotide PCR primers for construction of plasmid pS variants (see Table 1).
4. Plasmid pR (see Fig. 1B), designed and constructed as previously described (3) as a vector for expression of site-specific recombinase gene variants.
5. PCR fragment libraries of Cre gene variants, constructed as previously described (3) using a targeted mutagenesis strategy (see Note 4).
6. Oligonucleotide PCR primers for amplification of Cre gene variant libraries (see Table 1).

Table 1
Oligonucleotide Primers for PCR Amplification

Construction of Plasmid pS Variants	
Region #1	
pS#1Fwt	
5'-CTAATAGAAATTCAAATAACTTCGTATAGCATACATTATACGAAGTTATAACTTTAAG	
<i>EcoRI</i>	<i>loxP 1</i>
AAGGAGATATAC	
pS#1Rwt	
5'-TTGATCACCGTGCACCTTCTCATGTTTGACAGCTTATC	
<i>ApaLI</i>	
Region #2	
pS#2Fwt	
5'-GCAATCACCGTGCACCAAAAAACCCCTCAAGACCC	
<i>ApaLI</i>	
pS#2Rwt	
5'-TTCCCTCTAGAAAATAACTTCGTATAGCATACATTATACGAAGTTATAACTTTAAG	
<i>XbaI</i>	<i>loxP 2</i>
AAGGAGATATAC	
Amplification of Cre Gene Variant Libraries	
pR-CreN	
5'-GATCTTGGGCGTACGTAACAGGAGGAATTAACCATG	
<i>BstWI</i>	
pR-CreC	
5'-ACTGATATCCAGGTCGTTGGTCTAATCGCCATCTTCCAG	
<i>BstXI</i>	
Amplification of <i>Mj</i> YRS Gene Variant Libraries	
pBK- <i>Mj</i> YRSN	
5'-GAGGAATCCCATATGACGAATTTGAAATGATAAAGAG	
<i>NdeI</i>	
pBK-MJYRSC	
5'-CGTTTGAAACTGCAGTTATAATCTCTTCTAATTGG	
<i>PstI</i>	

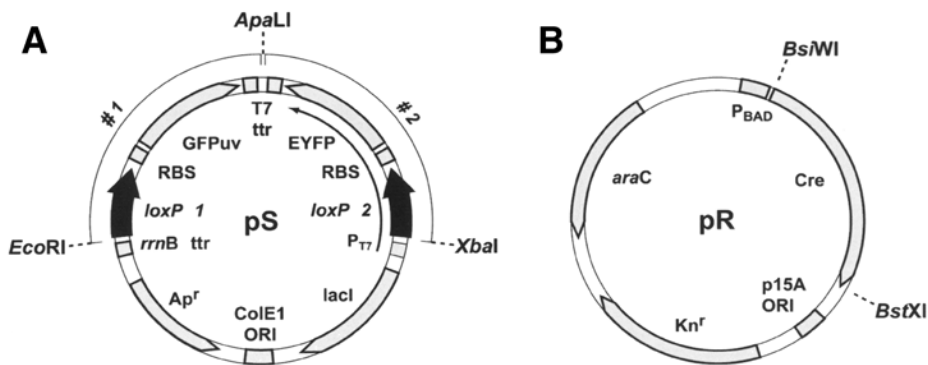


Fig. 1. Site-specific recombinase plasmid system for genetic screening (3). (A) plasmid pS contains, in its starting arrangement, two *loxP* sites (*lox* 1 and *lox* 2), the EYFP gene under control of the T7/lac promoter (P_{T7}), the GFPuv gene downstream of a transcription termination region (*rrnB* ttr), the *lacI* gene, which allows IPTG-inducible expression from P_{T7} , an ampicillin selectable marker (Ap^r), and a ColE1 origin of replication. The EYFP and GFPuv genes are flanked by ribosome binding sites (RBS) and T7 RNA polymerase transcription termination regions (T7 ttr). The pS-M plasmids are constructed as described under **Subheading 3.1.1.**, using the restriction sites shown, and have matching *loxM* variant sites at *lox* 1 and *lox* 2. Recombination at the *lox* 1 and *lox* 2 sites within pS and pS-M plasmids results in rearrangement of the plasmid and reorientation of P_{T7} upstream of the GFPuv gene. Recombination is reversible. (B) Plasmid pR contains the Cre gene under control of the *ara* promoter (P_{BAD}), the *araC* gene, which allows modulated expression from P_{BAD} , a kanamycin selectable marker (Kn^r), and a p15A origin of replication. The pS-C library plasmids are constructed as outlined under **Subheading 3.1.4.** using the restriction sites shown.

2.1.2. Aminoacyl-tRNA Synthetase Evolution

1. *E. coli* strain DH10B (Life Technologies).
2. Plasmid pREP/YC-JYCUA (see **Fig. 2A**), designed and constructed as previously described (4) as a reporter for activity of orthogonal aminoacyl-tRNA synthetase variants in *E. coli* (see **Notes 3** and **5**).
3. Plasmid pBK-JYA6 (see **Fig. 2B**), designed and constructed as previously described (5) as a vector for expression of aminoacyl-tRNA synthetase gene variants.
4. PCR fragment libraries of *M. jannaschii* tyrosyl-tRNA synthetase (*MjYRS*) gene variants were constructed as previously described (5) using a targeted mutagenesis strategy (see **Note 4**).
5. Oligonucleotide PCR primers for amplification of *MjYRS* gene variant libraries (see **Table 1**).

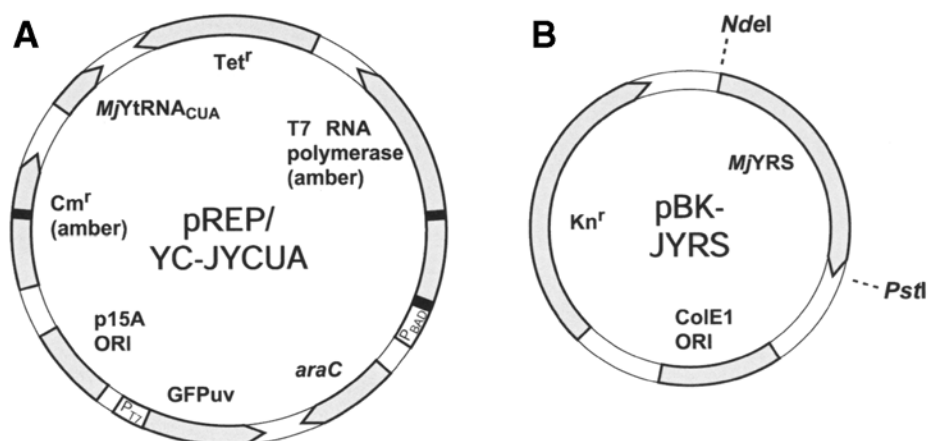


Fig. 2. Aminoacyl-tRNA synthetase plasmid system for selection and screening (4). (A) Plasmid pREP/YC-JYCUA. The amplifiable fluorescence reporter is used for FACS-based screening: T7 RNA polymerase, the gene for which is under control of the *ara* promoter (P_{BAD}), is produced upon suppression of the amber stop codons (black) and drives expression of the GFPuv gene. The chloramphenicol reporter (Cm^r) is used for positive selection, conferring bacterial resistance to chloramphenicol upon suppression of the amber stop codon (black). Plasmid pREP/YC-JYCUA contains the *MjYtRNA_{CUA}* gene, which encodes an orthogonal amber suppressor tRNA^{Tyr} derived from *M. jannaschii* (5), a p15A origin of replication, and a tetracycline selectable marker (Tet^r). (B) Plasmid pBK-JYRS (5) contains the constitutively-expressed tyrosyl-tRNA synthetase gene from *M. jannaschii* (*MjYRS*), a kanamycin selectable marker (Kn^r), and the ColE1 origin of replication. The pBK library plasmids are constructed as outlined under **Subheading 3.2.2.** using the restriction sites shown.

2.2. Reporter Variant and Library Plasmid Construction

1. Restriction Enzymes.
2. Calf intestinal alkaline phosphatase (CIP).
3. Reaction components for PCR. A thermostable DNA polymerase, PCR buffer, and deoxynucleotide triphosphates (dNTPs) (see **Note 6**).
4. PCR purification kit.
5. Gel extraction kit.
6. T4 DNA ligase.
7. Electroporator and 0.2-cm electroporation cuvettes.
8. Maxiprep plasmid purification kit.
9. Agarose and agarose gel electrophoresis equipment.
10. Tris-acetate EDTA (TAE) buffer: 40 mM Tris-acetate, 1 mM EDTA, pH 8.3.
11. Ethidium bromide.

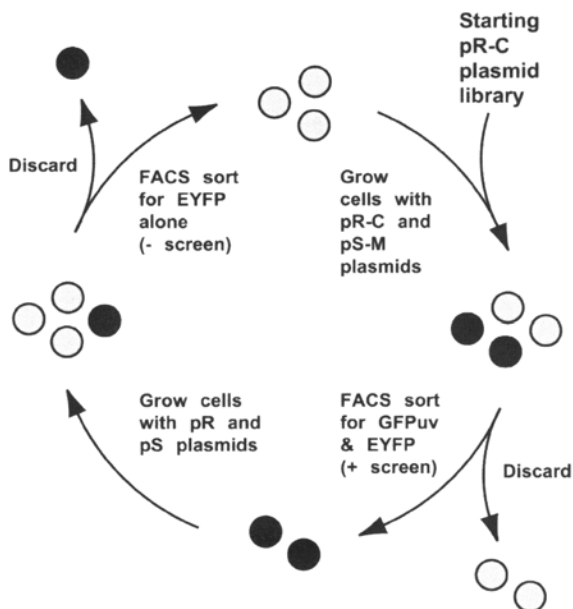


Fig. 3. Strategy for the evolution of a site specific recombinase using FACS-based screening. Screening may be done both positively, to identify recombinase variants that recombine a *loxM* variant site, and negatively, to identify recombinase variants that do not recombine the *loxP* site. Cells producing EYFP alone are in gray; cells producing both EYFP and GFPuv are in black.

2.3. General Reagents and Equipment

1. SOC media.
2. Luria-Bertoni (LB) media.
3. Glucose stock solution: 20% in water; sterile-filtered.
4. IPTG (isopropyl- β -D-thiogalactopyranoside): 1 mM in water; store at -20°C .
5. PBS (phosphate buffered saline): 10 mM phosphate, 0.14 M NaCl, 2.7 mM KCl, pH 7.4, at 25°C .
6. Miniprep plasmid purification kit.
7. Ampicillin stock solution: 100 mg/mL in water; should be stored at -20°C .
8. Kanamycin stock solution: 35 mg/mL in water; should be stored at -20°C .
9. Glycerol minimal media with leucine (GMML): 1% glycerol and 0.3 mM Leucine.
10. Tetracycline stock solution: 25 mg/mL in 75% EtOH; should be stored at -20°C .
11. Arabinose stock solution : 20% in water; sterile-filtered.
12. Unnatural amino acids stock solution: typically, 0.3 M in 0.3 M HCl or NaOH; should be stored at -20°C .
13. Glycerol: 10% in deionized water; sterile-filtered.
14. Fluorimeter and quartz cuvet.

3. Methods

3.1. Directed Evolution of a Site-Specific Recombinase

The following method describes the use of FACS to identify Crecombinase variants that efficiently recombine novel recombination sites but not the wild-type *loxP* site. The strategy uses a combination of positive screening to identify variants that recognize the novel recombination site, and negative screening to eliminate those variants that recognize both the novel site and *loxP* (Fig. 3, see Note 7).

3.1.1. Preparation of an *E. coli* pS-M Reporter Plasmid Variant Strain

This section outlines the production of competent DH10B-DE3 cells harboring a pS-M reporter plasmid variant that contains two novel sites, *loxM*. This strain is used along with FACS sorting to identify Cre variants within the library that can efficiently recombine the novel *loxM* site.

1. Restriction-digest ~3 μ g of plasmid pS with *EcoRI* and *XbaI* in the presence of CIP (see Note 8).
2. Purify the large (5.5 kbp) pS fragment by agarose gel electrophoresis followed by gel extraction (see Note 9). Quantify purified DNA by agarose gel electrophoresis.
3. PCR-amplify pS regions #1 and #2 (see Fig. 1A) with primers containing *loxP* variant sequences and the appropriate restriction sites (Table 1) (see Note 10).
4. Purify PCR products using a PCR purification kit.
5. Restriction-digest PCR fragments A and B using *EcoRI/ApaLI* or *ApaLI/XbaI*, respectively (see Note 8).
6. Purify digested PCR fragments by agarose gel electrophoresis followed by gel extraction (see Note 9). Quantify purified DNA by agarose gel electrophoresis.
7. Ligate 200 ng of the digested, purified pS fragment; 100 ng of the digested, purified PCR fragment #1; and 100 ng of the digested, purified PCR fragment #2 in a 5- μ L reaction (see Note 11).
8. Prepare electrocompetent *E. coli* DH10B-DE3 cells (see Note 12).
9. Transform 30 μ L of *E. coli* DH10B-DE3 electrocompetent cells with 2 μ L of the ligation reaction (see Note 13). Immediately add 200 μ L of SOC media and incubate the cells with gentle shaking (225 rpm) at 37°C for 1 h.
10. Plate recovered cells on LB agar containing 100 μ g/mL ampicillin; incubate at 37°C overnight.
11. Miniprep the DNA from eight individual clones and make a glycerol stock corresponding to each clone by mixing 750 μ L of culture with 250 μ L of 50% glycerol. Flash freeze the mixtures in dry ice and store them at -80°C.
12. Restriction-map the plasmid DNA corresponding to each individual clone. For each clone that maps correctly, analyze the sequences of regions #1 and #2 to verify that no errors have been introduced during plasmid construction.
13. From DH10B-DE3 (pS-M) glycerol stocks, prepare electrocompetent DH10B-DE3 (pS-M) cells (see Note 12).

3.1.2. Preparation of the *E. coli* pS Reporter Plasmid Strain

The following steps outline the production of electrocompetent DH10B-DE3 cells harboring the pS reporter plasmid, which contains two wild-type *loxP* sites. This strain will be used along with FACS sorting to remove from the library the variants of Cre that recombine the wild-type *loxP* site.

1. Prepare electrocompetent *E. coli* DH10B-DE3 cells (see **Note 12**).
2. Transform 25 μ L of *E. coli* DH10B-DE3 electrocompetent cells with 10 ng of plasmid pS (see **Note 13**). Immediately add 200 μ L of SOC media and incubate the cells with gentle shaking (225 rpm) at 37°C for 1 h.
3. Plate recovered cells on LB agar containing 100 μ g/mL ampicillin; incubate at 37°C overnight.
4. From a single colony, prepare electrocompetent DH10B-DE3 (pS) cells (see **Note 12**).

3.1.3. Functional Analysis of Reporter Strains

Before proceeding, it is important to test the DH10B-DE3 (pS) and (pS-M) reporter strains for proper function. This can be done by transforming each strain with plasmid pR, which expresses wild-type Cre, and analyzing the resulting pattern of fluorescence. Before the introduction of plasmid pR, both strains should produce only EYFP upon induction. Following transformation with pR, the pS strain should produce both EYFP and GFPuv upon induction, indicating that recombination has occurred (see **Fig. 4A**). Ideally, the pS-M/pR strain should produce EYFP and little or no GFPuv, indicating that wild-type Cre does not recombine the novel recombination site.

1. Transform 25 μ L each of DH10B-DE3 (pS) and (pS-M) with 10 ng of plasmid pR (see **Note 13**). Immediately add 200 μ L of SOC media and incubate the cells with gentle shaking (225 rpm) at 37°C for 1 h.
2. Plate recovered cells on LB agar containing 100 μ g/mL ampicillin and 35 μ g/mL kanamycin; incubate at 37°C overnight.
3. From single DH10B-DE3 (pS / pR) and (pS-M / pR) colonies, inoculate two 5-mL cultures containing LB, 100 μ g/mL ampicillin, 35 μ g/mL kanamycin, and 0.02% glucose. From glycerol stocks of DH10B-DE3 (pS) and (pS-M), inoculate two 5-mL cultures containing LB, 100 μ g/mL ampicillin, and 0.02% glucose (see **Note 14**). Allow the four cultures to grow to saturation with shaking (250 rpm) at 37°C (~12 h).
4. Pellet the cells by centrifugation at 5000g for 5 min. Decant the supernatant and resuspend the cells in 5 mL of LB containing 100 μ g/mL ampicillin and 100 nM IPTG (and 35 μ g/mL kanamycin for the pS / pR and pS-M / pR cultures) (see **Note 15**). Incubate the cultures with shaking (250 rpm) at 37°C for 4 h.
5. Pellet 200 μ L of each IPTG-induced cell culture by centrifugation at 10,000g for 1 min. Decant the supernatant. Resuspend the cells in 1 mL of PBS.

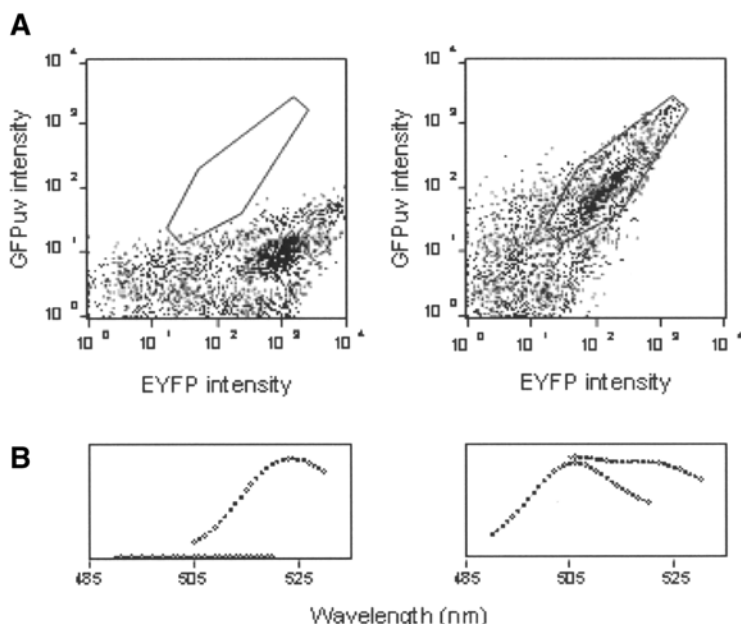


Fig. 4. Cre recombination in vivo can be followed by cytometry (**A**) or fluorometry (**B**) of *E. coli* cells. Cells containing pS alone (left) express only EYFP, whereas cells containing pR and pS (right) express roughly equal amounts of GFPuv and EYFP.

6. Transfer the suspended cells to a cuvet and analyze the cell population fluorimetrically. Determine the relative expression levels of GFPuv and EYFP by the relative emission intensities measured at 505 and 523 nm with excitation at 396 and 480 nm for GFPuv and EYFP, respectively (see **Fig. 4B**; see **Note 16**).

3.1.4. Preparation of a pS-M Reporter

Plasmid/pR-C Library Plasmid *E. coli* Strain

The following steps outline the construction of a plasmid library of Cre variants and their introduction into an *E. coli* pS-M reporter strain.

1. Use DNA oligonucleotide primers pR-CreN and pR-CreC (**Table 1**) to amplify Cre gene variant library fragments in four 100- μ L PCR reactions (see **Note 10**).
2. Purify DNA using a PCR purification kit.
3. Restriction-digest the purified PCR DNA using *Bsi*WI and *Bst*XI (see **Note 8**).
4. Purify digested PCR fragments by agarose gel electrophoresis followed by gel extraction (see **Note 9**). Quantify purified DNA by agarose gel electrophoresis.
5. Restriction-digest plasmid pR using *Bsi*WI and *Bst*XI (see **Notes 8** and **17**).

6. Purify digested vector by agarose gel electrophoresis followed by gel extraction (see **Note 9**). Quantify purified DNA by agarose gel electrophoresis.
7. Ligate vector and insert DNA in a molar ratio of 1 to 1.5, respectively, using at least 10 μg of vector in a 300- μL reaction for ~ 12 h at 16°C (see **Note 11**).
8. Following ligation, analyze a small amount of the reaction by agarose gel electrophoresis to verify that all of the starting material has been converted to larger products.
9. Purify the ligation products by extraction three times with 200 μL of phenol-chloroform and two times with 200 μL of chloroform, followed by ethanol precipitation. Redissolve the DNA pellet in 50 μL of water.
10. Carry out a pilot transformation of 25 μL of electrocompetent *E. coli* with 1 μL of the ligation product (see **Notes 13** and **18**). Plate three 10-fold serial dilutions of the transformed cells onto LB agar plates containing 35 $\mu\text{g}/\text{mL}$ kanamycin; incubate at 37°C overnight. Based on the number of colonies obtained, calculate the expected library size. Miniprep the plasmid DNA corresponding to 10–20 individual clones. Restriction map and sequence the plasmids to verify that a high percentage (ideally, greater than 90%) of the clones contain insert and that the distribution of mutations within the library is not excessively biased.
11. If results from the pilot transformation are acceptable, proceed with a large-scale transformation. Mix the purified ligation products with 500 μL of electrocompetent cells (do not dilute). Distribute 55 μL aliquots of the mixture into ten ice-cold 0.2-cm cuvettes and electroporate (see **Note 13**). Following each electroporation, immediately add 1 mL of SOC media. Transfer transformed cells to a 15-mL conical tube and allow them to recover with gentle shaking (225 rpm) at 37°C for 1 h.
12. Transfer recovered cells to 2 L of 2X YT media containing 35 $\mu\text{g}/\text{mL}$ kanamycin in a 4-L shaker flask. Immediately remove a 100- μL aliquot of the inoculated culture for use in estimating the number of independent transformants comprising the pR-C library (see **Note 19**).
13. Incubate the cells at 37°C overnight with shaking (250 rpm).
14. Maxiprep the pR-C plasmid DNA from 500 mL of the library culture. Redissolve the DNA in 200 μL of water.
15. Transform 200 μL of electrocompetent DH10B-DE3 (pS-M) cells with 5 μL of the maxiprep pR-C plasmid library DNA (~ 1 –2 μg) in four 0.2-cm cuvettes (see **Note 13**). Following each electroporation, immediately add 1 mL of SOC media. Transfer transformed cells to a 15-mL conical tube and allow them to recover with gentle shaking (225 rpm) at 37°C for 1 h.
16. Transfer recovered cells to 1 L of 2X YT media containing 100 $\mu\text{g}/\text{mL}$ ampicillin, 35 $\mu\text{g}/\text{mL}$ kanamycin, and 0.02% glucose in a 2-L shaker flask. Immediately remove a 100- μL aliquot of the inoculated culture for use in estimating the number of independent transformants (see **Note 19**). This number should be at least as large as the number of independent transformants obtained following library construction.
17. Incubate the cells at 37°C overnight with shaking (250 rpm).

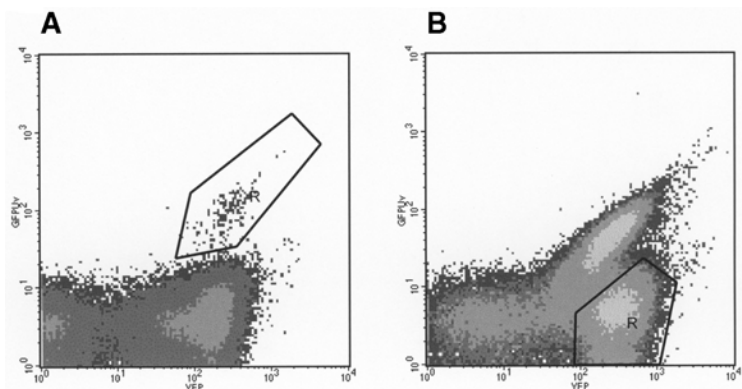


Fig. 5. FACS-based screening of Cre variants. **(A)** A typical initial cycle of positive screening. The boxed events are collected, corresponding to cells producing both EYFP and GFPuv. These cells contain Cre variants that can recombine the novel *loxM* site. **(B)** A typical initial cycle of negative screening. The boxed events are collected, corresponding to cells producing only EYFP. These cells contain Cre variants that cannot recombine the wild-type *loxP* site.

3.1.5. Screening of a Cre Variant Library Using FACS

An alternating series of positive and negative screens are carried out in order to screen the pR-C plasmid library for Cre variants that efficiently recombine a novel recombination site, *loxM*, but not the wild-type site, *loxP* (see Fig. 3). The pR-C plasmid library is shuttled between two strains. Introduction of the library into strain DH10B-DE3 (pS-M) allows positive screening (for recombination of *loxM*), while introduction of the library into strain DH10B-DE3 (pS) allows negative screening (against recombination of *loxP*). A total of five screens are carried out: three positive and two negative. The following is the general procedure for screening a library of Cre variants by FACS.

1. Pellet a 5-mL aliquot of a saturated culture of the pS-M / pR-C (for positive screening) or pS / pR-C (for negative screening) library strain by centrifugation at 5000g for 5 min. Decant the supernatant and resuspend the cells in 5 mL of LB containing 100 μ g/mL ampicillin, 35 μ g/mL kanamycin, and 100 nM IPTG. Incubate the culture with shaking (250 rpm) at 37°C for 4 h.
2. Pellet 200 μ L of the IPTG-induced cell culture by centrifugation at 10,000g for 1 min. Decant the supernatant. Resuspend cells in 1 mL of PBS.
3. FACS sort $\sim 10^8$ (for the initial positive screen) or $\sim 10^7$ (for the subsequent screens) cells. For positive screening, collect cells that have produced both GFPuv and EYFP (see Notes 20–22, Fig. 5A). For negative screening, collect cells that have produced only EYFP (see Fig. 5B).

4. Dilute collected cells into 100 mL of LB containing 100 $\mu\text{g/mL}$ ampicillin and 35 $\mu\text{g/mL}$ kanamycin and allow the cultures to grow to saturation at 37°C with shaking (250 rpm).
5. Miniprep the plasmid DNA from 5 mL of the saturated culture. Restriction-digest 25 μL of the miniprep DNA with *Pst*I and *Sac*I (in a 50- μL reaction containing NEB 1 buffer) to destroy the pS or pS-M plasmid (see **Notes 8** and **23**).
6. Transform 100 μL of electrocompetent DH10B-DE3 (pS) cells (if the next screening cycle will be negative) or DH10B-DE3 (pS-M) cells (if the next screening cycle will be positive) with 200 ng of digested DNA in two 0.2-cm cuvettes (see **Note 13**). Following each electroporation, immediately add 1 mL of SOC media. Transfer transformed cells to a 15-mL conical tube and allow them to recover with gentle shaking (225 rpm) at 37°C for 1 h. Transfer recovered cells to 500 mL of 2X YT media containing 100 $\mu\text{g/mL}$ ampicillin and 35 $\mu\text{g/mL}$ kanamycin in a 1-L shaker flask.
7. Repeat **steps 1–6** until a total three of cycles positive and two cycles negative screening have been carried out (see **Note 24**). After completing **step 5** of the final cycle of screening, proceed to **Subheading 3.1.6**.

3.1.6. *In Vivo Analysis of the Activity and Substrate Specificity of Evolved Cre Variants*

The following steps outline the procedure by which the *in vivo* activity of individual recombinase selectants may be characterized. The kinetic parameters of evolved Cre variants may be characterized using *in vitro* assays beyond the scope of this chapter.

1. Transform 25 μL of electrocompetent DH10B-DE3 (pS-M) cells with 10 ng the digested plasmid DNA prepared after the third cycle of positive FACS screening (see **Note 13**). Following recovery in SOC media, pellet the cells by centrifugation at 10,000g for 1 min and resuspend them in 100 μL of LB media. Plate a series of 1:10 dilutions of the transformed cells on LB agar plates containing 100 $\mu\text{g/mL}$ ampicillin and 35 $\mu\text{g/mL}$ kanamycin (see **Note 25**). Incubate the plates at 37°C for 48 h (see **Note 26**).
2. Examine the plates under a handheld long-wavelength ultraviolet light. Use a sterile toothpick to select 10–20 colonies that produce a homogeneous pattern of fluorescence (see **Note 27**) and inoculate a sterile 5-mL LB culture containing 100 $\mu\text{g/mL}$ ampicillin and 35 $\mu\text{g/mL}$ kanamycin. Allow the cultures to grow to saturation at 37°C with shaking (250 rpm).
3. Miniprep the plasmid DNA from the 5-mL saturated culture. Digest 25 μL of the miniprep DNA with *Pst*I and *Sac*I (in a 50- μL reaction containing NEB 1) buffer to destroy the pS-M plasmid (see **Notes 8** and **23**).
4. Transform 25 μL of electrocompetent DH10B-DE3 (pS) and (pS-M) cells with 10 ng of each digested plasmid preparation (see **Note 13**). Following each electroporation, immediately add 1 mL of SOC media, transfer cells to a 2-mL tube, and allow them to recover with gentle shaking (225 rpm) at 37°C for 1 h.

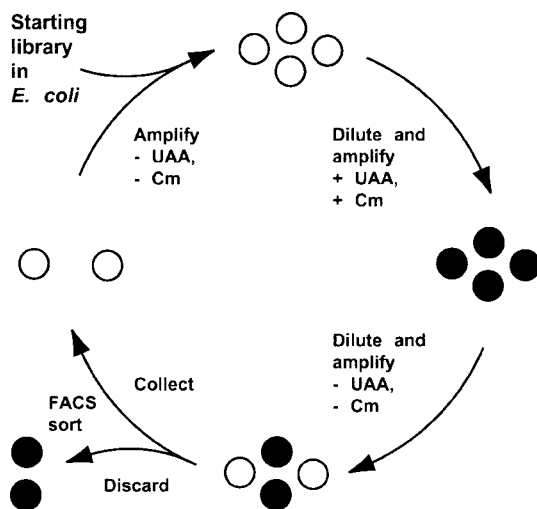


Fig. 6. Strategy for the evolution of an aminoacyl-tRNA synthetase using positive selection and negative FACS-based screening. Fluorescent and non-fluorescent cells are shown in black and white, respectively. ‘UAA’ refers to unnatural amino acid.

Transfer 100 μL of each sample of recovered cells to 5-mL cultures containing LB, 100 $\mu\text{g}/\text{mL}$ ampicillin, 35 $\mu\text{g}/\text{mL}$ kanamycin, and 0.02% glucose (*see Note 14*). Grow the cultures to saturation with shaking (250 rpm) at 37°C (~12 h).

5. Pellet the cells by centrifugation at 5000g for 5 min. Decant the supernatant and resuspend the cells in 5 mL of LB containing 100 $\mu\text{g}/\text{mL}$ ampicillin, (35 $\mu\text{g}/\text{mL}$ kanamycin for the pS/pR and pS-M/pR cultures) and 100 nM IPTG (*see Note 15*). Incubate the cultures with shaking (250 rpm) at 37°C for 4 h.
6. Pellet 200 μL of each IPTG-induced cell culture by centrifugation at 10,000g for 1 min. Decant the supernatant. Resuspend cells in 1 mL of PBS.
7. Transfer the resuspended cells to a cuvet and analyze the cell population fluorimetrically. Determine the relative expression levels of GFPuv and EYFP by the relative emission intensities measured at 505 and 523 nm with excitation at 396 and 480 nm for GFPuv and EYFP, respectively (*see Note 16*).

3.2. Directed Evolution of a Tyrosyl-tRNA Synthetase

The following method describes the use of a selection/screening system to identify tyrosyl-tRNA synthetase variants that efficiently and specifically charge an orthogonal tRNA with an unnatural amino acid. The strategy uses a chloramphenicol-based selection to positively enrich variants that recognize the novel amino acid and negative FACS-based screen to eliminate those variants that accept one of the natural amino acids (**Fig. 6**, *see Note 28*).

3.2.1. Preparation of the pREP/YC-JYCUA Reporter Plasmid *E. coli* Strain

The following steps outline the production of electrocompetent DH10B-DE3 cells harboring the pREP/YC-JYCUA reporter plasmid (see **Fig. 2A**).

1. Transform 25 μL of electrocompetent *E. coli* DH10B cells with 10 ng of plasmid pREP/YC-JYCUA (see **Note 13**). Immediately add 200 μL of SOC media and allow the cells to recover with gentle shaking (225 rpm) at 37°C for 1 h.
2. Plate recovered cells on LB agar containing 25 $\mu\text{g/mL}$ tetracycline; incubate at 37°C overnight.
3. From a single colony, prepare electrocompetent DH10B (pREP/YC-JYCUA) cells (see **Note 12**).

3.2.2. Preparation of a pREP/YC-JYCUA Reporter Plasmid/pBK Library Plasmid Strain

This subsection outlines the construction of a plasmid library of *MjYRS* variants and its introduction into the *E. coli* pREP/YC-JYCUA reporter strain.

1. Use DNA oligonucleotide primers pBK-MjYRSN and pBK-MjYRSC (see **Table 1**) to PCR-amplify *MjYRS* gene variant library fragments in four 100- μL PCR reactions (see **Note 10**).
2. Purify the DNA using a PCR DNA purification kit.
3. Restriction-digest purified PCR DNA using *NdeI* and *PstI* (see **Note 8**).
4. Purify the digested PCR fragments by agarose gel electrophoresis followed by gel extraction (see **Note 9**). Quantify purified DNA by agarose gel electrophoresis.
5. Restriction-digest the vector pBK-JYA6 using *NdeI* and *PstI* (see **Notes 8** and **17**).
6. Purify the digested vector by standard agarose gel electrophoresis followed by gel extraction (see **Note 9**). Quantify purified DNA by agarose gel electrophoresis.
7. Ligate the vector and insert DNA in a molar ratio of 1 to 1.5, respectively, using at least 10 μg of vector in a 300- μL reaction for ~12 h at 16°C (see **Note 11**).
8. Following ligation, analyze a small amount of the reaction by agarose gel electrophoresis to verify that all of the starting material has been converted to larger products.
9. Purify the ligation products by extraction three times with 200 μL of phenol-chloroform and two times with 200 μL of chloroform, followed by ethanol precipitation. Redissolve the DNA pellet in 50 μL of water.
10. Carry out a pilot transformation of 25 μL of electrocompetent *E. coli* with 1 μL of the ligation product (see **Notes 13** and **18**). Plate three 10-fold serial dilutions of the transformed cells onto LB agar plates containing 35 $\mu\text{g/mL}$ kanamycin and incubate at 37°C overnight. Based on the number of colonies obtained, calculate the expected library size. Miniprep the plasmid DNA corresponding to 10–20 individual clones. Restriction map and sequence the plasmids to verify that a high percentage (ideally, greater than 90%) of the clones contain insert and that the distribution of mutations within the library is not excessively biased.

11. If results from the pilot transformation are acceptable, proceed with a large-scale transformation. Mix the purified ligation products with 500 μL of electrocompetent cells (do not dilute). Distribute 55 μL aliquots of the mixture into ten cold 0.2-cm cuvetts and electroporate (*see Note 13*). Following each electroporation, immediately add 1 mL of SOC media. Transfer transformed cells to a 15-mL conical tube and allow them to recover with gentle shaking (225 rpm) at 37°C for 1 h.
12. Transfer recovered cells to 2 L of 2X YT media containing 35 $\mu\text{g}/\text{mL}$ kanamycin in a 4-L shaker flask. Immediately remove a 100- μL aliquot of the inoculated culture for use in estimating the number of independent transformants comprising the pR-C library (*see Note 19*).
13. Incubate the cells at 37°C overnight with shaking (250 rpm).
14. Maxiprep the pBK plasmid DNA from 500 mL of the library culture. Redissolve the DNA in 200 μL of water.
15. Transform 200 μL of electrocompetent DH10B (pREP/YC-JYCUA) cells with 5 μL of the maxiprepmed pBK supercoiled plasmid library DNA ($\sim 1\text{--}2\text{ }\mu\text{g}$) in four 0.2-cm cuvetts (*see Note 13*). Following each electroporation, immediately add 1 mL of SOC media. Transfer transformed cells to a 15-mL conical tube and allow them to recover with gentle shaking (225 rpm) at 37°C for 1 h.
16. Transfer recovered cells to 1 L of 2X YT media containing 25 $\mu\text{g}/\text{mL}$ tetracycline and 35 $\mu\text{g}/\text{mL}$ kanamycin in a 2-L shaker flask. Immediately remove a 100- μL aliquot of the inoculated culture for use in estimating the number of independent transformants (*see Note 19*). This number should be at least as large as the number of independent transformants obtained following library construction.
17. Incubate the cells at 37°C overnight with shaking (250 rpm).

3.2.3. Selection/Screening of an Aminoacyl-tRNA Synthetase Variant Library

A combination of selection and screening is used to identify MjYRS variants that have altered specificity with respect to the amino acid substrate (*see Fig. 6*). A chloramphenicol-based selection is used to enrich variants that are active in the presence of an unnatural amino acid. A negative FACS-based screen is used to eliminate variants that are active in the absence of the unnatural amino acid. The following is a general procedure for using selection and FACS-based screening to direct the evolution of an aminoacyl-tRNA synthetase.

1. Pellet 2 mL of *E. coli* (pREP/YC-JYCUA, pBK-lib) cells by centrifugation at 10,000g for 1 min. Discard the supernatant and resuspend the cells in 1 mL of GMML media.
2. To begin the first cycle of positive selection, use the resuspended cells to inoculate 500 mL of GMML containing 25 $\mu\text{g}/\text{mL}$ tetracycline, 35 $\mu\text{g}/\text{mL}$ kanamycin, and 1 mM unnatural amino acid. Incubate the cells for 3 h at 37°C with shaking at 250 rpm. Add chloramphenicol to a final concentration of 75 $\mu\text{g}/\text{mL}$ (*see Note 29*) and continue incubation until the cells reach stationary phase (~ 48 h; *see Note 30*).

3. To begin the second cycle of positive selection, use a 500- μ L aliquot of saturated culture from the first selection to inoculate a 100-mL GMML culture containing 25 μ g/mL tetracycline, 35 μ g/mL kanamycin, 75 μ g/mL chloramphenicol, and 1 mM unnatural amino acid. Incubate the cells at 37°C with shaking at 250 rpm until the cells reach stationary phase (~24–36 h; *see Note 30*).
4. To prepare for FACS-based negative screening, pellet a 100- μ L aliquot of cells from the second cycle of positive selection by centrifugation at 10,000g for 1 min. Discard the supernatant and resuspend the cells in 100 μ L of GMML media. Use the resuspended cells to inoculate a 25-mL GMML culture containing 25 μ g/mL tetracycline, 35 μ g/mL kanamycin, and 0.002% arabinose (*see Note 31*). Incubate the cells at 37°C with shaking at 250 rpm until the cells reach stationary phase (~24–36 h; *see Note 30*).
5. Pellet a 1-mL aliquot of the arabinose-induced cells by centrifugation at 10,000g for 1 min. Resuspend the cells in 3 mL of phosphate-buffered saline (PBS). Using FACS, sort $\sim 10^7$ – 10^8 cells for the lack of fluorescence (*see Fig. 7, see Notes 20 and 21*).
6. Dilute collected cells into 25 mL of LB media containing 25 μ g/mL tetracycline and 35 μ g/mL kanamycin and allow the cultures to grow to saturation at 37°C with shaking (250 rpm). Pellet a 100- μ L aliquot of the amplified cells by centrifugation at 10,000g for 1 min. Resuspend the cells in 100 μ L of GMML.
7. To begin the third cycle of positive selection, use the resuspended cells to inoculate 25 mL of GMML containing 25 μ g/mL tetracycline, 35 μ g/mL kanamycin, and 1 mM unnatural amino acid. Incubate the cells for 3 h at 37°C with shaking at 250 rpm. Add chloramphenicol to a final concentration of 75 μ g/mL (*see Note 29*) and continue incubation until the cells reach stationary phase (~48 h; *see Note 30*).

3.2.4. *In Vivo Analysis of the Activity and Substrate Specificity of Evolved MjYRS Variants*

The following steps outline the procedure by which the *in vivo* activity and specificity of individual synthetase selectants may be characterized fluorimetrically.

1. Dilute cells from the third cycle of positive selection into GMML to a density of ~ 50 cells/ μ L and plate 10- μ L aliquots of the dilution on eight GMML/agar plates containing 25 μ g/mL tetracycline, 35 μ g/mL kanamycin, 0.002% arabinose, 0 or 1 mM unnatural amino acid, and 0, 35, 75, or 100 μ g/mL chloramphenicol. Incubate the plates at 37°C for 48 h.
2. Using a handheld long-wavelength ultraviolet light, count the number of fluorescent and non-fluorescent colonies on each plate (*see Note 32*).
3. From the plate containing the highest chloramphenicol concentration for which a significantly greater number of fluorescent colonies formed in the presence vs the absence of unnatural amino acid, pick 10–20 fluorescent colonies. From each colony, inoculate 4 mL of GMML media containing 25 μ g/mL tetracycline, 35 μ g/mL kanamycin, and 0.002% arabinose. Transfer 2 mL of each inoculated sample to a separate tube and add the unnatural amino acid to a final concentration of 1 mM.

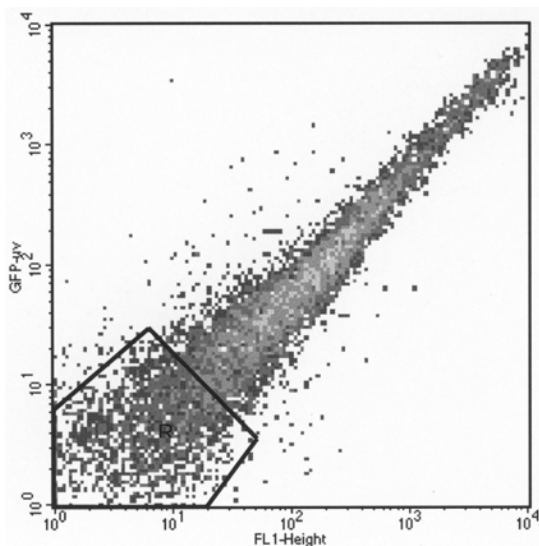


Fig. 7. FACS-based negative screening of *MjYRS* variants. The boxed events are collected, corresponding to cells producing little or no GFP_{yuv}. These cells, which were grown in the absence of the unnatural amino acid, contain *MjYRS* variants that cannot utilize as substrates any of the natural amino acids within *E. coli*.

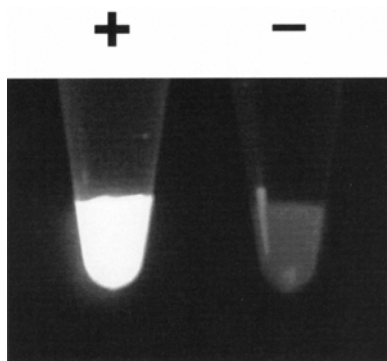


Fig. 8. Long-wavelength ultraviolet illumination of cells containing an *MjYRS* variant that accepts only an unnatural amino acid substrate. Cells were grown in either the presence (+) or absence (–) of the unnatural amino acid.

Incubate all cultures at 37°C with shaking (250 rpm) until the cells reach stationary phase (~24–36 h; see **Note 30**).

4. Pellet 200 μ L of cells from each culture by centrifugation at 10,000g for 1 min. Decant the supernatant (see **Note 33**, **Fig. 8**). Resuspend the cells in 1 mL of PBS.
5. Measure the cell optical density (at 600 nm) of each resuspended cell mixture.

6. Transfer 200 μ L of each cell mixture to a cuvet and use a fluorimeter to measure its fluorescence emission intensity at 505 nm with excitation at 396 nm.
7. Normalize the cellular fluorescence by dividing the fluorescence intensity of each cell mixture by its OD⁶⁰⁰. Determine the unnatural amino acid-dependent fluorescence corresponding to each *MjYRS* variant by calculating the ratio of normalized cellular fluorescence values for cells grown in the presences vs the absence of the unnatural amino acid (see **Note 34**).

4. Notes

1. *E. coli* DH10B-DE3 cells are λ -DE3 lysogens, which allow isopropyl β -D-1-thiogalactopyranoside (IPTG)-inducible expression of plasmid-borne genes that are under the control of the T7 promoter (**6**).
2. Plasmid pS (see **Fig. 1A**) has a ColE1 origin of replication, which allows it to replicate simultaneously in *E. coli* with plasmid pR (and variants, **Fig. 1B**), which has the p15A origin of replication.
3. While the design and construction of a genetic reporter system is an important component of FACS-based directed evolution, it is beyond the scope of this chapter.
4. In principle, PCR fragment libraries can be made either by targeted mutagenesis of specific codons within the gene, based on three-dimensional structural data, or by random mutagenesis. The former offers the advantage of concentrating the mutations at sites that are most likely involved in determining substrate specificity.
5. Plasmid pREP/YC-JYCUA (see **Fig. 2A**) has the p15A origin of replication, which allows it to replicate simultaneously in *E. coli* with plasmid pBK-JYRS (and variants, **Fig. 2B**), which has the ColE1 origin of replication. It contains a chloramphenicol acetyl transferase (CAT) reporter that is used as the basis for positive selection and a T7 RNA polymerase (T7 RNAP) / green fluorescent protein (GFPuv) reporter system that is used with FACS to screen against synthetase variants that accept natural amino acids. The fluorescence reporter system also is used to visually and fluorimetrically evaluate synthetase activity based on amino acid incorporation.
6. Although *Pfu* DNA polymerase was used for the methods described here, the Expand kit from Roche has been found to give higher PCR yields, especially for longer PCR products.
7. The objective of the method outlined here is to change the specificity of Cre by applying both positive and negative selection pressure (see **Fig. 3**). Positive pressure is obtained by screening for Cre variants that can recombine the novel *loxM* site, while negative pressure is obtained by screening against those variants that can recombine the wild-type *loxP* site. The application of positive selection pressure alone may be used if one wishes to broaden the specificity of Cre.
8. Standard conditions for restriction enzyme digestion and CIP treatment are as described by New England Biolabs.
9. Standard agarose gel electrophoresis (**7**) is performed using a 1% agarose gel with TAE buffer containing 0.5 μ g/mL ethidium bromide.

10. DNA is visualized under long-wavelength ultraviolet light, excised using a sterile razor blade, and removed from the gel slice by gel extraction.
11. Standard PCR conditions for a 100 μL reaction: 10 μL 10X PCR buffer, 10 μL dNTPs (2 mM each), 4 μL each primer (10 μM each), ~ 10 ng template, and 1.5 μL DNA polymerase (*see Note 6*). Typically, 20 cycles of PCR are carried out using the following cycle: 95°C for 1 min, 50°C for 1 min, and 72°C for 2 min.
12. Standard ligation reaction conditions are as described by New England Biolabs.
13. High competency in the transformation of *E. coli* is essential for efficient plasmid construction, especially when large numbers of transformants are required. Electroporation is a convenient method for transforming *E. coli*; the preparation of electrocompetent *E. coli* strains with transformation efficiencies of 10^8 – 10^{10} colony-forming units (cfu)/ μg of supercoiled plasmid DNA is routine. Keep in mind that for non-supercoiled and nicked plasmid DNA (as obtained after ligation), efficiencies will be at least an order of magnitude lower. For making libraries, it is convenient to use commercially-available electrocompetent DH10B cells (Life Technologies) for the initial transformation, as these cells have a guaranteed transformation efficiency of 10^{10} cfu/ μg of supercoiled plasmid DNA. Supercoiled DNA can be subsequently prepared and introduced into a non-commercial strain. The general method for preparation of electrocompetent *E. coli* is as follows:
 - a. From a single colony or glycerol stock, inoculate a 5-mL LB starter culture containing the appropriate antibiotics (if any) and incubate at 37°C with shaking at 250 rpm overnight.
 - b. From the starter culture, inoculate a 1-L 2XYT culture containing the appropriate antibiotics and grow to an optical density (OD) at 600 nm of 0.5.
 - c. Transfer culture to two ice-cold, 0.5-L GS3 tubes and centrifuge at 1°C for 5 min at 10,000g. Decant the supernatant.
 - d. Resuspend the cells in 1 L of ice-cold 10% glycerol and centrifuge at 1°C for 5 min at 7500g. Decant the supernatant.
 - e. Repeat **step 12d**.
 - f. Quickly resuspend the cells in the residual 10% glycerol and keep them on ice. Transform the cells immediately or flash-freeze them on dry ice before storing them at -80°C .
14. Use the conditions that are recommended by the electroporator manufacturer. It is extremely important that the cells remain cold at all times prior to transformation. Also, it is important that the cells be electroporated as quickly as possible after thawing on ice, as they will lose competency over time.
15. Addition of glucose acts to repress production of T7 RNA polymerase as well as transcription from the T7/lac promoter. This prevents premature expression of the fluorescent reporter proteins.
16. Addition of IPTG induces the production of T7 RNA polymerase and transcription from the T7/lac promoter.
17. For cells containing pS and wild-type Cre, GFPuv fluorescence was $\sim$ six-fold higher than EYFP fluorescence, which reflects differences between the two pro-

teins in expression efficiency, extinction coefficient, and quantum yield. The relative abundance of the two reporter arrangements can be calculated by adjusting EYFP intensity by a factor of six.

18. It is helpful to use a parent vector containing a “stuffer fragment” that is long enough to allow doubly- and singly-digested vector DNA fragments to be resolved. Do not treat the vector with CIP; although CIP treatment increases the fraction of clones that contain insert, it significantly decreases transformation efficiency.
19. A pilot transformation is useful to check the efficiency of the ligation reaction before proceeding with a large-scale transformation.
20. To estimate the number of independent transformants, make three 10-fold serial dilutions of the 100- μ L aliquot removed from the freshly-inoculated culture. Plate 10 μ L of each dilution (including the original aliquot) onto a series of LB agar plates containing the appropriate antibiotics. Based on the resulting number of colonies, calculate the total number of transformants in the culture.
21. These experiments are carried out using a BDIS FACVantage cytometer with a TSO option. Laser excitation is performed using a Coherent Enterprise II 421 water-cooled argon ion laser, emitting 351 and 488nm lines (30 and 250 mW, respectively). GFPuv is excited at 351 nm and produces emissions that are collected using a 519/20 nm bandpass filter. EYFP is excited at 488 nm and produces emissions that are collected using a 585/45 nm bandpass filter. Comparable systems should give similar results.
22. The cytometer is specially configured to trigger on scatter from small particles. Both forward scatter (FSC) and median angle side scatter (SSC) are acquired on a log scale. The system is triggered by a SSC threshold to avoid the low level noise from FSC at high sensitivity. A 70 μ m nozzle is used with a system pressure of $\sim$ 30 psi. Cells are typically sorted at a rate of $\sim$ 10,000/s.
23. GFPuv excitation and emission spectra overlap to some extent with the excitation and emission wavelengths used to detect EYFP (*see* Fig. 4). Therefore, it is difficult to distinguish cells that produce both EYFP and GFPuv from those that produce only GFPuv. However, in these experiments, the important variable is the production of GFPuv, which indicates that recombination has occurred. It is relatively easy to distinguish cells that have produced GFPuv from those that have not.
24. Reporter plasmids are removed at this stage to prevent their transformation, along with the pR-C plasmid library, into the new reporter strain in the following step. If reporter plasmids are not removed, they can be propagated at a low frequency into the next cycle of screening and can interfere by producing false positive or negative signals. It is not necessary to purify the digested DNA before transformation, as the digestion is carried out in the low-salt NEB 1 buffer, which will does not significantly affect transformation efficiency.
25. The success or failure of the experiment should become evident as the cycles of screening progress (*see* Fig. 5). If the experiment is successful, the majority of cells analyzed during the second cycle of negative screening should produce more EYFP than GFPuv and the majority of cells analyzed during the third cycle of positive screening should produce approx equal amounts of both GFPuv and EYFP.

26. Plating a series of cell dilutions ensures that at least one of the plates will contain an appropriate density of colonies.
27. Incubation of the plates at 37°C for 48 h is necessary and sufficient to induce transcription from the T7/lac promoter and production of EYFP and GFPuv. Addition of IPTG to the plates does not appear to facilitate induction.
28. Long-wavelength ultraviolet light excites only the GFPuv fluorophore and not EYFP. If recombinase evolution was successful, the majority of colonies on the plate should express GFPuv after incubation for 48 h at 37°C, which is indicative of reporter plasmid recombination. When picking colonies for analysis, it is important to note the pattern of GFPuv fluorescence within each colony. Colonies with a homogeneous pattern of fluorescence are likely to contain the most active Cre variants. Colonies with a striated pattern of fluorescence are likely to contain a Cre variant with an activity that is slow on the timescale of colony growth.
29. In principle, directed evolution of an aminoacyl-tRNA synthetase can be carried out entirely by FACS-based screening. For such a strategy, the chloramphenicol-based positive selection is replaced with a positive screen in which fluorescent cells grown in the presence of an unnatural amino acid are collected using FACS.
30. The optimal chloramphenicol concentration depends on the activity of synthetases in the initial library. Chloramphenicol is bacteriostatic rather than bacteriocidal, so selection efficiency should increase with increasing chloramphenicol concentration without loss of population diversity. In practice, the use of an arbitrarily high concentration of chloramphenicol often produces selection artifacts. Conversely, a chloramphenicol concentration that is too low may result in insufficient selection stringency. *A priori*, it is impossible to predict whether an evolution experiment will be successful, much less how active the best synthetase in a population of variants will be. A chloramphenicol concentration of 75 µg/mL is used because it has been shown to be effective in enrichment experiments (8) and lies somewhat below the IC₅₀ supported by the majority of the *MjYRS* variants that have been identified by directed evolution thus far. (5,9,10) Although it is possible that a different chloramphenicol concentration will be optimal for a given evolution experiment, we consider 75 µg/mL an appropriate concentration for initial experiments.
31. *E. coli* grown in GMML media with sufficient aeration will saturate at an OD (600 nm) of ~1–2.
32. An arabinose concentration of 0.002% w/v has been optimized to allow controlled expression of the amber stop codon-containing T7 RNA polymerase gene within pREP/YC-JYCUA. This results in a robust fluorescence signal (in the presence of a suitably-charged suppressor tRNA) with minimal effects on the growth rate of the *E. coli* host.
33. If the evolution experiment is successful, there should be a greater number of fluorescent colonies on the plates containing the unnatural amino acid than on plates lacking the unnatural amino acid.
34. At this point, it is possible to use a handheld long-wavelength ultraviolet light to observe the visible fluorescence from each cell pellet (see Fig. 8). Cells exhibiting

no visible difference in fluorescence as a result of growth in the presence of the unnatural amino acid are likely to contain an *MjYRS* variant that accepts a natural amino acid; such cells need not be characterized further.

35. An alternative option for analysis of synthetase activity and specificity is to measure the chloramphenicol IC₅₀ for cell growth on GML/agar plates in the presence versus the absence of the unnatural amino acid (4).

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Calmodulin-Tagged Phage and Two-Filter Sandwich Assays for the Identification of Enzymatic Activities

Christian Heinis, Julian Bertschinger, and Dario Neri

1. Introduction

Directed evolution has proven to be a powerful route to enhance the stability, catalytic efficiency, or substrate specificity of enzymes. Large repertoires of enzyme mutants are generated, followed by the isolation of those enzyme variants with the desired catalytic properties. In this chapter, we outline two general methods for the efficient isolation of enzymatic activities from very large repertoires of protein variants.

In the first methodology, enzymes are displayed on filamentous phage (*1–8*). The reaction substrate (and after catalysis the reaction product) is anchored on the calmodulin-tagged phage-enzymes by means of a calmodulin binding peptide. Phage displaying a catalytically active protein are physically isolated by means of affinity reagents specific for the product of reaction (*9,10*). In the second methodology, enzyme mutants are expressed in bacterial colonies on a porous ‘master’ filter (*11–15*). The enzymes are released and diffuse to a second ‘reaction’ filter that closely contacts the master filter. The catalytic activity of the enzyme variants is assayed on the reaction filter (*16*). The biotin ligase BirA will be used as a model enzyme to illustrate both the selection and screening methodology.

2. Materials

2.1. Isolation of Enzymatic Activities by Calmodulin-Tagged Phage

1. Phagemid pSD4 (*10*).
2. Gene of biotin ligase BirA.
3. *E. coli* strain TG1.
4. Helper phage VCS M13 (Stratagene).

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5. Hyper-phage (Progen).
6. Oligonucleotide primers.
7. Restriction enzymes, *Taq* polymerase, T4 DNA ligase.
8. Agarose and DNA gel equipment.
9. DYT (double yeast tryptone) media.
10. Ampicillin (100 µg/mL medium), kanamycin (25 µg/mL medium).
11. PBS (phosphate buffered saline): 50 mM phosphate, pH 7.2, 100 mM NaCl in water.
12. TBS (tris buffered saline): 50 mM Tris-HCl, pH 7.4, 100 mM NaCl in water.
13. TBSC (tris buffered saline with calcium): 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM CaCl₂ in water.
14. MPBS (phosphate buffered saline with milk): 50 mM phosphate, pH 7.2, 100 mM NaCl, 2% (w/v) milk powder in water.
15. TBSE (tris buffered saline with EDTA): 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 20 mM EDTA in water.
16. PEG solution (20% (w/v) polyethylene glycol 6000, 2.5 M NaCl).
17. Inorganic pyrophosphatase (Sigma).
18. Bovine serum albumin, BSA (Sigma).
19. Calmodulin-binding substrate/product peptides.
20. Streptavidin coupled M-280 Dynabeads (Dyna).
21. Magnetic tube rack (Dyna).
22. SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) equipment.
23. Nitrocellulose filter (Schleicher & Schuell).
24. Anti-pIII murine monoclonal antibody (Mo Bi Tec).
25. Anti-M13 antibody-HRP (Amersham Biosciences).
26. Horseradish peroxidase (HRP) conjugated streptavidin (Amersham Biosciences).
27. Detection reagents for Western Blot and Dot blot using Chemiluminescence : ECL (Amersham Biosciences).
28. Streptavidin-coated Plates (Boehringer).
29. MicroTest III flexible assay plates (Falcon).
30. HRP substrate solution, e.g., BM blue POD substrate (Boehringer).

2.2. Isolation of Enzymatic Activities by the Two-Filter Sandwich Assay

1. Plasmids pQE12 (Qiagen), pGEX-4T-2 (Amersham Biosciences).
2. Genes of BirA and albumin binding domain.
3. *E. coli* strain TG1.
4. Oligonucleotide primers.
5. Restriction enzymes, *Taq* polymerase, T4 DNA ligase.
6. Agarose and DNA gel equipment.
7. DYT (double yeast tryptone) media.
8. Ampicillin (100 µg/mL medium).
9. PBS (phosphate buffered saline): 50 mM phosphate, pH 7.2, 100 mM NaCl in water.

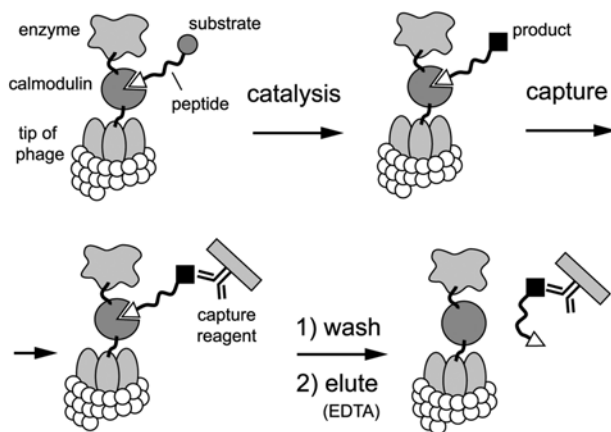


Fig. 1. Calmodulin-tagged phage enzyme for the selection of enzymatic activity. High-affinity calmodulin-binding peptides can be used for the non-covalent stable anchoring of reaction substrates and products on the surface of filamentous phage, and for the selection of active phage enzymes with anti-product affinity reagents. The calmodulin/ligand complex can be dissociated by addition of calcium chelators.

10. TBS (tris buffered saline): 50 mM Tris-HCl, pH 7.4, 100 mM NaCl in water.
11. TBSC (tris buffered saline with calcium): 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM CaCl_2 in water.
12. Reaction buffer: 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 12 mM MgCl_2 , 10 mM ATP, 100 μM D-biotin, 1 mg/mL pyrophosphatase (Sigma), 1 mM CaCl_2 , 0.1% v/v Tween-20 in water.
13. Master filter: PVDF Durapore membrane filter type GVWP, 0.22 μm (Millipore).
14. Reaction filter: PVDF Immobilon-P filter (Millipore).
15. Nitrocellulose filter (Schleicher & Schuell).
16. Horseradish peroxidase (HRP) conjugated streptavidin (Amersham Biosciences).
17. Detection reagents for Western Blot and Dot blot using Chemiluminescence : ECL (Amersham Biosciences).

3. Methods

3.1. Calmodulin-Tagged Phage for the Isolation of Enzymatic Activities

The methods to isolate catalytic activities with calmodulin-tagged phage (see Fig. 1) are described below. The gene of the enzyme of interest is inserted upstream to the calmodulin-gIII fusion gene in a phagemid (e.g., pSD4 [10]; Fig. 2). Phage particles displaying the calmodulin-enzyme fusion protein are produced and characterized. Active phage-enzyme are selected using a substrate linked to the calmodulin binding peptide and an anti-product affinity reagent.

3.1.1. Vectors for Displaying Enzyme-Calmodulin Fusions on Phage

3.1.1.1. PHAGEMID PLASMID pSD4

The vector pSD4 is used to display enzyme and calmodulin on the minor coat protein pIII of filamentous phage. It is a derivative of pHEN1 (17) and contains the phage origin of replication (M13 ori, allowing the genome to be packaged into the phage particles), the *E. coli* origin of replication (colE1 ori, enabling the phagemid to replicate in the *E. coli* host cells), an ampicillin resistance gene (for selection of phagemid-containing colonies), the lacZ-promotor (for regulation of expression with promotion by IPTG and inhibition by glucose), a peptide leader (pel B, for secretion of phage protein III fusion protein to the periplasm of the bacteria), a multiple cloning site (MCS), a 10 amino acid residue linker and the calmodulin gene fused to gIII (Fig. 2). The gene of the enzyme can be linked upstream to calmodulin gene by inserting it into the multiple cloning site. pSD4 is a phagemid vector (rather than a phage vector) and needs co-infection with a helper phage for phage production. Co-infection with a newly developed hyperphage (rather than helper phage) that lacks the genetic information for protein III appears to improve enzyme display on phage dramatically. Alternatively, phage vectors (as fd-tet-DOG1 [18]) are used to display calmodulin-enzyme on phage. However, transformation of host cells is inefficient with large plasmids.

3.1.1.2. CLONING STRATEGY

The gene of the enzyme is PCR amplified and ligated into the multiple cloning site in frame with the leader sequence and the gene III according to standard recombinant DNA methodologies (19). The ligated vectors are electroporated into TG1 *E. coli* cells. The presence of the enzyme gene in the plasmid is confirmed by PCR screening. Cells harboring the correct plasmid are stored as glycerol (15% glycerol) at -80°C .

3.1.2. Production of Calmodulin-Tagged Phage Enzyme

Phage displaying calmodulin-enzyme using the phagemid plasmid pSD4 are expressed and purified according to the protocols shown below.

3.1.2.1. PHAGE PRODUCTION

1. Inoculate the glycerol stock into 10 mL DYT (ampicillin 100 $\mu\text{g/mL}$, glucose 1%).
2. Grow to OD_{600} 0.4–0.5 at 37°C (about 1–1.5 h).
3. Infect the 10 mL culture with 10 μL of 10^{12} t.u./mL helper phage VCS-M13 or hyper-phage (the titer of the helper phage can be lower if monoclonal phage enzyme is used but should exceed the number of different clones if a library of phage enzyme is used). Infections are carried out at 37°C in a water bath for at least 30 min.

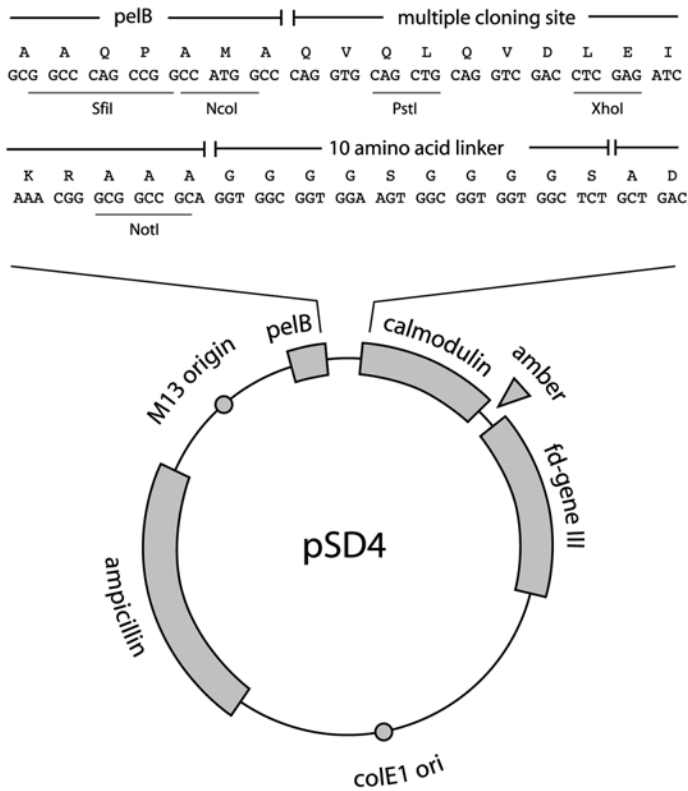


Fig. 2 Schematic representation of the phagemid pSD4, for the display of calmodulin-tagged enzymes on filamentous phage. Genes of enzymes can be inserted into the multiple cloning site in frame with the pelB leader sequence and the calmodulin gene.

4. Spin down the infected bacteria at 3300g for 10 min. Gently resuspend the pellet in 500 mL of DYT (ampicillin 100 µg/mL, kanamycin 25 µg/mL).
5. Incubate with shaking 30°C overnight.

3.1.2.2. PHAGE PURIFICATION

1. Spin down the culture at 10,800g for 10 min, transfer the phage supernatant to a tube, and add 125 mL PEG solution.
2. Mix well and leave for a minimum of 1 h at 4°C or at least 40 min on ice.
3. Spin 4000g for 15 min.
4. Resuspend the pellet in 40 mL water, and add 1/5 volume PEG/NaCl (e.g., 10 mL PEG/NaCl to 40 mL).
5. Mix and leave for a minimum of 20 min at 4°C.

6. Spin 4000g for 15 min and discard the supernatant.
7. Respin briefly and aspirate off any remainings of PEG/NaCl.
8. Resuspend the pellet in 2 mL PBS. Phage yields are normally 10^{11} – 10^{13} t.u./mL phage suspension.
9. Spin 3300g for 10 min or 11,600g for 2 min to remove any residual bacterial cell debris.
10. Store the phage supernatant either at 4°C for short term storage or in PBS-15% glycerol for longer term storage at –20°C.
11. Titrate phage: Add a molar excess of TG1 cells in DYT at a density of 4×10^8 cells/mL ($OD_{600}=0.4$) to the phage and incubate at 37°C for 30 min. Plate the cells on ampicillin-containing plates and incubate them overnight at 37°C.

3.1.3. Characterization of Calmodulin-Enzyme Displaying Phage

Good levels of functional enzyme display on phage are essential in order to achieve selection for catalysis. The presence of the calmodulin-enzyme fusion-protein on the tip of phage can be investigated by Western blot or by phage ELISA (enzyme-linked immunosorbent assay). Several other methods have been used to quantitate the protein display on phage as pIII fusion (*see Note 1*).

3.1.3.1. WESTERN BLOT ANALYSIS OF PIII FUSION PROTEIN

Western blot analysis of phage proteins using an anti-pIII murine monoclonal antibody (**20**) reveals whether the pIII fusion-proteins has the correct molecular size:

1. Denature a total of 5×10^8 infective phage by heating at 90°C for 5 min in the presence of reducing SDS-sample buffer.
2. Run the sample on a 10% polyacrylamide gel and then electrophoretically transfer proteins onto a nitrocellulose filter.
3. Block the filter for 1 h at room temperature in MPBS.
4. Add a pre-blocked anti-pIII murine monoclonal antibody (1:1000 diluted in MPBS) to the filter and incubate for 1 h.
5. Wash three times with PBS containing 0.1% v/v Tween-20.
6. Add pre-blocked secondary anti-mouse HRP-goat IgG antibody (1:1000 diluted in MPBS) to the filter and incubate for 1 h.
7. Wash three times with PBS containing 0.1% v/v Tween-20 and three times with PBS.
8. Peroxidase activity is detected using an ECL kit by mixing equal amounts of reagent 1 with reagent 2. Pour onto the blot and detect electrochemiluminescence with a light sensitive film.

3.1.3.2. ELISA ANALYSIS OF PIII FUSION PROTEIN

The presence of calmodulin on phage can easily be detected by phage ELISA. The phage are immobilized via a biotinylated calmodulin binding pep-

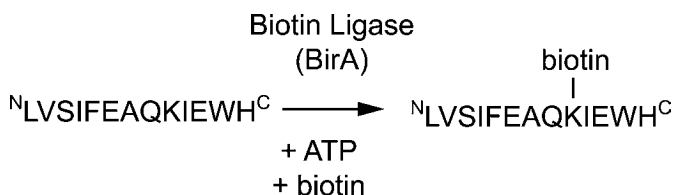


Fig. 3. Schematic representation of the biotin ligase (BirA) catalyzed reaction. Biotin is ligated to the lysine amino acid residue of the biotin acceptor peptide (BAP).

tide (*see Note 2*) and detected with anti-M13 antibody according to the following protocol:

1. Incubate streptavidin-coated plates with 100 μL of 10^{-7} M solution of biotin-labeled calmodulin binding peptide biotin-CAARWKKAFIAVSAANRFFKIS in TBSC for 10 min.
2. Wash wells three times with TBSC.
3. Block with 2% (w/v) milk powder in TBSC for 2 h at room temperature.
4. Add to each well 20 μL TBSC containing 10% (w/v) milk powder and 80 μL phage (10^{12} t.u./mL) and incubate for 30 min at room temperature.
5. Wash wells five times with TBSC with 0.1% (v/v) Tween-20 and five times with TBSC.
6. Add horseradish peroxidase-labelled anti-M13 antibody (diluted 1:2000 in 2% milk dissolved in TBSC) and incubate for 20 min at room temperature.
7. Wash wells as in **step 5**.
8. Detect the plate-bound peroxidase with HRP substrate solution, e.g., add 100 μL ready-to-use BM blue POD substrate (Boehringer) to each well and leave at room temperature for 10 min.
9. Add 50 μL of 1 M H_2SO_4 to each well.
10. Read the OD at 690 nm and at 450 nm. Subtract OD 690 from OD 450.

3.1.4. Selection of Active Phage Enzyme

The isolation of enzymatic activities by calmodulin-tagged phage is described in **Subheadings 3.1.4.1.–3.1.4.3**. As an illustrative example, selection procedures with the biotin ligase BirA are reported here (**Fig. 3**). The procedures include (a) the immobilization of substrate on phage, (b) the isolation of active phage-enzyme, and (c) the propagation of isolated phage. These methods can be used to perform ‘model’ selection experiments in which the performance of selection for catalysis is investigated with monoclonal phage-enzyme (*see Note 3*). The same methods can be used to isolate improved catalysts from polyclonal phage-enzyme libraries.

3.1.4.1. IMMOBILIZATION OF SUBSTRATE ON PHAGE

High-affinity calmodulin binding peptides (*see Note 2*) (**21**) are used for the non-covalent but stable ($k_{\text{off}} < 10^{-4}$ s) anchoring of reaction substrates and products on the surface of phage. The reaction substrate is covalently bound on either terminus to the calmodulin binding peptide ($^{\text{N}}$ CAAARWKKAFIAVSAANRFKKIS $^{\text{C}}$). The Ca^{2+} dependent calmodulin/peptide complex is stable at pH values between 4 and 10. The following protocol describes the anchoring of the substrate peptide for the biotin ligase $^{\text{N}}$ GAAARWKKAFIAVSAANRFKKISTS GGGPGGLVSIFEAQKIEWH $^{\text{C}}$ (substrate moiety in bold [**22,23**]) on phage:

1. Block calmodulin-enzyme displaying phage (10^9 t.u.) in a final volume of 100 μL TBSC containing 3% (w/v) BSA for 30 min at room temperature.
2. Add phage to 100 μL of 10^{-7} M BirA substrate peptide ($^{\text{N}}$ GAAARWKKAFIAVSAANRFKKISTS GGGPGGLVSIFEAQKIEWH $^{\text{C}}$) in TBSC with 0.1 M biotin, 10 mM ATP, 12 mM MgCl_2 , 1 mg/mL inorganic pyrophosphatase, and 0.06% Tween-20.
3. Incubate the reaction for 20 min at room temperature to allow conversion of calmodulin-bound substrate into product by the enzyme.

3.1.4.2. ISOLATION OF ACTIVE PHAGE ENZYME

Phage displaying a catalytic activity are physically isolated by means of affinity reagents specific for the product of reaction. The product of the catalytic reaction of the biotin ligase (BirA) is a biotinylated peptide and can be captured by streptavidin coated beads.

1. To the reaction mixture add 1/4 volume PEG solution, mix, and incubate on ice for 15 min.
2. Spin down phage at 10,000g for 2 min and aspirate off the supernatant. Resuspend the phage pellet in 200 μL TBSC.
3. Add 1/4 volume PEG solution (50 μL), mix, and incubate on ice for 15 min.
4. Spin down phage at 10,000g for 2 min and aspirate off the supernatant. Resuspend the phage pellet in 200 μL TBSC-BSA 3% (w/v).
5. Prepare 50 μL streptavidin-coated M280 dynabeads by capturing the beads and aspirating the buffer. Block beads in 50 μL TBSC-BSA 3% (w/v).
6. Add reaction mixture to 50 μL of pre-blocked streptavidin-coated M280 dynabeads, mix, and incubate for 10 min at room temperature.
7. Capture magnetic beads with an appropriate magnet and aspirate off the supernatant.
8. Wash five times with TBSC and 0.1% (v/v) Tween-20.
9. Wash three times with TBSC.
10. In order to elute phage, add 500 μL TBSE, mix, and incubate for 5 min at room temperature.
11. Aspirate off the supernatant and saturate EDTA with 100 μL 1 M CaCl_2 solution.

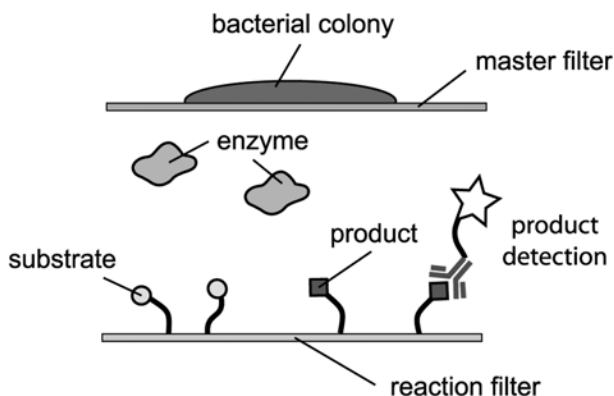


Fig. 4. Schematic representation of the filter “sandwich” assay for the biotin ligase BirA. Bacteria on the master filter release biotin ligase (BirA) to the reaction filter which is coated with a fusion protein of GST and the biotin acceptor peptide (BAP) which represents the substrate of the reaction. The enzyme converts the reaction substrate into the product, detected with streptavidin-HRP and a chemiluminescent substrate.

3.1.4.3. PROPAGATION OF ISOLATED PHAGE

1. Add phage to 1 mL exponentially growing TG1 *E. coli* cells (OD_{600} 0.4–0.5) and incubate at 37°C in a water bath for 30 min.
2. Plate cells on DYT (ampicillin 100 µg/mL, glucose 1%) plates. In order to count the number of isolated infective phage, dilute infected cells 1:100, 1:10⁴ and 1:10⁶ and plate on DYT (ampicillin 100 µg/mL, glucose 1%) plates.
3. Incubate overnight at 30°C.
4. Scratch the cells from the DYT plate and use them as starting material to produce new phage (as described in **Subheading 2.1.1.1.**) for further selection rounds.

3.2. Isolation of Enzymatic Activities by Two-Filter Sandwich Assays

Two different in vitro screening methods for the isolation of enzymatic activities from a large repertoire of protein variants based on two-filter sandwich assays are described in **Subheadings 3.2.1.** and **3.2.2.** In both of the two screening methods, bacterial cells expressing enzyme are grown on a porous “master filter,” receiving nutrients from an agar plate by diffusion. This filter loaded with colonies is then laid on top of a second filter (termed “reaction filter”). Enzymes released from the bacterial colonies can turn suitable substrates on the reaction filter into products. The product is detected and the corresponding catalytically active colonies are identified. The biotin ligase BirA is used as a model enzyme to illustrate the principles of the filter screening methods (see **Fig. 4** and **5**).

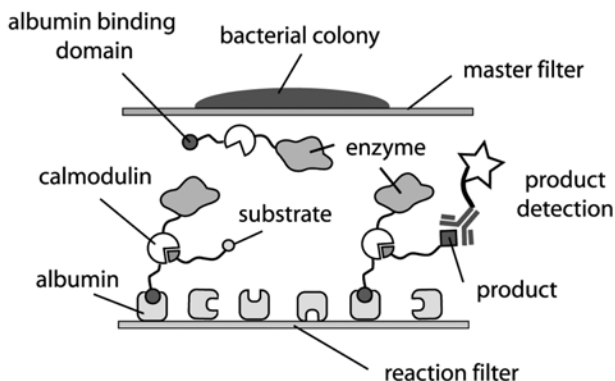


Fig. 5. Schematic representation of the filter “sandwich” assay for the biotin ligase BirA. Fusion protein of BirA-calmodulin-ABD is released from the bacteria and immobilized on the reaction filter coated with human serum albumin (HSA) by the non-covalent interaction of the albumin binding domain (ABD) and HSA. The substrate peptide, linked to a high-affinity calmodulin-binding peptidic moiety, is then added to BirA-CaM-ABD and binds to calmodulin. BirA biotinylates the peptide and the product is detected with streptavidin-HRP.

3.2.1. Two-Filter Sandwich Activity Assay with Immobilized Substrate

In the first two-filter sandwich assay (**Fig. 4**), the reaction filter is coated with the reaction substrate (in this case with the biotin acceptor peptide (BAP) linked to GST). The enzyme (biotin ligase BirA) secreted from bacterial colonies on the master filter converts the immobilized substrate into the product (in this case a biotinylated peptide). Regions on the reaction filter with reaction product are detected with anti-product affinity reagents (in this case with streptavidin-HRP conjugate and a chemiluminescent substrate). Described below are (a) the DNA plasmid for biotin ligase (BirA) expression, (b) the production and immobilization of the biotin acceptor peptide (BAP) and (c) the two-filter sandwich activity assay.

3.2.1.1. ENZYME EXPRESSION VECTOR

The pQE12 vector system is used for cytoplasmic expression of the enzyme. The PCR-amplified biotin ligase gene is inserted into the cloning sites EcoRI/BglII of the pQE12 plasmid (see for DNA-primer design) by standard recombinant DNA methods. The resulting vector pCHH20 contains the *E. coli* origin of replication, *colE1*, an ampicillin resistance gene (for selection of plasmid containing colonies), and the biotin ligase gene under the control of the *lacZ*-promotor/operator. Other DNA vectors can be used for cytoplasmic expression of enzymes.

3.2.1.2. SUBSTRATE PRODUCTION AND IMMOBILIZATION ON REACTION FILTER

The BirA substrate, the biotin acceptor peptide (BAP; ^NLVSIFEAKQIEWH^C; [22,23]) is immobilized as fusion peptide of glutathione-S-transferase (GST) on a hydrophobic membrane filter. The GST-BAP fusion protein is over-expressed in TG1 *E. coli* cells using the vector pGEX-4T-2 and purified on glutathione-sepharose resin according to standard techniques. Other reaction substrates may be chemically linked via a linker arm to carrier proteins (e.g., GST). The substrate-protein conjugate is coated onto a hydrophobic membrane as described below:

1. Incubate a 5-cm diameter PVDF membrane (Immobilon-P) in PBS containing 0.5 mg/mL GST-BAP at room temperature overnight.
2. Wash the filter three times with TBS with 0.1% (v/v) Tween-20.
3. Block the filter in 1% milk/TBS for 1 h at room temperature.
4. Incubate the filter in reaction buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 12 mM MgCl₂, 10 mM ATP, 100 μM D-biotin, 1 mg/mL pyrophosphatase, 1 mM CaCl₂, 0.1% v/v Tween-20 in water) for 10 min at room temperature.

3.2.1.3. TWO-FILTER SANDWICH ACTIVITY ASSAY

Described below are the steps to assay enzymatic activity with the two-filter sandwich method using a substrate coated membrane filter:

1. Grow *E. coli* TG1 bacterial cells harboring the expression plasmid pCHH20 in liquid DYT media containing 100 μg/mL ampicillin until OD₆₀₀ = 0.6.
2. Spread a suitable dilution of the bacterial cells on a DYT (ampicillin 100 μg/mL) agar plate which is covered with a 4.7-cm diameter PVDF Durapore membrane filter (GVWP, 0.22 μm).
3. Incubate the plate at 30°C until very small colonies (about 0.2-mm diameter) are visible.
4. Transfer the PVDF durapore filter (master filter) on a new DYT agar plate containing 100 μg/mL ampicillin and 1 mM IPTG for the induction of protein expression.
5. Incubate the membrane filter/agar plate at room temperature overnight.
6. Place the substrate coated reaction filter (see **Subheading 3.2.1.2.**) into an empty Petri dish and dry the filter carefully with blotting paper (see **Note 4**).
7. Cover the semi-dry reaction filter with the master filter carrying the bacterial colonies.
8. Freeze and thaw the “filter-sandwich” three times in order to lyse the cells and release the biotin ligase.
9. Incubate the “filter-sandwich” for 1 h at 37°C to allow the released enzyme to biotinylate the BAP.
10. Remove the master filter and wash the reaction filter four times with TBS/0.1% (v/v) Tween-20.
11. Block the reaction filter in 1% milk/TBS for 30 min at room temperature.

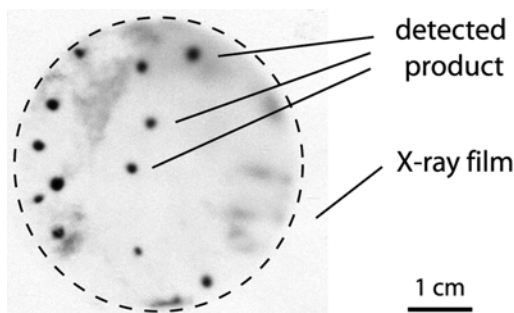


Fig. 6. The figure shows an X-ray film exposed to a reaction filter treated with streptavidin-HRP and the chemiluminescence ECL detection kit. Black spots indicate regions, which were covered in the filter “sandwich” with bacterial colonies expressing active enzyme.

12. Incubate the reaction filter in 1% milk/TBS containing HRP-streptavidin conjugate (1:1000).
13. Wash five times with TBS/0.1% (v/v) Tween-20.
14. Peroxidase activity is detected using an ECL kit by mixing equal amounts of reagent 1 with reagent 2. Pour onto the blot and detect electrochemiluminescence with a light sensitive film (*see Fig. 6*).
15. Identify and isolate the catalytically active colonies by superposition of the X-ray film with a replica of the master filter carrying the colonies.

3.2.2. Two-Filter Sandwich Activity Assay with Immobilized Substrate and Enzyme

In the second two-filter sandwich assay (*see Fig. 5*), the reaction filter is coated with human serum albumin (HSA). The enzyme is expressed as fusion protein of biotin ligase, calmodulin and albumin binding domain (ABD) (24,25) and is immobilized on the reaction filter by binding of the ABD to human serum albumin (HSA). The biotin acceptor peptide (which represents the substrate) linked to a high-affinity calmodulin binding peptide is then immobilized by binding to calmodulin and can be biotinylated by the enzyme. The product of the reaction is detected with a streptavidin-horseradish peroxidase conjugate.

3.2.2.1. ENZYME-CALMODULIN-ALBUMIN BINDING DOMAIN EXPRESSION VECTOR

The fusion protein of biotin ligase, calmodulin and albumin binding domain (ABD) is expressed in bacterial cells harboring the plasmid pCHH19. The plasmid contains the *E. coli* origin of replication, *colE1*, an ampicillin resistance gene (for selection of plasmid containing colonies) and the fused genes of biotin ligase, calmodulin and albumin binding domain under the control of the *lacZ*-promotor/operator. Spacer sequences of 13 amino acid residues

(GGSGGGGSGGGGS) and a 8 amino acid residues (TSGGGPGG) link the proteins biotin ligase/calmodulin and calmodulin/ABD respectively. The genes of the three proteins were PCR assembled with suitable DNA primers (*see [16]* for primer design) and ligated EcoRI/BglIII into the pQE12 plasmid by standard recombinant DNA methods (*19*).

3.2.2.2. TWO-FILTER SANDWICH ACTIVITY ASSAY

Described below are the procedures to assay enzymatic activity with the two-filter sandwich method using the albumin binding domain/albumin complex for enzyme immobilization and the calmodulin binding peptide/calmodulin complex for substrate immobilization:

1. Grow *E. coli* TG1 bacterial cells harboring the expression plasmid pCHH19 in liquid DYT media containing 100 µg/mL ampicillin until OD₆₀₀ = 0.6.
2. Spread a suitable dilution of the bacterial cells on a DYT (ampicillin 100 µg/mL) agar plate which is covered with a 4.7-cm diameter PVDF Durapore membrane filter (GVWP, 0.22 µm).
3. Incubate the plate at 30°C until very small colonies (about 0.2-mm diameter) are visible.
4. Transfer the PVDF durapore filter (master filter) on a new DYT agar plate containing 100 µg/mL ampicillin and 1 mM IPTG for the induction of protein expression.
5. Incubate the membrane filter/agar plate at room temperature overnight.
6. Incubate a 5-cm diameter PVDF membrane (Immobilon-P) in PBS containing 1 mg/mL human serum albumin (HSA) at room temperature overnight.
7. Wash the filter three times with TBS with 0.1% (v/v) Tween-20.
8. Block the filter in 1% milk/TBS for 1 h at room temperature.
9. Place a human serum albumin (HSA) coated reaction filter into an empty Petri dish and dry the filter carefully with blotting paper (*see Note 4*).
10. Cover the semi-dry reaction filter with the master filter carrying the bacterial colonies.
11. Freeze and thaw the “filter-sandwich” three times in order to lyse the cells and release the fusion protein (biotin ligase/calmodulin/albumin binding domain).
12. Incubate the “filter-sandwich” for 30 min at 37°C to allow the released fusion protein to bind to human serum albumin (HSA)
13. Remove the master filter and wash the reaction filter with TBSC and block with 1% milk/TBSC for 30 min.
14. Incubate the filter with 10⁻⁸ M (final concentration) substrate peptide ^NGAAARWKKAFIAVSAANRFKKISTSGGGPGGLV**SIFE**AQK**IEW**H^C (substrate moiety in bold) for 30 min at room temperature.
15. Wash the reaction filter three times in TBSC with 0.1% (v/v) Tween-20.
16. Incubate the filter in reaction buffer for 1 h at 37°C.
17. Wash the reaction filter four times with TBSC/0.1% (v/v) Tween-20.
18. Block the reaction filter in 1% milk/TBSC for 30 min at room temperature.

19. Incubate the reaction filter in 1% milk/TBSC containing HRP-streptavidin conjugate (1:1000).
20. Wash five times with TBSC/0.1% (v/v) Tween-20.
21. Peroxidase activity is detected using an ECL kit by mixing equal amounts of reagent 1 with reagent 2. Pour onto the blot and detect electrochemiluminescence with a light sensitive film (*see Fig. 6*).
22. Identify and isolate the catalytically active colonies by superposition of the X-ray film with a replica of the master filter carrying the colonies.

4. Notes

1. The level of calmodulin-enzyme display on phage can also be characterized by:
 - a. Determination of phage titer before and after a chromatographic phage purification using affinity reagents specific for the enzyme or the calmodulin moiety.
 - b. Quantitative determination of catalytic activity of phage enzymes.
 - c. Phage ELISA in a microtiter plate coated with antibody specific for the enzyme and phage detection with anti-M13 antibody.
2. The calmodulin binding peptide binds to calmodulin with high affinity ($K_d = 2 \text{ pM}$). The interaction is Ca^{2+} dependent. It is important to use buffers with 1 mM CaCl_2 in all steps in which the peptide is bound to calmodulin. The calmodulin/peptide complex can be dissociated by addition of buffers containing a molar excess of EDTA (20 mM).
3. 'Model' selection experiments are performed in order to monitor biopanning efficiency. In selection experiments with the biotin ligase BirA, biotinylated calmodulin-binding peptide simulating the reaction product is used in control reactions. In negative controls, either biotin or the substrate peptide are not added to the reaction mixture.
4. It is important that the reaction filter is slightly dried before the filter "sandwich" is assembled. If the filter sandwich is too wet, the released enzyme or enzyme fusion protein will diffuse horizontally in the filter "sandwich". However, to allow the enzyme to catalyze the reaction or the fusion protein to be immobilized, the filter membrane should not be dried completely.

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High-Throughput FACS Method for Directed Evolution of Substrate Specificity

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and George Georgiou

1. Introduction

Directed evolution is a powerful technology for the isolation of enzyme variants with enhanced properties such as organic solvent tolerance (1), thermostability (2), glutaraldehyde resistance (3), and altered substrate specificity (4,5). Typically, enzyme variants are isolated using low throughput screening techniques such as agar plate or microtiter well plate assays. Recently, Fluorescence Activated Cell Sorting (FACS) has emerged as a tool offering significant promise for the high-throughput screening of very large libraries of mutants with single cell resolution (6). Arising from the simultaneous collection of multiple parameters, FACS is inherently capable of rapidly evaluating libraries for several different catalytic activities simultaneously with no increase in time required for library screening. We have exclusively used a Becton Dickinson FACSCaliber instrument, perhaps the most popular low-end sorter commercially available.

Substrate design is a critical component for a successful FACS-based cell sorting strategy. Fluorogenic probes that are non-fluorescent until a specific catalytic activity unmask the fluorescent moiety, can be synthesized based upon Fluorescence Resonance Energy Transfer (FRET). FRET is a distance dependent phenomenon whereby an excited donor fluorophore can transfer energy to an attached quenching molecule, without the emission of a photon. FRET can be used to monitor an enzymatic cleavage event, by positioning the donor on one side of the target scissile bond, with the quencher on the opposite side. After the cleavage event takes place, the donor and quencher fragments are free to diffuse away from each other, and the emission of the donor fluorophore can

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consequently be observed. For monitoring catalytic activity at the single cell level, it is essential that the separated donor fluorophore becomes quantitatively linked to the cell expressing the enzyme that catalyzed the cleavage event. A general way to establish such a physical link between fluorescent product formation and the cell is to capitalize on electrostatic interactions with the cell surface (see **Fig. 1A**). Because the surface of bacteria (and of most other cells) is highly negatively-charged, molecules carrying at least three positive charges become adsorbed in low ionic strength conditions. In our method, a string of positive charges are appended to a FRET substrate. Cleavage of the target scissile bond not only separates the donor and quencher onto different fragments capable of diffusion, but also results in a positively charged fragment that contains the donor fluorophore. Thus, cell fluorescence is proportional to the turnover of the substrate allowing the discrimination of clones expressing catalysts from non-catalysts.

Typically, the protein of interest must be displayed on the cell surface, by fusing it to a proper display signal. Several methods for protein display in bacteria and yeast have been developed (7–10). Since the retention of the fluorescent product on the cell surface relies on electrostatic interactions, the cells and substrate must be incubated in a low ionic strength, isotonic solution. For this purpose, we have routinely used 1% sucrose solutions. Under these conditions we observe good retention of the fluorescent product for about 45 min.

For simplicity, our lab has focused on the directed evolution of the substrate specificity of the native *E. coli* surface protease OmpT. OmpT is a very stable and highly active endopeptidase that exhibits strong preference for cleavage between two basic amino acids such as Arg-Arg sequences. A flow cytometric FRET substrate possessing an Arg-Val target scissile bond, with BODIPY® as the donor and tetramethylrhodamine as the quencher were used for detection of the catalytic activity to be evolved. In addition, a second flow cytometric reagent, employing only a single fluorophore, tetramethylrhodamine, was used to monitor cleavage of an Arg-Arg containing (wild-type) peptide (see **Fig. 1B**). This second substrate is neutral until cleavage at the target scissile bond, upon which a positively charged fragment with the tetramethylrhodamine fluorophore is unmasked, resulting in electrostatic capture of the fluorescent product by the catalytic bacterial surface. The ratio of the BODIPY® emission (from the Arg-Val containing FRET substrate), and tetramethylrhodamine emission (originating from the single fluorophore substrate that is cleaved within the Arg-Arg sequence) is used to sort for clones exhibiting a desired catalytic selectivity. Note that on the cell surface BODIPY® and tetramethylrhodamine are sufficiently spatially resolved so that no fluorescence quenching of the BODIPY® is observed.

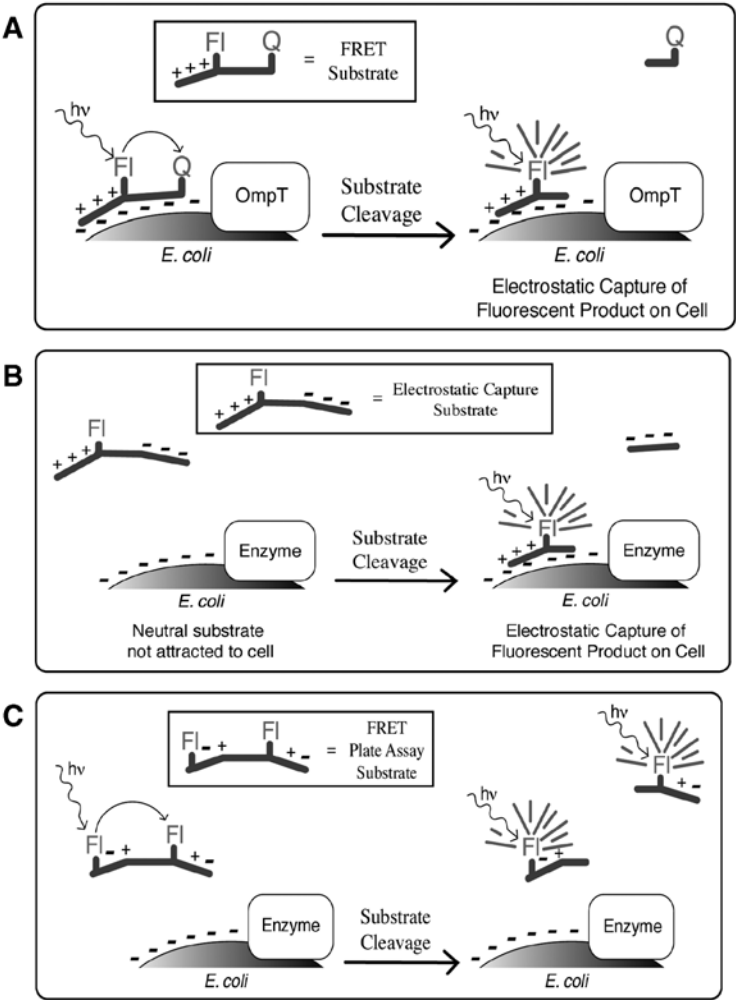


Fig. 1. Schematic showing the mechanism of (A) FRET substrates that upon hydrolysis lead to the generation of a positively charged substrate which is electrostatically retained on the cell surface; (B) electrostatic capture single fluorophore substrates; and (C) the FRET substrate (non-cell associated) used for secondary assays. FRET occurs because of proximity of two self-quenching fluorophores.

Clones isolated by flow cytometry must be examined via a secondary assay to confirm the pattern of catalytic activity. Since a typical cell sorting experiment will result in the isolation of $10\text{--}10^3$ clones, the secondary assay is best carried out using a microtiter-well plate format. The secondary assay allows the elimination of artifacts that result in aberrant fluorescence, either because

of improper interactions between the fluorophores and cell surface components or from alternative substrate cleavage sites. We use a homogeneous FRET assay where the BODIPY[®] fluorophore is used both as the acceptor and the donor (*see* **Fig. 1C**). The use of a single fluorophore greatly simplifies substrate synthesis. As with the sorting substrates above, enzymatic cleavage disrupts FRET between the two BODIPY[®] molecules resulting in a fluorescence increase. For the experiments aimed at changing the substrate specificity of OmpT from Arg-Arg to Arg-Val, the clones isolated by flow cytometry are grown in culture tubes or in microtiter wells on duplicate plates and incubated with appropriate bis-BODIPY[®] Arg-Arg or Arg-Val peptide substrates. The ratio of the fluorescence emission from these two FRET substrates provides an accurate measurement of the substrate specificity of the enzyme variant.

2. Materials

2.1. Bacterial Strain Growth and Flow Cytometer Preparation

1. *E. coli* cell lines suitable for a particular screening application. For the screening of OmpT libraries, we used strain *UT5600* (*F*⁻, Δ *ompT-fepC26*, *ara-14*, *leu-B6*, *azi-6*, *LacY1*, *proC14*, *tsx-67*, *entA403*, *trpE3*, *rfbD1*, *rpsL109*, *xyl-5*, *mtl-1*, *thi-1*) and derivatives (*see* **Note 1**).
2. Library of random OmpT variants under the control of the native OmpT promoter (*see* **Note 2**).
3. Becton-Dickinson FACSCalibur flow cytometer equipped with cell sorting module and workstation (*see* **Note 3**).
4. Luria-Bertani (LB) media from Difco without glucose.
5. 125-mL Ehrlenmeyer shake flasks.
6. Ampicillin (100 mg/mL).
7. Falcon polystyrene tubes for FACS sample injection port.
8. Sterile plastic conical 50-mL tubes.
9. 4 L of autoclaved, room temperature 0.1% glycerol.
10. 1 L of sterile, filtered 1% sucrose.
11. 3 L of 2% bleach.
12. 6 L of autoclaved, room temperature double distilled water.
13. UV/Vis spectrophotometer.
14. 100 mL 70% ethanol.

2.2. Flow Cytometric Population Gating and Sorting

1. Custom peptides fluorescently labeled with BODIPY[®] (BY) and tetramethylrhodamine (TMR). As an example the peptide substrates used for sorting OmpT libraries and for the secondary screening assays are shown below (*see* **Note 4**). The scissile bond is shown in bold: substrate **1** Glut-EEG**GR**RGK(TMR)-CONH₂, substrate **2** Ac-C(TMR)AR**V**GK(BY)GRGR-CONH₂, substrate **3** BY-EEG**GR**RGK(BY)-CONH₂, substrate **4** Y-EEG**RV**GRGK(BY)GR-CONH₂.
2. Sterile toothpicks.

3. Filtration devices and sterile 0.22- μ m, 47-mm diameter Millipore grid filters.
4. Culture tubes.
5. Agar plates containing 100 μ g/mL Ampicillin.

2.3. Verification of Catalytic Activity Using a Secondary Assay

1. Fluorescence plate reader with fluorescein compatible filters for excitation and emission.
2. Opaque Falcon 96-well plates with clear plastic covers.
3. Qiagen plasmid isolation kit.
4. Cryovac tubes.
5. Sterile 50% glycerol.

3. Methods

The methods below describe the 1) cell growth and flow cytometer preparation, 2) flow cytometric population analysis and sorting, and 3) verification of putative mutants based upon substrate specificity.

3.1. Bacterial Strain Growth and Flow Cytometer Preparation

1. Four 125-mL Erlenmeyer shake flasks are charged with 5 mL of sterile LB media containing 100 μ g/mL Ampicillin. Ten microliter aliquots of positive control cells, negative control cells and library cells are inoculated into individual flasks.
2. Cultures are incubated with shaking at 37°C for 14–16 h, and the OD₆₀₀ of each flask is measured (*see Note 5*).
3. Cells are harvested by centrifugation, washed with 1% sucrose, resuspended in 1% sucrose at an OD₆₀₀ = 1, and left on ice until needed.
4. The flow cytometer is turned on, communication with the Apple workstation verified, and 1 L of sterile, room temperature double distilled water is placed in the sheath. 2 mL of water should be placed in a polystyrene tube on the sample injection port (SIP), and should be present at all times during the cleaning operation. The cytometer settings are as follows: FSC = E01, SSC = 400, FL1-4 = 700. Threshold should be on SSC = 200, although this value may vary from cytometer to cytometer depending on the background event rate and the age of the instrument. All settings should be in log, and not in linear mode. Acquisition dotplots of SSC/FSC, FL1/FL2 (X/Y) should be arranged, and single parameter histograms of FL1 and FL2 should be visible. The status window and event counter of the cytometer should be visible at all times to detect any malfunction of the instrument. Pressurize the system, press the prime button, and set the cytometer to run. Observe the particulate pattern (*see Note 6*).
5. Three 50-mL plastic conical vials should be placed under the collection ports, and a region of high SSC/FSC values should be drawn, where events fall every 10–30 s, and set the sort gate to collect the sort region just defined. Each tube should collect between 40–50 mL of water over the course of 30 min. This procedure verifies that the instrument is functioning correctly, and that the operator has the opportunity to observe instrument malfunction in a timely manner. The

initial collection of water in the tubes verifies that all tube lines from the sorting mechanism to the collection tubes are clear of obstructions and salt crystals.

6. Following the collection of 50 mL sample in each tube, the water in the sheath should be discarded, and 2.0 L of a 2% bleach solution introduced into the sheath container. The sheath line running from the tank should not be plugged into the normal sheath socket, but should be plugged into the filter socket. Two milliliters of 70% ethanol can be placed in the SIP, and 70% ethanol from a squirt bottle should be used to sterilize the collection port nozzles. Again, a sort window in the upper right corner of the SSC/FSC dotplot should be used to collect events. When the bleach reaches the flowcell, the event rate will change substantially. The sorting mechanisms should not be exercised more than 1 time per second, and may require adjustment of the sorting window. All three collection ports should have between 40–50 mL liquid following the 30 min sterilization run.
7. Following sterilization, the sheath container must be removed from cytometer, and the inlet port should be carefully handled to avoid contamination, as all following operations must be sterile. The bleach should be returned to the proper vessel, and 100 mL of sterile water should be used to rinse the inside of the sheath reservoir. Following the rinse, 3 L of sterile, room temperature water should be placed in the sheath container, and the sheath container replaced in the cytometer. The filter by-pass should remain, due to bleach in the line between the reservoir and the socket. The machine should be run, and about 20 mL collected in collection port 1. At this time, the line from the sheath container should be replaced in the normal socket, and the filter reconnected. 50 mL of sterile, double distilled water should be collected in each 50-mL plastic conical flask.
8. The sheath should be emptied, and 3 L sterile 0.1% glycerol added to the sheath container in a sterile manner. Sorting should be continued, and the event rate should return to baseline values. If the event rate remains elevated, continue collecting 0.1% glycerol in the collection ports until the values return to normal (*see Notes 7 and 8*).
9. After the cytometer returns to baseline event values, the sorting should be discontinued, and the machine set to standby.

3.2. Flow Cytometric Population Analysis and Sorting

1. Reactions of peptides and cells are set-up in 1.5-mL Eppendorf tubes, with 78 μ L 1% sucrose, 20 μ L of the appropriate bacteria, and 1 μ L of each probe to be used. 20 μ L aliquot of the reaction mixture should be added to a polystyrene Falcon tube containing 1 mL of 1% sucrose. All reactions should be incubated for 10 min, and analyzed immediately by FACS after being placed in the Falcon tube.
2. It is critical to establish proper controls and gating windows for flow cytometric screening. Control bacteria not expressing enzyme and incubated without substrate are used to define a very broad SSC/FSC rectangle labeled R1. The objective of this window is to include all viable cell particles. Then the accept gate for the cytometer should be set to R1, meaning that all other particles outside of R1 are excluded from consideration. Secondly, a very small oval gate should be drawn

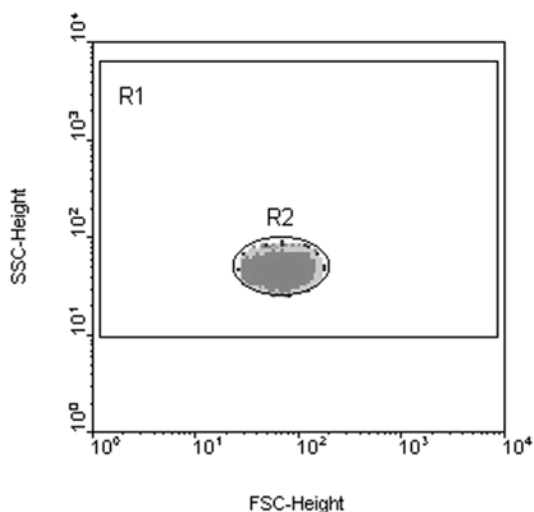


Fig. 2. FSC/SSC sort region used for identification of the candidate bacterial population.

to include the region of SSC/FSC to include the majority of events for healthy, control cells. This oval window is labeled R2, and is helpful for the isolation of cell populations exhibiting a higher cell viability, and for guaranteeing that the desirable fluorescence events are due to probe fluorescence rather than aberrant biological fluorescence. All subsequent gates should be set to include only those cells that fall inside R2 (*see Fig. 2*).

3. Each reaction described above should be analyzed using the following protocol: A 20 μL aliquot of the cell reaction should be added to 1 mL of 1% sucrose, and placed on the SIP immediately for analysis. Sample data is shown in **Fig. 3–5**.
4. Next, the library reaction should be set up, with 78 μL of 1% sucrose and 20 μL of library cells diluted to an $\text{OD}_{600} = 1$, and 1 μL of 10 μM stocks of substrates **1** and **2**. After 10–20 min of incubation, 20 μL of the library reaction should be placed in 1 mL of 1% sucrose, and analyzed. The strict R2 gate may need to be adjusted, but should not be enlarged. Substrate specificity regions can be drawn as a polygon, R3, to favor FL1 and thus cleavage of the preferred substrate activity (e.g., hydrolysis of Arg-Val by OmpT mutants) over FL2, and the non-preferred substrate (substrates containing the dipeptide Arg-Arg preferred by wild-type OmpT). Different libraries and incubation time points can also be explored, and low error-rate versus high error-rate libraries can be directly compared for the frequency of desirable clones as shown in **Fig. 6**, panels **A** and **B**, respectively.
5. The sort gate should be defined as R2 and R3, using Boolean gate definitions. The sort gate should be validated by setting the sort purity to the “recovery” mode, and initiating the sorting sequence. The sort region R3 may need to be adjusted to achieve the target frequency (*see Note 9*).

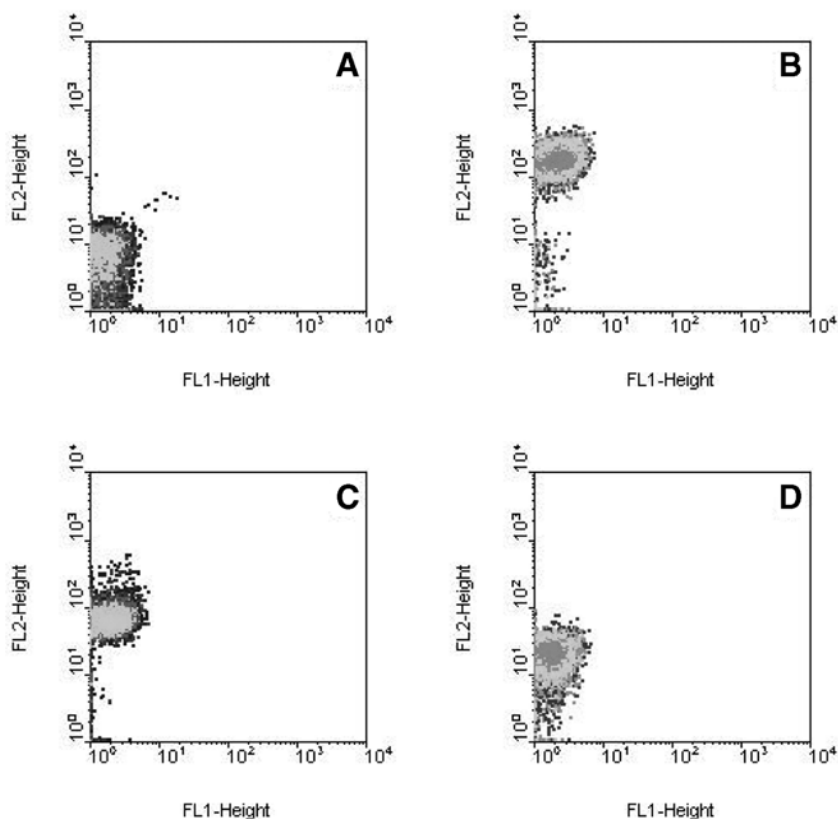


Fig. 3. Flow cytometric analysis of cells incubated with substrate **1** with (A) UT5600 (no OmpT activity); (B) UT5600/pML19 (OmpT expressed from a multicopy plasmid); (C) UT5600/pC5 (cells expressing the OmpT mutant C5 which exhibits 60-fold higher Arg-Val cleavage activity relative to the wild-type enzyme); and (D) UT5600/pG11 (a mutant OmpT displaying low activity towards Arg-Arg and Arg-Val peptides). The C5 and G11 mutants are described in (6).

6. Once the sort region has been tested, sorting of the library should commence. Cell recovery may vary over the course of the experiment, and the sort region R3 may need to be adjusted. The BD FACSCalibur sort cycle is ~30 min long, corresponding to three 10-min intervals in which the collected cells during that time frame are collected into sterile 50-mL plastic conical collection tubes 1–3.
7. Once a tube has been filled and the cytometer is collecting liquid into the next tube, the finished tube should be filtered onto a sterile Millipore filter, and placed on LB agar plates containing 100 μ g/mL Ampicillin.
8. The plates should be incubated at 37°C for 12 h, followed by periodic observation.

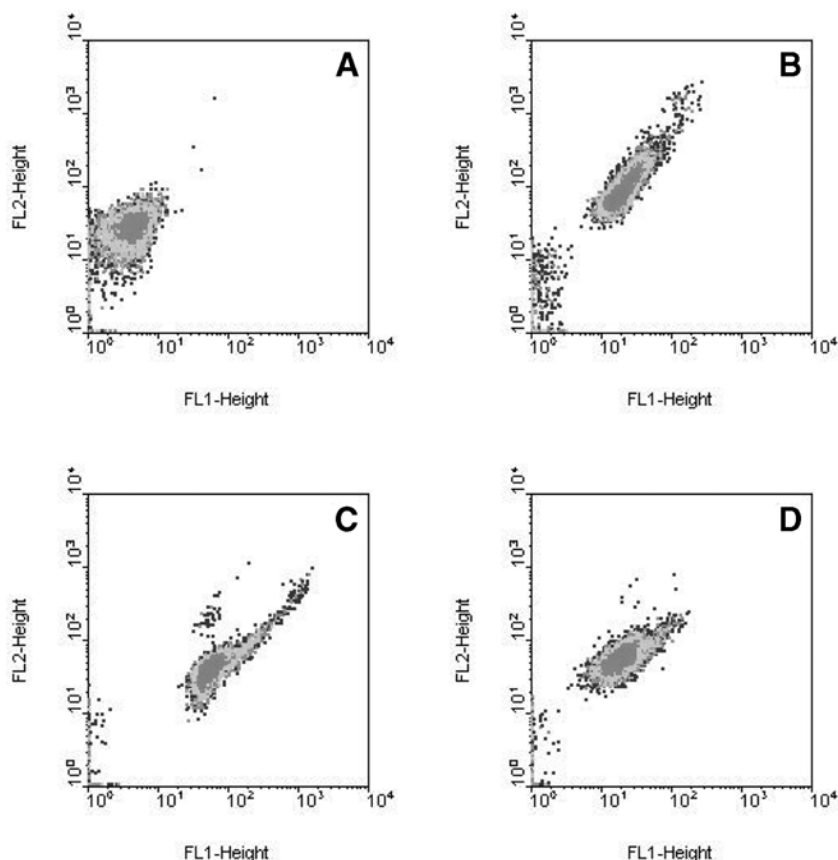


Fig. 4. Flow cytometric analysis of substrate **2** with (A) UT5600, (B) UT5600/pML19, (C) UT5600/pC5, and (D) UT5600/pG11. The proteins expressed from the various strains are described in the legend to **Fig. 2**.

3.3. Verification of Catalytic Activity Using a Secondary Assay

1. When the colonies are large enough to be seen by the naked eye, sterile toothpicks can be used to inoculate the colony into a culture tube with 2 mL of LB with 100 $\mu\text{g/mL}$ ampicillin. The tubes should be labeled, and incubated at 37°C for 12–18 h. At the same time, control cells should be inoculated into culture tubes with 2 mL of LB media with the appropriate concentration of antibiotics (see **Note 10**).
2. Once the cells have grown for 12–18 h, aliquots should be archived by removing 800 μL from the culture tube into cryovac vials, and adding 200 μL 50% sterile glycerol for storage at -80°C for future reference.

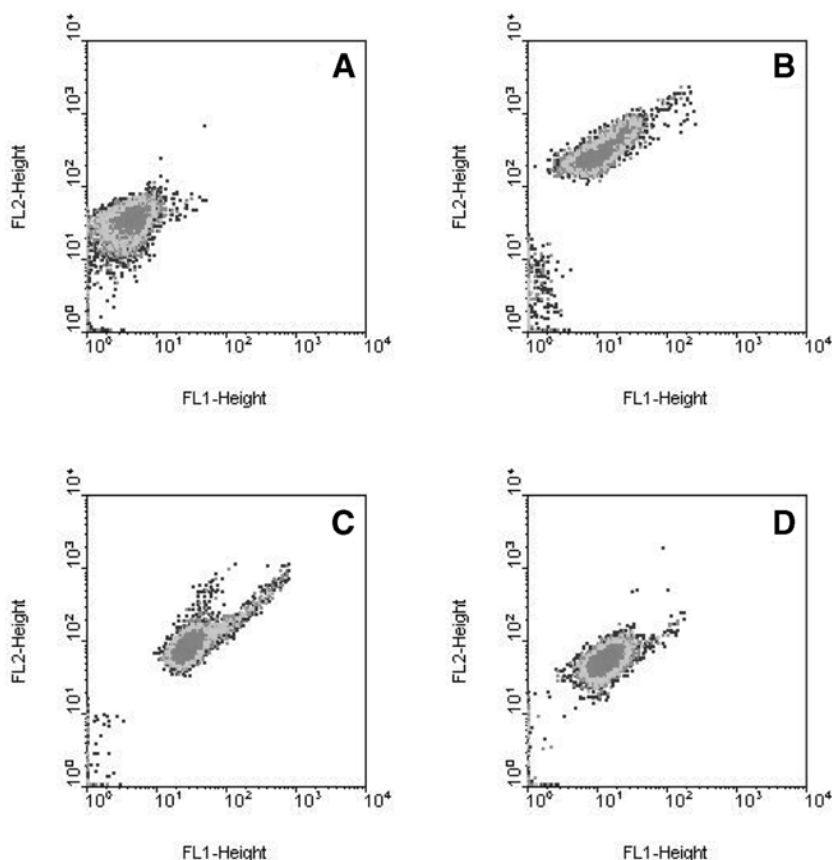


Fig. 5. Flow cytometric analysis of substrates **1** and **2** with (A) UT5600, (B) UT5600/pML19, (C) UT5600/C5, and (D) UT5600/G11.

3. A second aliquot of 100 μL should be removed, pelleted by centrifugation, washed with 1% sucrose, resuspended at $\text{OD}_{600} = 1$ in 1% sucrose, and the cells stored on ice until needed.
4. A sterile, opaque 96-well plate should be prepared by adding 70 μL of 1% sucrose to each well. 20 μL of each clone should be added into the duplicate wells with proper labeling, and 10 μL of a 100 μM stock of either substrate **3** or **4** (or other FRET substrates as required for a specific purpose) should be added rapidly to the proper wells. Only one plate should be prepared at a time (see **Note 11**).
5. Each plate should be immediately placed in a fluorescence plate reader. Excitation and emission should both be from the top, and the mode should be kinetic and the time points should be 15 min apart. The time-point sampling separation and overall time for the kinetic run will depend on the catalytic activity of the protein.

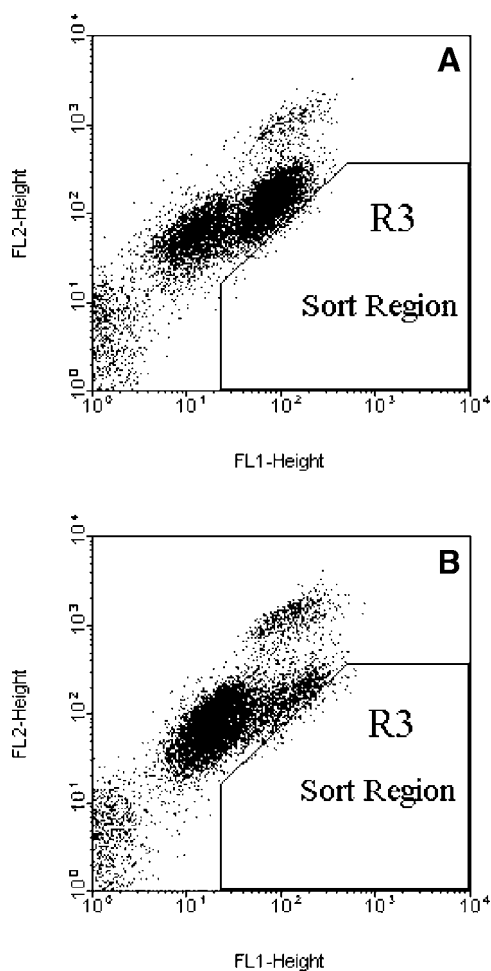


Fig. 6. Comparison of (A) Low error-rate and (B) High error-rate libraries, with substrates **1** and **2** and the sort region R3 for isolation of clones possessing the greatest specificity for Arg-Val relative to Arg-Arg cleavage activity.

- Following completion of all the fluorescence measurements, substrate specificity of the clones can be easily evaluated by comparing the fluorescence emission for the same clone obtained with substrates **3** and **4**. Clones displaying the desired fluorescence profile are identified.
- Identified clones can then be further preserved by returning to the culture tube, and removing the remaining media and cells, followed by plasmid isolation using a Qiagen plasmid isolation kit.

4. Notes

1. Control cells used for illustrating the method are UT5600, which is *ompT*⁻, pML19 is a plasmid that contains full length OmpT including the wild-type promoter, C5, an enzyme variant possessing high Arg-Arg and moderate Arg-Val cleavage, and G11, which possess very low Arg-Arg and low Arg-Val cleavage activity (6).
2. The Diversify™ Mutagenesis kit from Clontech has worked well in our hands for error-prone PCR amplification. For illustration purposes, the libraries depicted here are based upon the C5 enzyme variant discussed above, and have either low (0.1%) or high (1.0%) error rates as determined by DNA sequencing.
3. The following procedure has been written for a Becton-Dickinson FACSCalibur or FACSsort. The method may also work with a Partec PAS III, but will not work with any droplet based cell sorter, such as a Coulter EPIC, a BD FACStar or FACS Vantage, or a Cytomation MoFlo, due to the use of ionic electrolyte for droplet charge deflection.
4. Substrates are synthesized by conventional peptide synthesis and fluorophore conjugate labeling, and have been omitted owing to space. Excellent protocols for solid phase peptide synthesis (SPPS) are available, and fluorophore conjugation information is available from Molecular Probes at www.probes.com. Glut refers to glutaric anhydride capping of the amino terminus, and Ac refers to acetyl capping of the *N*-terminus. BY refers to the fluorophore BODIPY® FL, and TMR refers to tetramethylrhodamine. Substrate **1** is based on electrostatic principles for detection of wild-type Arg-Arg cleavage activity by flow cytometry, substrate **2** is a FRET-based substrate for the flow cytometric detection of Arg-Val cleavage activity, substrates **3** and **4** are FRET substrates employing BODIPY® self-quenching for plate-based determination of Arg-Arg and Arg-Val cleavage activities respectively.
5. OD₆₀₀ provides a point of reference for predicting the number of events. *E. coli* expressing heterologous protein, or over-expressing surface displayed protein are known for having lower events per OD₆₀₀ than would otherwise be expected.
6. Particular attention should be paid to the event counter and status windows. A normal background event rate for a clean BD FACSCalibur/FACSsort flow cytometer is ~200 events/s, although lower values can be observed. Typical sorts are done at rates of 1500 events/s, and the volume of reaction cells added to the SIP tube may need to be adjusted to fall in the ideal range. Simple scanning rates can vary from 500–2000 events/s.
7. If the event rate does not return to normal in 30 min, check the sheath and add more sterile water if required. Older cytometers may need up to 90 min to return to normal event values. However, if this does not occur, replace the SIP tube with a fresh tube containing 2 mL of the cleanest, most particulate free water available. Repeat three times.
8. For many flow cytometric protocols, but not this one, PBS is used as the sheath fluid. However, the electrostatic nature of the substrates employed in this method are salt sensitive, so a non-ionic sheath must be used. Be certain to completely

rinse the entire system for 90 min with double distilled water after the conclusion of sorting to remove all traces of the non-ionic sheath buffer.

9. Target cell frequency is a complicated function of the richness of the library being sorted, cell viability, and the number of cells to be analyzed post-sorting. Typical cell viability is 10–50%. Cell sorting frequency can also be altered by selecting the more stringent exclusion sorting mode for increased purity of the target cells. Recovery is used to guarantee all possible target cells are isolated, whereas exclusion sorting mode is used to guarantee that all isolated cells fell within the sort region specified.
10. 96-well plates may also be used when a large number of sorted clones are expected, however protein expression is always better using culture tubes due to superior oxygenation of the growth media
11. Different 96-well plates are available. Completely transparent plates result in unpredictable fluorescence reflections that can make positive identification problematic. Opaque plates with clear bottoms are also available, and can be used to both determine the optical density of a culture grown in a 96-well plate, as in **Note 10**, and to measure the fluorescence emission of the plate to substrates **3** and **4**.

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Improving Protein Folding Efficiency by Directed Evolution Using the GFP Folding Reporter

Geoffrey S. Waldo

1. Introduction

Recombinant protein expression in heterologous hosts such as *Escherichia coli* (*E. coli*) can provide large amounts of a protein of interest. Often, expression can result in the accumulation of the recombinant protein as inactive, insoluble inclusion bodies (1). When attempts at refolding inclusion bodies fail (2,3), directed evolution methods can provide an alternative route to stable, correctly-folded proteins (4–6). In directed evolution methods, typically a library of genetic variants is screened for improved folding and solubility. When high-throughput function or activity screens are unavailable for the protein of interest, a folding reporter assay can be used (7). Folding reporter assays typically couple the folding of the test protein with that of a protein with an easily-detectable function, such as an antibiotic resistance protein or a fluorescent protein such as green fluorescent protein (GFP) (7). A cyclical process of DNA recombination, mutagenesis, and subsequent rescreening can produce variants with further improvement (4–7). The GFP method has been used to improve the folding of several proteins while preserving the test protein enzymatic function and native-like structure (7–10).

2. Materials

1. GFP-UV plasmid incorporating Cramer variant “cycle 3” (Clontech).
2. *E. coli* strains DH10B (Invitrogen) and BL21(DE3) (Novagen).
3. Oligonucleotide primers.
4. Restriction enzymes.
5. Thermostable DNA polymerases Pfu and Pfu exo- (Stratagene).
6. DNase-I (Invitrogen).

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7. $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (Sigma).
8. IPTG (isopropyl- β -D-thiogalactopyranoside) (Sigma).
9. Calf intestinal phosphatase, T4 DNA ligase (New England Biolabs).
10. T4 DNA ligase 5X Buffer (Invitrogen).
11. Agarose gel equipment.
12. Polyacrylamide electrophoresis gel equipment.
13. Centrisep gel filtration spin columns (Princeton Separations).
14. QIAprep plasmid preparation kit, QIAquick PCR cleanup kit, QG gel solubilization buffer (QIAGEN).
15. 150-mm and 100-mm petri plates, velveteen replicator tool (Biorad).
16. Supported nitrocellulose membranes (130 mm and 82 mm) (Osmonics).
17. LB (Luria Bertani) nutrient media.
18. Plasmids pET28(a) and pET21(a) (Novagen).
19. Kanamycin and ampicillin antibiotics (Sigma).
20. Electroporator and electroporation cuvettes (1-mm path) (Biorad).
21. Plating (spreading) tool, Petri plate turntable, and plating beads (i.e., ColiRollers[®], Novagen).
22. 8-channel pipettors, Boekel 96-pin replicator (optional)
23. Illumination source (488 nm), observation filter (520 nm long-pass) (LightTools Research).
24. Digital camera (DC290, Kodak).
25. NIH Image (Mac users) or Scion Image (Windows users).
26. Replicator tool and velveteen squares, PGC Scientifics Corp.

3. Methods

The methods described below outline 1) the construction of the GFP fusion expression plasmid and C-terminal 6HIS expression plasmid, 2) creation of the cDNA GFP fusion expression plasmid library, 3) plating and expression of the GFP fusion expression library in *E. coli*, and 4) screening of the library and selection of optimized folding variants using GFP fusion fluorescence.

3.1. GFP Fusion Protein Folding Reporter Expression Plasmid and C6HIS Expression Plasmid

The vectors described in this section are available from the authors. Otherwise, these can be created using the following detailed protocols. The construction of the GFP fusion expression plasmid is described in **Subheading 3.1.1–3.1.4**. This includes 1) the description of the C-terminal GFP fusion expression vector, 2) the replacement of the Xba-1::BamH-1 fragment of pET28 by the corresponding fragment from pET21, 3) the insertion of a BamH-1::Xho-1 stuffer containing a flexible linker and EcoR-1 and Kpn-1 restriction sites, and 4) insertion of an Nde-1::BamH-1 frame-shift stuffer containing multiple translational stops.

The construction of the GFP variant to be inserted into the folding reporter vector is described in **Subheading 3.1.5.1–3.1.5.3**. This includes 1) creation of the F64L, S65T variant (**11,12**) of the “cycle 3” GFP from Crameri et al. (**13**), 2) silencing internal restriction sites in the GFP, and 3) ligation of the GFP into the folding reporter vector. The construction of the C6HIS expression plasmid is described in **Subheading 3.1.6**. This includes excision of the GFP from the folding reporter plasmid and replacement by a 6HIS motif (*see Note 1*).

3.1.1. Description of the GFP Folding Reporter Plasmid

The GFP fusion expression plasmid described below is based on the pET-28 vector, a component of the T7 expression system developed by Studier (**14**). Sequences inserted into the Nde-1::BamH-1 cloning site of the GFP folding reporter vector can be expressed as GFP fusion proteins under the control of the T7 promoter when transformed into *E. coli* DE3 cells, which contain an IPTG (isopropyl- β -D-thiogalactopyranoside) inducible T7 RNA polymerase gene. A protocol for creating a vector for expressing proteins with C-terminal GFP fusions is given, but knowledgeable users can modify the protocol as needed. Essentially any expression vector can be used that satisfies the following criteria: 1) the GFP fusion protein must not be expressed prior to induction, 2) the fusion protein must be strongly expressed after induction, 3) the fusion protein is expressed with a C-terminal GFP (i.e., X-L-GFP) where L is a flexible linker, such as the amino acids AGSAAGSG (**7**), and 4) the GFP folds well when expressed by itself i.e., variants such as described by Crameri et al. (**13**) are suitable. The structure of the cloning sites within the folding reporter vector is: Nde-1 XXX BamH-1-Linker-EcoR-1 GFP (Stop codon) Kpn-1 Xho-1. The genes of interest are cloned into the Nde-1::BamH-1 site without a stop codon, and in-frame with the flanking restriction sites. The Nde-1 (CATATG) contains the initiator methionine, and the BamH-1 (GGATCC) is translated gly-ser.

3.1.2. Modification of Xba-1::BamH-1 Fragment of pET28 Vector

1. The Xba-1::BamH-1 fragment of pET28(a) is replaced with the Xba-1::BamH-1 fragment from vector pET21(a). Plasmids pET28 and pET21 (1 μ g each) are separately double-digested in 50 μ L reactions with 5 units each of Xba-1 plus BamH-1 restriction enzymes (BamH-1 buffer), supplemented with bovine serum albumin (BSA).
2. The reaction is precipitated using 700 μ L of 90% ethanol containing 0.5 mM MgCl, the supernatant aspirated by pipet, the DNA pellet dried, and resuspended in 20 μ L of Tris-HCl, and resolved on 1.5% agarose gels.
3. The approx 300 bp fragment is excised from the pET21 digest gel, the approx 5 kb fragment is excised from the pET28 digest gel, the DNA recovered using the

QIAGEN gel extraction kit, ligated in a 20 μ L reaction with T4 ligase (New England Biolabs, NEB) according to manufacturer instructions, and 1 μ L electroporated into DH10B, and plated on LB agar plates containing 35 μ g per mL kanamycin (LB-Kan agar plates). Single colonies are frozen in LB media containing 20% glycerol (v/v) and stored at -80°C .

4. Plasmids are prepared, cleaned using QIAGEN plasmid prep kit, and screened for correct insert using restriction digestion followed by agarose gel size analysis.

3.1.3. Modification of BamH-1::Xho-1 Fragment of pET28 Vector

1. A plasmid is prepared from the modified pET28 **Subheading 3.1.2.** vector from an overnight 3 mL culture from freezer stock, 1 μ g is digested in a 50 μ L digest with 5 units each of BamH-1 and Xho-1 restriction enzymes for 2 h 37°C with BamH-1 reaction buffer (NEB), supplemented with BSA, 0.5 units of calf intestinal phosphatase added, the digest continued for 1 h, then cleaned using the QIAquick PCR cleanup kit.
2. A BamH-1::Xho-1 stuffer cassette is prepared by thermally annealing 5'-phosphorylated primer 1: 5' P-GATCCGCTGGCTCCGCTGCTGGTTCTGGCGAATT CGGTACCC 3' and primer 2: 5' P-TCGAGGGTACCGAATTCGCCAGAA CCAGCAGCGGAGCCAGCG 3' in restriction enzyme buffer 2 (NEB). 20 ng of the BamH-1 plus Xho-1 digested plasmid and 100 ng of the annealed cassette ligated in a 20 μ L reaction with T4 ligase, 1 μ L electroporated into DH10B, and plated on LB-Kan agar plates.
3. Single colonies are frozen in LB media containing 20% glycerol (v/v) and stored at -80°C . These are used to inoculate 3-mL LB-Kan liquid cultures.
4. Plasmids are prepared and screened for correct insert using restriction digestion followed by agarose gel size analysis.

3.1.4. Modify Nde-1::BamH-1 Fragment in pET28 Derivative Vector

1. A plasmid is prepared from a 3 mL culture of strain from **Subheading 3.1.3.**, then 1 μ g is digested in a 50 μ L reaction with 5 units each of Nde-1 and BamH-1 restriction enzymes supplemented with BSA for 2 h 37°C , 0.5 units of calf intestinal phosphatase added, the digest continued for one additional hour, then cleaned using the QIAquick PCR cleanup kit.
2. A phosphorylated Nde-1::BamH-1 stuffer cassette is prepared by thermally annealing primer 3: 5' P-TATGTGTTAACTGAGTAG 3' and primer 4: 5' P-GATCCTACTCAGTTAACACA 3' in restriction enzyme buffer 2 (NEB). 20 ng of the BamH-1 and Xho-1 digested plasmid and 100 ng of the annealed cassette are ligated with T4 ligase in a 20 μ L reaction and 1 μ L is electroporated into DH10B.
3. Freezer stocks are prepared from single colonies in LB media containing 20% glycerol (v/v) and stored at -80°C . These are used to inoculate 3-mL cultures in LB supplemented with 35 μ g/mL kanamycin.
4. Plasmids are prepared and screened for correct insert using restriction digestion followed by agarose gel size analysis.

3.1.5. Preparation of Modified GFP F64L+S65T “Cycle 3” Variant and Ligation into Modified pET 28 Vector

3.1.5.1. CREATION OF F64L+S65T MUTATIONS

1. Clontech GFP-UV vector, containing the “cycle-3” variant of Crameri et al. (**13**), is used as a template for two separate PCRs using proofreading Pfu polymerase. Fragment 1 (~ 200 bp) is obtained by PCR with the EcoR-1 GFP primer: 5' TTCTGGCGAATTCAGCAAAGGAGAAGAAGCTTTTCACT 3' and the downstream mutagenic primer 5' AAAGCATTGAACACCATAGGTCAGAGTAGTGACAAGTGTGG 3'. Fragment 2 (~ 520 bp) is obtained by PCR using the upstream mutagenic primer 5' CCAACACTTGTCCTACTCTGACCTATGGTGTTCATGCTTT 3' and the downstream Kpn-1 GFP primer: 5' GATATAGGTACCTTATTTGTAGAGCTCATCCATGCCATG 3'.
2. The two fragments are gel purified using the QIAquick gel extraction protocol and 5 ng of each are recombined at high concentration in a single 20 cycle PCR using the external EcoR-1 GFP and Kpn-1 GFP primers. The 720 bp fragment is resolved by agarose gel and recovered using the QIAquick gel purification kit.

3.1.5.2. SILENCING INTERNAL NDE-1 RESTRICTION SITE IN GFP

1. The cleaned DNA **Subheading 3.1.5.1.** is used as a template for PCR to remove the internal Nde-1 site present in the original GFP-UV cassette. Fragment 1 (~ 230 bp) is obtained by PCR with the EcoR-1 GFP primer: 5' TTCTGGCGAATTCAGCAAAGGAGAAGAAGCTTTTCACT 3' and the downstream mutagenic primer 5' AGTCATGCCGTTTCATGTGATCCGGATAACGGG 3'. Fragment 2 (~ 510 bp) is obtained by PCR using the upstream mutagenic primer 5' CCCGTTATCCGGATCACATGAAACGGCATGACT 3' and the downstream Kpn-1 GFP primer: 5' GATATAGGTACCTTATTTGTAGAGCTCATCCATGCCATG 3'.
2. The two fragments are gel purified using the QIAquick gel extraction protocol and 5 ng of each are recombined in a single 20 cycle PCR using the external EcoR-1 GFP and Kpn-1 GFP primers. The reassembled 720 bp fragment is gel purified using the QIAquick gel purification kit.

3.1.5.3. SILENCING INTERNAL BAMH-1 RESTRICTION SITE IN GFP AND LIGATION INTO MODIFIED pET28 VECTOR TO CREATE GFP FOLDING REPORTER VECTOR

1. The cleaned DNA **Subheading 3.1.5.2.** is used as a template for PCR to silence the internal BamH-1 site present in the original GFP-UV cassette. Fragment 1 is obtained by PCR with the EcoR-1 GFP primer: 5' TTCTGGCGAATTCAGCAAAGGAGAAGAAGCTTTTCACT 3' and the downstream mutagenic primer 5' CTGCTAGTTGAACGGAACCATCTTCAATGTTGT 3'. Fragment 2 is obtained by PCR using the upstream mutagenic primer 5' ACAACATTGAAGATGGTTCCGTTCAACTAGCAG 3' and the downstream Kpn-1 GFP primer: 5' GATATAGGTACCTTATTTGTAGAGCTCATCCATGCCATG 3'.

2. The two fragments are gel purified using the QIAquick gel extraction protocol and 5 ng of each are recombined in a single PCR using the external EcoR-1 GFP and Kpn-1 GFP primers.
3. The PCR product is digested with EcoR-1 and Kpn-1, and gel purified.
4. The sized, cleaned product is ligated into the modified pET28 vector, and transformed into DH10B. Freezer stocks are prepared from single colonies in LB media containing 20% glycerol (v/v) and stored at -80°C .
5. A small amount of freezer library is used to inoculate 3-mL culture in LB supplemented with 35 $\mu\text{g/mL}$ kanamycin.
6. Plasmids are prepared and screened for correct insert using restriction digestion followed by agarose gel size analysis, and DNA sequencing.

3.1.6. Creation of C-6HIS-Modified pET28 Vector

1. The folding reporter plasmid from DNA 3.1.4.3. is digested with BamH-1 + Kpn-1 (using BamH-1 buffer) and ligated with a cassette created by thermally annealing phosphorylated primers Primer 1: 5' Phos-GATCCACCATCATCACCACC ATTAATGGTAC and Primer 2: 5' Phos-CATTAATGGTGGTGATGATG GTGG, and transformed into DH10B.
2. Freezer stocks are prepared from single colonies in LB media containing 20% glycerol (v/v) and stored at -80°C . These are used to inoculate 3-mL cultures in LB supplemented with 35 $\mu\text{g/mL}$ kanamycin.
3. Plasmids are prepared and screened for correct insert using restriction digestion followed by agarose gel size analysis, and DNA sequencing. The resulting plasmid can be used to clone inserts using Nde-1 + BamH-1 restriction sites. Proteins cloned into the Nde-1::BamH-1 site will be expressed with the C-terminal tag GSHHHHHH.

3.2. Overview of 6-Day Directed Evolution Process

Day 1: Subheadings 3.2.1–3.2.4.

- PCR of target gene to obtain linear amplicon.
- Digestion with DNase-I to produce fragment pool.
- Reassembly of gene by primerless PCR.
- PCR with cloning primers.

Day 2: Subheadings 3.2.5–3.2.8.

- Restriction digest and gel purification.
- Ligation into folding reporter plasmid.
- Transformation into high-efficiency DH10B by electrotransformation.

Day 3: Subheadings 3.2.9–3.2.10.

- Prepare plasmids from plate wash.
- Transform into expression strain BL21(DE3).

Day 4: Subheading 3.2.11.

- Prepare freezer library from viable cell wash of BL21(DE3) plate.
- Plate out diluted expression library for GFP fusion expression screening.

Day 5: Subheadings 3.2.12–3.2.14.

- Induce expression plates with IPTG.
- Pick clones to master plate.
- Prepare and outgrow replica plates.

Day 6: Subheadings 3.2.15–3.2.17.

- Archive master plate.
- Induce replica plates.
- Photograph and analyze.
- Prepare plasmids from optima by vacuum aspiration of cell mass from master.

The steps are repeated until there is no further improvement in fluorescence. This process will take about three weeks to repeat three times. Individual optima are subcloned into a non-fusion vector **Subheading 3.1.18.** to express protein alone. Solubility is verified by fractionation into pellet and soluble fractions and SDS-PAGE analysis (**7.15**).

3.2.1. Creation of Target Gene GFP Fusion Expression Plasmid

1. PCR the target gene to be evolved using gene-specific primers with appropriate restriction sites. For the GFP folding reporter vector, the upstream primer should contain an Nde-1 site, and the downstream primer should contain a BamH-1 site (no stop codon). The restriction sites should be in-frame with the gene of interest, and should contain 5–7 nt of flanking sequence 5' to the restriction site.
2. Clone the digested PCR fragment into similarly digested GFP folding reporter plasmid (*see Note 2*) by ligation and chemical transformation into BL21(DE3). Plate out on nitrocellulose membranes on LB-Kan plates.
3. After 10–12 hr at 34°C, move the membrane to a fresh LB-Kan plate containing 1 mM IPTG, and induce until fluorescence is visible (4–6 h 37°C). Archive cell mass from some of the average “consensus” colonies in 20% glycerol-LB stocks and store at –80°C.
4. A small sample from each freezer library is scraped from the frozen stock and used to inoculate 3-mL cultures in LB supplemented with 35 µg/mL kanamycin.
5. Plasmids are prepared and screened for correct insert using restriction digestion agarose gel size analysis, and DNA sequencing. Plasmids containing the correct insert are used to transform DH10B.
6. A freezer stock is prepared from a single colony in LB media containing 20% glycerol (v/v) and stored at –80°C.

3.2.2. PCR of Target Gene Using Vector-Specific Flanking Primers

1. Use DH10B propagated plasmid from product in **Subheading 3.2.1.** as template for PCR. Appropriate flanking primers for the GFP folding reporter vector are upstream: 5' TCGAGATCTCGATCCCGCGAAATTAATACGACT 3', and downstream: 5' CCGTATGTAGCATCACCTTCACCCTCTCCA 3' (*see Note 3*). The first primer anneals about 80 nt upstream from the Nde-1 cloning site. The

second primer anneals about 80 nt down from the EcoR-1 restriction site and within the GFP gene. Perform a 100 μ L PCR, sufficient to yield 3–5 μ g of purified DNA.

2. Clean using the Qiaquick PCR cleanup kit, and elute with 100 μ L 10 mM Tris-HCl buffer, pH 8.5 (no EDTA). Verify the product by agarose gel analysis (lane 2, **Fig. 1**).

3.2.3. DNase-I Digest of PCR Amplicon

1. The DNase-I fragmentation is that used by Waldo et al. (7) and is a modified version of that described by Arnold and co-workers (6) in which cobalt is used as the counterion *in lieu* of manganese (see **Note 4**). Briefly, 90 μ L of cleaned PCR product (~ 5 μ g) is combined with 10 μ L of 0.5 M Tris-HCl, pH 7.5, 2 μ L of 100 mM CoCl₂ in a 100 μ L thermal cycling PCR tube, mixed by pipetting, and incubated for 1 min at 15°C in a thermocycler.
2. 0.15 units of DNase-1 is added, mixed by pipetting, and the sealing cap applied.
3. The tube is incubated for 3 min at 15°C for digestion, then 5 min at 90°C to inactivate the DNase-I.
4. The sample is cleaned by size exclusion on a Centriscap spin column previously equilibrated with 10 mM Tris-HCl, pH 8.5, according to manufacturer instructions. The eluted DNA fragments should average 20 bp in length (agarose gel size analysis, lane 3, **Fig. 1**).

3.2.4. Primerless “Shuffling PCR” (Stemmer Protocol) and Reamplification with Outside Primers

1. Assemble two 25 μ L reactions in PCR tubes on ice. Reaction 1 “high concentration” contains 17.5 μ L of the eluted DNA fragments. Reaction 2 “low concentration” contains 5 μ L of the eluted DNA fragments and 12.5 μ L of ddH₂O. The PCR protocol uses no outside primers and is based on the method published by Arnold and co-workers (6) with the modification that *Pfu* exo-(non-proofreading) polymerase is used for the reassembly step. The 72°C extension step starts with a 25 s extension step, and is increased by 5 s per PCR cycle for a total of 35 cycles.
2. Analyze both reactions by agarose gel to determine the yield and extent of reassembly. Select the product that has a modulus near that of the starting PCR template (see **Note 5**, **Fig. 1**, lane 4).
3. Assemble a 100 μ L PCR on ice, using 3 μ L of the reassembled product as template. Use the same outside primers from **Subheading 3.2.2**. Run a diagnostic 1.5% agarose gel to confirm amplification. Clean with PCR Qiaquick according to manufacturer’s instructions, eluting with 75 μ L 10 mM Tris-HCl, pH 8.5. Yield should be approx 5 μ g total (lane 5, **Fig. 1**).

3.2.5. Restriction Digest of Target and Gel Purification

1. The 50 μ L (~ 3 μ g) of cleaned PCR product is restricted in a double-digest with 25 units each of Nde-1 and BamH-1 restriction enzymes (BamH-1 reaction buffer, NEB) supplemented with bovine serum albumin (BSA) according to manufacturer recommendations, and incubated at 37°C for 3 h.

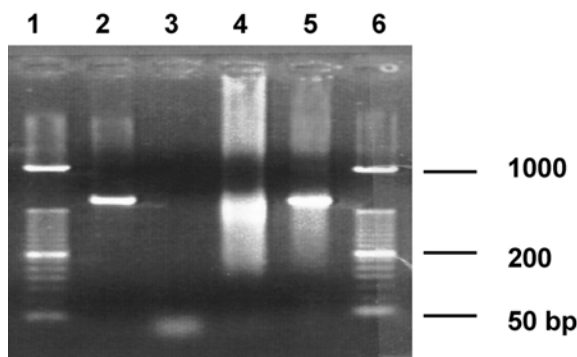


Fig. 1. Gene shuffling. The full-length gene pool of a 600 bp target gene is amplified by PCR (2); cut into small approx 20 bp fragments with DNase-I (3); reassembled by primerless PCR until nearly full-length (4); and reamplified to yield the full-length gene (5). Molecular weight standards (1 and 6). Only the low concentration reassembly reaction is shown in lane 4.

2. The DNA product is ethanol precipitated by adding 750 μL of a solution of 0.5 mM MgCl_2 and 90% ethanol in a 1.5-mL eppendorf tube, and incubated at room temperature ($\sim 20^\circ\text{C}$) for 15 min. The precipitated DNA is centrifuged for 15 min at 13,000g, decanted, dried and resuspended with 20 μL 10 mM Tris-HCl, pH 8.5.
3. The sample is resolved by preparative agarose gel electrophoresis (*see Note 6*). The band is excised and cleaned with the QIAquick gel extraction kit according to manufacturer instructions (*see Note 7*).

3.2.6. Vector Preparation

1. Assemble in 0.5-mL eppendorf 50 μL of a plasmid prepared from the DH10B strain containing the folding reporter, 7.2 μL 10 BamH-1 buffer, 3.6 μL of 20 BSA (diluted from 100 NEB stock), 3.6 μL each of Nde-1 and BamH-1 restriction enzymes. Vortex and centrifuge briefly. Incubate for 2 h at 37°C .
2. Vortex and centrifuge briefly, then add 0.2 μL of calf intestinal phosphatase (NEB) and continue the 37°C incubation additional 1 h (*see Note 8*).
3. Clean with QIAquick PCR cleanup kit and elute with 75 μL of 10 mM Tris-HCl, pH 8.5.

3.2.7. Library Ligation

1. Assemble in a thermal cycling PCR cycling tube: 38 μL of gel-purified insert (~ 500 ng), 9 μL of cleaned digested vector (~ 100 ng), 9 μL of 5X T4 DNA ligase buffer (Gibco BRL), and 4 μL T4 DNA ligase (NEB). Mix by pipetting. Incubate 3 h 30°C .
2. Transfer ligation from ligation PCR tube to 1.5-mL eppendorf. Wash walls of PCR ligation tube with 10 μL of 10 mM Tris-HCl, pH 8.5, until all DNA has been resuspended (*see Note 9*). Add this to the 1.5-mL eppendorf.

3. Ethanol precipitate by adding 700 μL of 0.5 mM MgCl_2 in 85% ethanol, vortex, centrifuge briefly, incubate 15 min room temperature, centrifuge 13,000g for 15 min, decant supernatant, dry pellet, and resuspend carefully with 10 μL 10 mM Tris-HCl, pH 8.5 (see **Note 10**).

3.2.8. Large-Scale Transformation into DH10B by Electroporation

1. Thaw 100 μL aliquot of DH10B cells on ice (10–15 min).
2. Pre-chill 100 μL ddH₂O on ice (10 min), pre-chill 3 e-transformation cuvetts (2 mm gap) per library.
3. Combine 100 μL thawed DH10B and 50 μL pre-chilled ddH₂O, add the diluted DH10B cells to same tube on ice containing the 10 μL resuspended, ligated DNA, flick to gently mix, incubate 5 min on ice.
4. Transfer 50 μL of transformation mix in e-cuvet. Set electroporator to 2.5kV, 25 uFD, 200 ohms pulse shaper (4 uS pulse τ ; if τ was < 3.5, repeat).
5. Recover each of the three transformations by immediately resuspending in 1 mL SOC (**16**) in 12 mL culture tube with shaking at 37°C for 1.5 h.
6. Centrifuge the recovered cultures in 1.5-mL eppendorf tubes for 1 min 13,000g.
7. Leave 200 μL of supernatant over the pelleted cells (remove ~ 800 μL supernatant). Resuspend cells by pipetting.
8. Pool the 3 tubes into one 1.5-mL eppendorf. Plate the library onto a large Kirby-Bauer (150 mm diameter) selective media LB plate supplemented with 35 $\mu\text{g}/\text{mL}$ kanamycin.
9. On a separate LB-Kan agar plate, plate 1/500th of library. Incubate 12–16 h at 37°C. Expect >500 colonies on counting plate. Library should be a lawn. Libraries containing at least 30,000 individuals are acceptable.

3.2.9. Library Plasmid Recovery

1. Add 12 mL LB to Kirby Bauer DH10B library plate from plates in **Subheading 3.2.8.**, resuspend with spreader.
2. Transfer suspension to 15-mL Falcon tube, vortex to resuspend.
3. Perform QIAgen plasmid prep on 750 μL of cell suspension. Cell mass prepped should be equivalent to 3 mL of overnight LB culture, i.e., approx 50 mg pellet.

3.2.10. Expression Strain Transformation

1. Perform four 50 μL chemical transformations of BL21(DE3), each using 4 μL of the plasmid.
2. Recover each transformation in 1 mL SOC for 1.5 h at 37°C.
3. Pool transformations by centrifugation, resuspend the combined cell mass in approx 800 μL SOC, and plate on a single 150 mm diameter LB-Kan plate.
4. Plate a counting plate (1/400th of library) by transferring 2 μL of the 800 μL recovery suspension to a small 80 mm diameter Petri plate (counting plate). Expect a lawn on the master plate, and approx 500–2000 clones on the counting plate. Grow the plates at 32°C overnight to prevent overgrowth.

3.2.11. Preparation and Outgrowth of Expression Library Screening Plates

1. The following morning, resuspend the cells from the expression library plates (**Subheading 3.2.10.**) using 12 mL LB media.
2. Dilute to 1.0 OD (600 nm) in LB containing 20% glycerol, freeze in 100- μ L aliquots, and store at -80°C .
3. Label five 142 mm polyester reinforced nitrocellulose membranes (Osmonics), wet with sterile water, apply membranes to LB-Kan 150-mm diameter agar plates, and allow membrane/plate sandwiches to dry thoroughly in a laminar flow hood (~ 0.5 h).
4. Thaw an aliquot of the 1.0 OD (600 nm) expression library, vortex, and perform two sequential 320-fold serial dilutions into SOC.
5. Apply 800 μ L of the diluted cell suspension on each of the screening plates, spread uniformly with a plating tool or with plating beads, and allow to dry in a laminar flow hood for approx 10 min until no longer damp. Incubate 10–15 h 32°C (*see Note 11*).
6. When plating the expression library for screening, also prepare any control GFP fusion clones (i.e., your wild-type gene cloned into the GFP folding reporter, as well as any optima from previous rounds) by streaking freezer stocks for single colonies directly onto a LB-Kan plates (no nitrocellulose membrane).

3.2.12. Induction of Expression Library Screening Plates

In order to complete induction, picking to master plate, and replica plating in one d, induction of the overnight growth plates must be started early in the day. The overnight growth plates **Subheading 3.2.11.** should have colonies approx 1–1.5 mm diameter.

1. Early in the morning, pre-warm five LB-Kan 150 mm diameter plates (supplemented with 1 mM IPTG) at 37°C for 30 min, then transfer the membranes bearing colonies (face up) to the IPTG induction plates using blunt forceps. Make sure to exclude bubbles.
2. Incubate approx 4–5 h at 37°C or until fluorescence is clearly visible by eye using an appropriate illumination source and observation filter (*see Note 12, Fig. 2*).

3.2.13. Picking Optima to Master Plate

1. Prepare a gridded plate by drawing 40 guide marks on the back of a standard 80 mm Petri dish plate containing the appropriate antibiotics (no nitrocellulose, no IPTG). Also include a row of guide marks for transferring control clones (*see Note 13*).
2. Pick about 40 of the brightest clones from the induced library expression plate (approx 8 per each of the 5 plates) onto the gridded plate using a sterile needle. Take care to exclude truncation artifacts (*see Note 14*).
3. Transfer some colony mass from the Petri plates bearing the wild-type GFP fusions as well as any top optima from previous rounds of evolution.
4. Incubate approx 5–6 h at 37°C until approx 1–2 mm diameter.

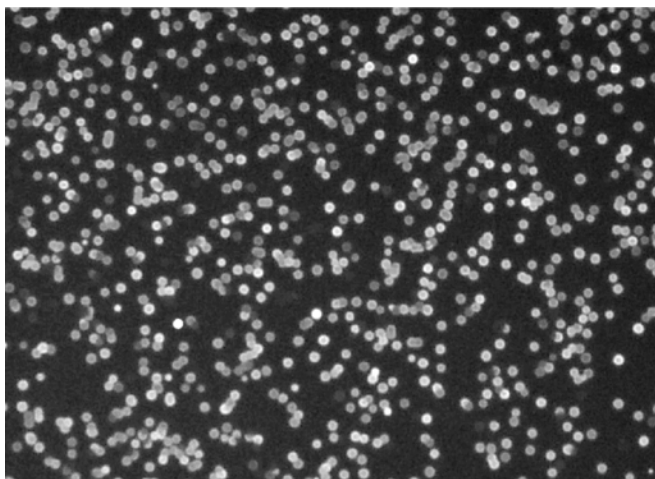


Fig. 2. Appearance of *E. coli* colonies expressing a mutated library of *Pyrobaculum aerophilum* nucleoside diphosphate kinase as GFP fusion proteins (488 nm excitation, 520 nm long-pass emission filter, imaged with a digital camera Kodak DC-290). Several desirable clones with greater than average fluorescence are visible. One such clone is just below and slightly to the left of the center of image. An outlier artifact (too bright relative to the continuum population distribution) is visible to the left of the image. The artifact was subsequently verified to be expressing a truncated peptide (by SDS-PAGE).

3.2.14. Preparing Replica Plates

1. Label two 80 mm diameter nitrocellulose membranes, one to be induced at 27°C and the other at 37°C.
2. Moisten the membranes with sterile water and transfer the membranes to two 100 mm Petri plates (LB-Kan). Allow the plates bearing the membranes to dry thoroughly (approx 0.5 h) in a laminar flow hood.
3. Perform a velvet colony lift from the overnight master (**Subheading 3.2.13.**) using a replicator tool according to manufacturer's instructions. Transfer to each of the replica plates using moderate pressure. Apply pressure uniformly to the entire plate to avoid incomplete transfer.
4. Grow out the replicas and master approx 6–7 h at 37°C until cell mass is visible.
5. Wrap the master and store at 4°C for later recovery of optimal colonies. The master can be stored for up to two wk.

3.2.15. Inducing Replica Plates

1. To induce the replicas, transfer the membranes face up on to two LB-Kan plates containing 1 mM IPTG. Incubate one plate at 27°C for 7–8 h, the other at 37°C for 5–6 h until fluorescence is clearly visible (**Fig. 3**, see **Note 15**).

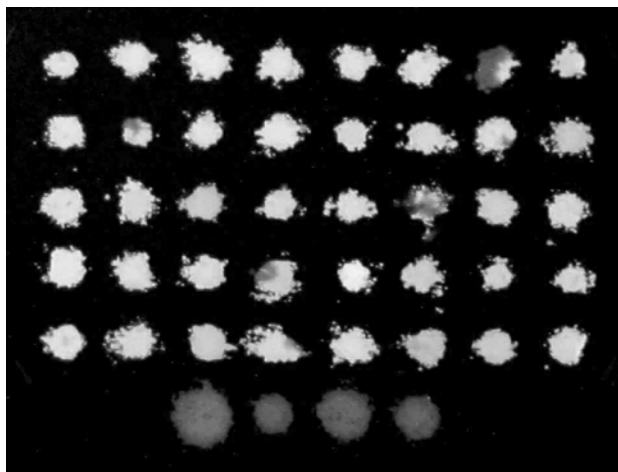


Fig. 3. Velvet replica plate induced at 37°C. Top 5 rows: 40 brightest clones expressing GFP fusions from round 1 of evolution of nucleoside diphosphate kinase (NDP-K) from *P. aerophilum*. Lowest row: four wild-type NDP-K-GFP fusion controls. Note mixed colonies (blotched appearance) in row 1, column 7; row 2 columns 2 and 7; row 3 column 6; row 4 column 4; and row 5 column 4. Such artifacts occur when cell mass from two or more colonies are transferred from the library induction plate in a single pick.

2. Using a multichannel pipettor or Boeckel pin replicator, transfer some cell mass from each colony on the master plate to a 96-well 20% glycerol-LB stock tissue culture plate, approx 175 μ L, for archiving. Store at -80°C .

3.2.16. Imaging and Analysis of Induced Replica Plates

1. Image the plates using a digital camera and appropriate excitation and emission filters. Bracket the exposure time to avoid saturating the pixel depth. The appropriate exposure time will vary depending on the range of fluorescence levels (*see Note 16*).
2. Using image processing software such as NIH Image or Scion Image, extract the green channel. Use thresholding and “blob picking” functions to select the colony images and determine the average intensity of each colony, including the optima and controls.
3. Export the data to a spreadsheet program such as Excel to facilitate analysis. Desirable clones will have two essential characteristics: 1) they should be brighter than the wild-type or optimized control clones from previous round(s), and 2) each should be as bright or brighter at 27°C relative to 37°C (*see Note 17*). Typically, the top 1/3 of the set of optima is used for the subsequent round of shuffling and screening.

3.2.17. Preparation of Pooled Plasmid for Further Round(s) of Shuffling and Screening

1. Prepare a pooled mix of cells from the desired optimized clones. Cells can be pooled by aspiration from the master plate, or by combining samples from overnight liquid cultures. The amount of combined cell mass should be equivalent to a standard 3 mL overnight liquid culture (~ 30 mg cell mass).
2. Prepare a plasmid pool from the pelleted cell mass using the QIAprep kit.
3. Use the pooled plasmid as the template for PCR in the next round of shuffling (**Subheading 3.2.2.**).

3.2.18. Completion of Cycles of Shuffling and Verification of Protein Expression

After 3–4 rounds of directed evolution, there will be no further improvement in fusion fluorescence, and the trajectory is complete.

1. Subclone the top evolved optimized clones from the GFP fusion vector using standard molecular biology techniques (**16**), into the C6HIS expression vector from **Subheading 3.1.6.** (*see Note 18*). Appropriate primers flanking the insert are described in **Subheading 3.2.2.**
2. The expressed proteins can be assessed for solubility by sonication in an appropriate buffer system (for *P. aerophilum* NDP-K 0.1 M Tris-HCl, pH 8.0, 0.15 M NaCl, 10% glycerol), fractionation into soluble and insoluble fractions by centrifugation, and SDS-PAGE followed by gel densitometry analysis (**7,15**).
3. The clones should be sequenced to determine identity of the mutations. Typically, 3–6 point mutations are observed.

4. Notes

1. It is important to note that many proteins are less soluble when expressed as N-terminal fusions with GFP, than when expressed alone. This is because even well-folded proteins will interfere with the folding of GFP to some extent (**7**). Hence, GFP fusion fluorescence is correlated with non-fusion folding and solubility. In other words, the fluorescence of cells expressing a test protein as a fusion with GFP is correlated with the folding yield of the test protein expressed alone (without the GFP moiety). The solubility of a test protein expressed alone and with GFP attached are not necessarily equivalent.
2. Other restriction sites can be used if the gene has internal restriction sites that are incompatible with the Nde-1 + BamH-1 sites used here. An adaptor cassette can be created using appropriate phosphorylated primers (**16**). These would have the new internal site(s) in-frame with the flanking Nde-1::BamH-1 sites. For example, suppose the target gene contains an internal BamH-1 site, but no Spe-1 site. A new internal Spe-1 cloning could be created by ligating an Nde-1::Spe-1::BamH-1 stuffer into the folding reporter vector. The insert could then be cloned into Nde-1::Spe-1. Note that the gene and restriction sites must not disrupt the reading frame.

3. Flanking sequences of approx 30–50 bp are necessary so that the entire gene can be mutated. Using gene-specific primers to amplify the template for shuffling will prevent mutagenesis in the footprint of the primers during subsequent reamplification.
4. Manganese can oxidize at alkaline pH.
5. Analyze the products by 1.5% agarose gel electrophoresis. The ideal reaction will yield a product whose modulus is at or slightly below the starting template MW (**Fig. 1**). Too high a starting concentration of fragments will yield a smear product band that is too short. Insufficient concentration of fragments will yield a product that is faint and too short. Occasionally, the product will be heavier than the expected mass (greater than that of starting template). This can arise from some single-stranded product formation during reassembly, or ramification. This product is still satisfactory for the next step (fillout with outside primers).
6. This crucial step removes small DNA fragments that would otherwise interfere with screening the full-length product.
7. Be sure to add 1X gel volume of isopropanol, vortex but DO NOT pellet prior to loading on column. Perform 2 additional washes with 500 μ L QG (gel dissolution buffer) to remove traces of agarose. If agarose is not removed, it will inhibit the subsequent ligation.
8. Do not omit the treatment by calf intestinal phosphatase. Omission of the phosphatase will lead to an excessively high background from self-ligated vector.
9. Wash walls of PCR ligation tube with 10 μ L of Tris-HCl, pH 8.5, one quadrant at a time until all DNA has been dissolved. Buffer will adhere to adsorbed DNA on walls of tube. When DNA is removed, buffer will no longer adhere to walls.
10. Place in airflow for 5–10 min (depending on ambient humidity) to remove ethanol (pellet will be slightly damp). Do not overdry, or DNA will be difficult to dissolve.
11. It is much easier to achieve a uniform distribution of clones using glass beads to spread the cell suspension by shaking the plate to move the beads around. Discard the beads while the plate is still wet, and allow the plate to dry. Novagen sells suitable plastic-coated glass beads (“Coli Rollers”). The specified dilution should yield approx 5000 clones per Kirby-Bauer plate.
12. Excite the red-shift GFP using an appropriate filter, such as a 488 nm, 50.8 mm square filter (Edmund Scientific, H43168). This can be inserted into a standard 35 mm slide projector. For observing or photographing the emitted fluorescence, use a 520 nm 50.8-mm square filter (Edmund Scientific, H43173) or similar. Complete illumination and visualization systems are available, such as the Illumatool Model LT-9500, LightTools Research.
13. It is helpful to produce the grid pattern in the standard 96-well plate spacing, to facilitate subsequent manipulation of the clones using a multichannel pipettor or replicator tool. A plastic guide from a pipet tip box is helpful as a stencil.
14. A small population of extremely bright clones can occur if aberrantly small DNA fragments, coding for small soluble peptides, are inadvertently cloned in-frame with the C-terminal GFP reporter (*see Fig. 2*). Normally, the gel-purification step (**Subheading 3.2.5.**) rejects these small fragments, but a few (up to 10 per

10,000 clones) can occasionally be seen. These form a small population of exceptionally bright outliers, but are easy to avoid, since they are many times brighter than the authentic clones, while the normal distribution of clone brightness forms a smooth histogram.

15. Expression rate is lower at 27°C than 37°C, and the induction times are designed to give complete induction without compromising cell viability. Induction is complete when there is no further increase in brightness with time.
16. We use a KODAK DC-290 digital camera, on a photographic boom from Edmund Scientific. You can also jury-rig a ring-stand and clamp with a 1/4-20 right-angle screw. Use 1/32, 1/8, 1/4, 1/2, 1, 2, and 4 s exposure times to bracket full range of intensities, picking the image with counts approx mid-range of 256-bit image (i.e., 128 counts). Do not exceed linear dynamic range of CCD camera for best results.
17. Many proteins fold better at lower temperatures. Thus, the same clone will generally be brighter on the 27°C plate relative to the 37°C plate.
18. It is important that the protein solubility be assessed by expression without the attached GFP. Even well-folded proteins will generally interfere with the folding of GFP to some extent (7), causing the effective folding yield (and solubility) of the fusion protein to be lower than that of the protein expressed alone. A variety of cloning vectors can be used for subcloning the evolved inserts, as long as the restriction sites of the destination vector are compatible with those used in the folding reporter vector (Nde-1 + BamH-1 in this example).

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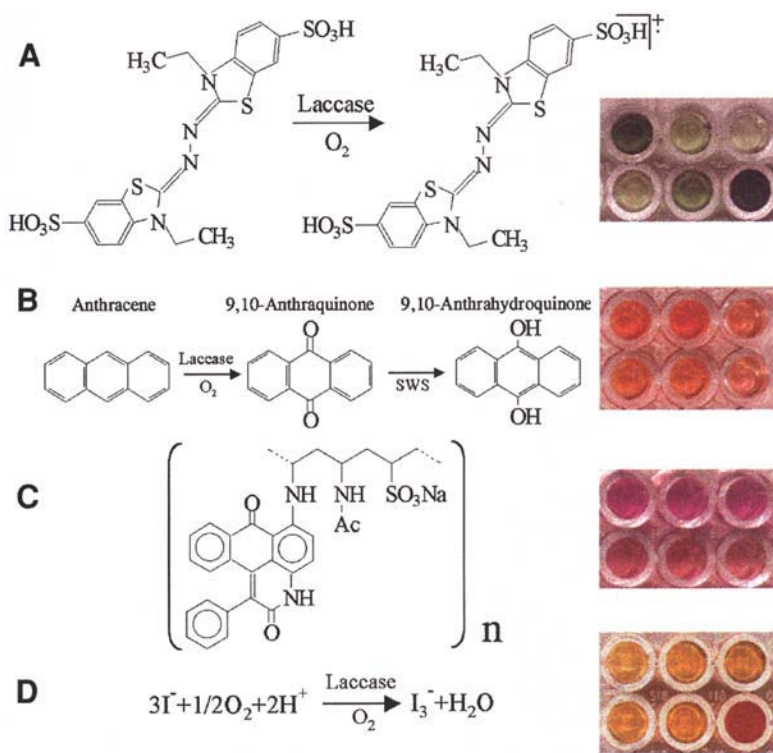
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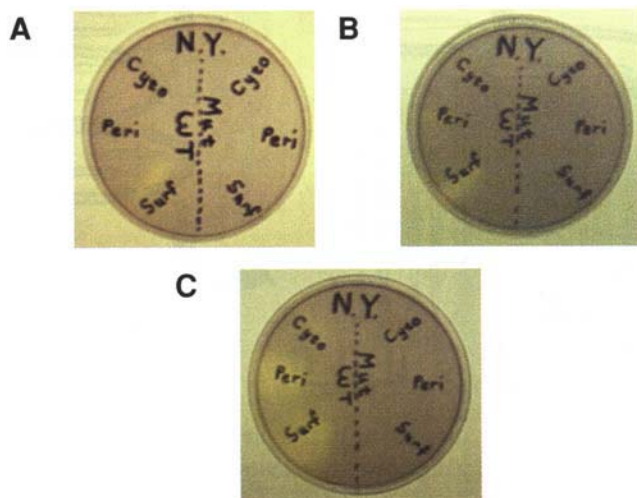
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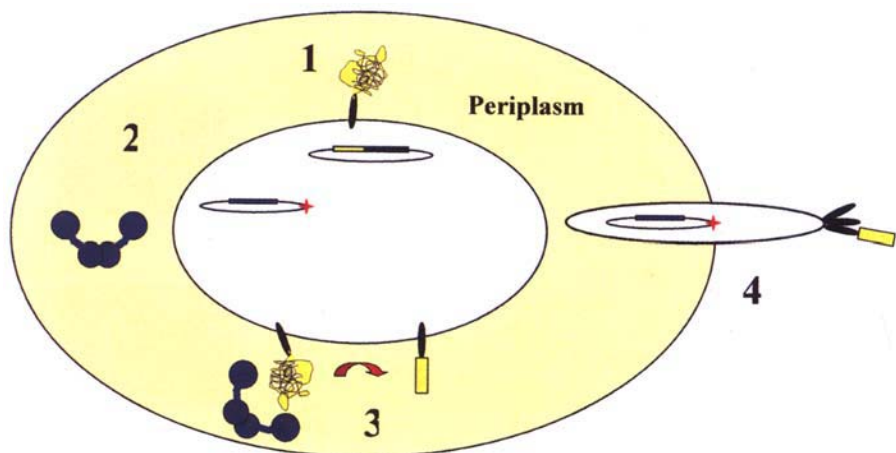
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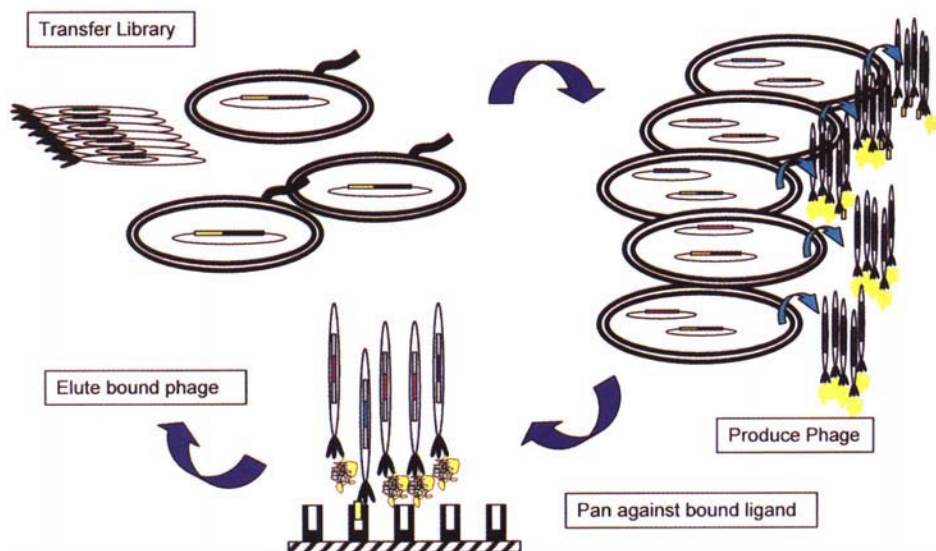
Color Plate 1, Fig. 1. (see discussion in Chapter 20, and full caption on p. 196). Colorimetric assays for screening laccases.



Color Plate 2, Fig. 1. (see discussion in Chapter 21, and full caption on p. 205). Fig. 1. pH sensing plate assay of wild-type (WT, on left) and S42-A mutant (Mut, on right) cutinase.

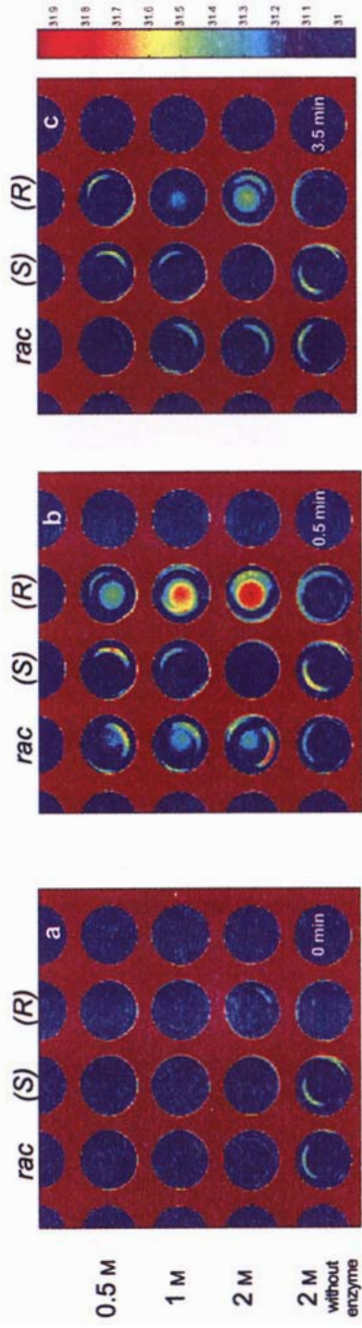
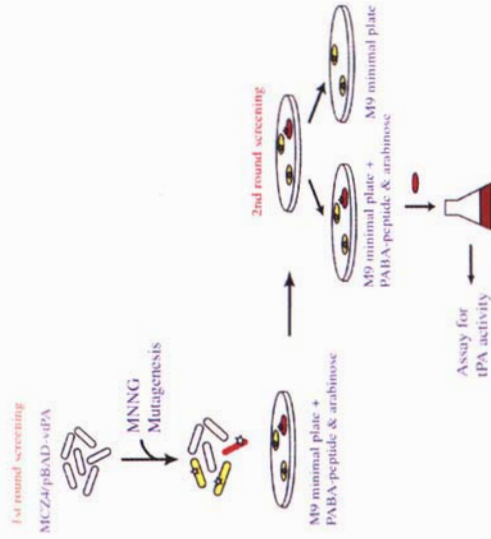


Color Plate 3, Fig. 3. (see discussion in Chapter 25, and full caption on p. 246). Illustration of the major schematic concepts of phage display platform.



Color Plate 4, Fig. 4. (see discussion in Chapter 25, and full caption on p. 247). Schematic diagram of cycle of phage display selection.

Color Plate 5, Fig. 5. (*see discussion in Chapter 25, and full caption on p. 253*). Illustration of genetic selection methodology.





Color Plate 7, Fig. 1. (see discussion in Chapter 27, and full caption on p. 285). Pipet robot *Genesis* (Tecan GmbH, Maennedorf, Switzerland) with integrated carousel (right).

Directed Enzyme Evolution

Screening and Selection Methods

Edited by

Frances H. Arnold*California Institute of Technology, Pasadena, CA***George Georgiou***University of Texas at Austin, Austin, TX*

Directed evolution, the application of evolutionary design to enzyme engineering, requires effective screening strategies to isolate those proteins that perform a desired function from the libraries generated by the techniques. In *Directed Enzyme Evolution: Screening and Selection Methods*, seasoned practitioners from many leading laboratories describe their leading and readily reproducible screening strategies for isolating useful clones. These techniques have been optimized for sensitivity, high throughput, and robustness, and are of proven utility for directed evolution purposes. The assays presented use a variety of techniques, including genetic complementation, microtiter plates, solid-phase screens with colorimetric substrates, and flow cytometric screens. There are also representative examples of how phage libraries may be interrogated for enzymatic activity. Each protocol contains detailed step-by-step instructions and many notes on how best to deal with the problems that may occur. An accompanying volume, *Directed Evolution Library Creation: Methods and Protocols* (ISBN 1-58829-285-1), describes readily reproducible methods for the creation of mutated DNA molecules and DNA libraries.

Taken together, *Directed Enzyme Evolution: Screening and Selection Methods* and *Directed Evolution Library Creation: Methods and Protocols* capture for newcomers and more experienced investigators alike all the key methods for using directed protein evolution to better understand protein structure-function relationships, to discover new enzymes and therapeutic proteins, and to design new assays suitable for specific applications.

FEATURES

- Cutting-edge methods for screening DNA libraries to isolate specific proteins
- Easy-to-follow protocols covering all aspects of directed evolution
- Accessible to scientists and engineers with a minimal background in molecular biology
- Microtiter plate assays for several important classes of enzymes

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Part I Genetic Selection. Genetic Complementation Protocols. Use of *Phi* I-D deficient *E. coli* for Functional Complementation of DNA Polymerase. Selection of Novel Eukaryotic DNA Polymerases by Mutagenesis and Genetic Complementation of Yeast. Autogene Selections. Selection for Soluble Proteins via Fusion with Chitosan-Bindable Acetyltransferase. Phage: A Phage-Based Method for Selecting Thermostable Proteins. Minimization of Proteins by Random Fragmentation and Selection. **Part II Screens for Enzymes.** Evaluating a Screen and Analysis of Mutant Libraries. Screening Mutant Libraries in *Saccharomyces cerevisiae*. Solid-Phase Screening Using Digital Image Analysis. Screening for Thermostability. High-Throughput Screening of Mutant α -Amylase Libraries for Increased Activity at 125°C. High-Throughput Carbon Monoxide Binding Assay for Cytochromes P450. High-Throughput Screen for Aromatic Hydroxylation. Colorimetric Screens for Aliphatic Hydroxylation by Cytochrome P450 Using *p*-Nitrophenyl-Substituted Alkalies. High-Throughput Screens Based on NAD(P)H Depletion. High-Throughput Tetramethylbenzidine (TMB) Screen for Peroxidases. Screen for Oxidases by Detection of Hydrogen Peroxide with Horseradish Peroxidase. Colorimetric Dehydrogenase Screen Based on NAD(P)H Generation. Colorimetric Assays for Screening Laccases. pH Sensing Agar Plate Assays for

Enzymatic Enzyme Activity. A pH-Indicator-Based Screen for Hydrolytic Halocalkane Dehalogenase. Detection of Aromatic α -Hydroxyketones with Tetraazulium Salts. Selection of Heat-Stable *Clostridium cellulovorans* Cellulases After *In Vitro* Recombination. Screening and Selection Strategies for Disulfide Isomerase Activity. An Overview of High-Throughput Screening Systems for Enantioselective Enzymatic Transformations. Select Protocols of High-Throughput *in-Screening* Systems for Assaying Enantioselective Enzymes. Directed Evolution of the Substrate Specificities of a Site-Specific Recombinase and an Antisense-*tRNA* Synthetase Using Fluorescence-Activated Cell Sorting (FACS). Calmodulin-Tagged Phage and Two-Filter Sandwich Assays for the Identification of Enzymatic Activities. High-Throughput FACS Method for Directed Evolution of Substrate Specificity. Improving Protein Folding Efficiency by Directed Evolution Using the GFP Folding Reporter. Index.

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