

Combinatorial protein design: strategies for screening protein libraries

Huimin Zhao and Frances H Arnold*

Powerful strategies for screening protein libraries further strengthen the arguments for applying 'irrational' approaches to understanding and designing new proteins. Developments during the past year include the application of functional complementation and automation to reduce screening loads, as well as the use of computerized data acquisition to characterize whole protein libraries rather than just selected individuals.

Addresses

Division of Chemistry and Chemical Engineering 210-41 California Institute of Technology, Pasadena, CA 91125, USA
*e-mail: frances@cheme.caltech

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Abbreviations

BsLDH	<i>Bacillus stearothermophilus</i> L-lactate dehydrogenase
CSDL	cell surface display libraries
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence activated cell sorting
GFP	green fluorescent protein
NAD	nicotinamide adenine dinucleotide
PCR	polymerase chain reaction
SGPB	<i>Streptomyces griseus</i> protease B
SPA	scintillation proximity assay

Introduction

Protein engineers are becoming increasingly impatient with rational design approaches and the extensive structural and mechanistic information required to guide such efforts. Identifying the amino acids responsible for existing protein functions and those that might give rise to new functions remains an often overwhelming challenge. This, together with the growing appreciation that many protein functions are not confined to a small number of amino acids but are affected by residues far from active sites, has prompted a growing number of research groups to turn to 'irrational' design approaches, such as random mutagenesis and *in vitro* evolution, to engineer novel proteins [1] as well as to better understand existing ones [2,3]. These irrational approaches involve the generation and selection or screening of molecular repertoires with sufficient diversity for the altered function to be represented. Such approaches have been used to create novel functional nucleic acids [4], peptides and other small molecules [4], antibodies [4], as well as enzymes and other proteins [1-6,7*]. Given an intelligent approach to generating the mutant library (i.e. one that considers the enormous size of protein sequence space), the development of efficient methods to search protein libraries for desired properties is

probably the single most important element determining the success of these experiments.

The prerequisite to selection is the generation of a function that confers a growth or survival advantage to the host organism. Selection can be a very efficient search mechanism, allowing an exhaustive search of libraries of 10^6 or more protein variants. The disadvantage of a biological selection is that the property or protein of interest cannot be decoupled from a biological function. Thus, exploring novel functions such as stability or activity in non-natural environments (e.g. organic solvents) or activity on non-natural substrates can be difficult or even impossible [5]. The creative researcher who is tempted to apply a 'synthetic' selection approach should also know that these can be extremely tedious to develop and are often ineffectual because cells find alternative ways to solve the problem posed. *In vitro* selections have been developed in an attempt to mimic the power of natural selection, including a variety of selections based on column binding or 'panning' [4,6] and a recently reported chemical selection for catalysis [8]. Thus member(s) with desired functions can be selected directly from the rest of the library by either preferential binding or covalent interaction due to the accomplishment of a chemical feat. When choosing a search strategy, it is useful to remember the principle, "You get what you select (screen) for" [9*]. Many selections do not yield the desired result! Selection approaches are discussed in a separate review by Kast and Hilvert (pp 470-479) in this section on engineering and design.

For many problems, and especially those of practical interest, libraries of variants must be screened rather than selected, which means that the library members must be examined separately (often one at a time). A typical screening strategy thus involves the construction of an arrayed protein library and the application of a rapid assay that is sufficiently sensitive and specific to identify positives. The screen can be more or less sensitive, depending on the willingness of the researcher to accept false positives (and to apply additional tests). In this review, we focus on recent developments in screening protein libraries for specific functions.

Semi-quantitative visual screens

Simple visual screens are widely used when the function of interest can generate a visible signal. Green fluorescent protein (GFP) variants are easily identified in visual screens based on the intrinsic fluorescence of GFP under UV illumination [10-12]. GFP libraries generated by error-prone polymerase chain reaction (PCR) [11,12] and

DNA shuffling [10] have been expressed in *Escherichia coli* and arrayed on agar plates. Visual screening of 10^4 – 10^5 clones yielded variants with increased intensity of the fluorescence signal [10,11] and suppressed thermosensitivity [12].

Simple colorimetric assays are also widely used in screening [13,14,15*,16–18]. Brunet *et al.* [13] have assessed the role of turns in stabilizing the structure of an α -helical protein by completely randomizing a tripeptide turn of cytochrome *b-562* using cassette replacement mutagenesis. 45 clones have been screened on the basis of the observation that cells expressing mutants that fold correctly will bind heme and yield bright red periplasmic extracts, whereas cells expressing mutants that fold incorrectly yield colorless extracts. More recently, a similar approach has been applied to studying a β turn in a β -barrel protein, plastocyanin, which is blue in its native, folded state and colorless when denatured [14]. Because of the need for concentration and other pretreatments, however, this approach is not yet suitable for screening large libraries. Hawrani *et al.* [15*] have completely randomized two amino acids in a solvent-exposed surface loop of *Bacillus stearothermophilus* L-lactate dehydrogenase (*BsLDH*) with the goal of engineering new substrate specificities. The expressed *BsLDH* library is immobilized spatially on nitrocellulose membranes after cell lysis and several washing steps and tested for each variant's ability to reduce NAD^+ in high and low concentrations of a target substrate (to detect substrate inhibition), and in the presence and absence of an allosteric activator (to find variants that do not require the activator). Enzyme activity is detected by coupling the hydrogen transfer system of NADH/NAD^+ to further redox reactions involving phenazine methosulphate and nitroblue tetrazolium, which yields a blue insoluble dye on reduction.

Two *in vivo* color screening strategies have been described recently [19*,20]. Bacterial membrane permeability increases after synthesis of the poliovirus protein 3AB. Lama and Carrasco [19*] have developed a screen in which permeabilizing wild-type 3AB and nonpermeabilizing 3AB mutants are differentiated because of the different rate of entry of a chromogenic β -galactosidase substrate, X-Gal. *E. coli* clones with wild-type 3AB are stained dark blue, whereas the mutants lacking pore-forming activity are stained light blue. To isolate α -factor pheromone receptor mutation(s) that constitutively signal in the absence of α -factor, Konopka *et al.* [20] have constructed a yeast strain which contains the pheromone-responsive *FUS1-lacZ* reporter gene. Constitutive receptor mutants are detected as blue colonies on an agar plate containing X-Gal, as a result of induced expression of *lacZ*-encoded β -galactosidase. Approximately 600,000 colonies have been screened.

Clones secreting active proteases produce a zone of clearing or 'halo', the size of which is proportional to the hydrolytic activity when grown on agar plates

containing casein or skim milk proteins. Utilizing this well-known visual screen, You and Arnold [9*] have applied sequential rounds of random mutagenesis and screening to significantly enhance the expression level of subtilisin E and further increase its specific activity in aqueous organic solvent. Sidhu *et al.* [21] have varied seven residues involved in conferring primary specificity of *Streptomyces griseus* protease B (SGPB) by DNA cassette mutagenesis. An *E. coli* expression library containing 29,952 possible SGPB mutants has been screened for secretion of active protease by halo formation on agar plates containing skim milk. Sidhu *et al.* [21] have found that the substrate specificity of recombinant SGPB is constrained by the sequence at the promature junction, and active protease production is dependent on the efficiency of self-processing by pro-mature junction cleavage. Easily observed colony phenotypes are also exploited in rapid screening methods [22,23].

Screening in 96-well plate formats suitable for automation

Although visual screening on the basis of color or halo formation is rapid and efficient, its limitations are also obvious: visual screens are nonquantitative and often insensitive to small changes in properties, and they are not generally applicable. Designing a visual screen for protein functions such as catalysis of a specific reaction or of a specific substrate may be difficult or impossible. Digital imaging spectroscopy has been developed to increase the sensitivity and throughput of filter and agar plate based screens [24]; however, the 96-well microtitre plate remains the standard format for automated, high-throughput screening [7*,25*,26]. Screening automation and quantification of the results are highly desirable; 96-well plates appear to be the format most compatible with currently available robotic arms, liquid handling systems and plate readers.

Data collection and analysis are greatly facilitated by computerized data acquisition. Assay automation and computerized data acquisition are being exploited extensively by researchers at Recombinant Biocatalysis (La Jolla, CA), who are using high-throughput 96-well plate assays to identify enzyme catalysts from genomic libraries, to characterize their substrate specificities, and to further evolve specific properties [26]. Useful information about the protein library can be retrieved from the large amount of data generated during screening. We have used library screening to distinguish functional from nonfunctional and deleterious mutations in a laboratory-evolved thermostable subtilisin E, 1E2A [27*]. The evolved gene has been randomly recombined with the wild-type gene to create a library of all possible mutation combinations. The resulting library has been screened for thermostability using normalized residual activity after incubation at high temperature in 96-well plates (Figure 1). The fact that roughly 25% of the clones exhibit thermostability comparable with that of 1E2A immediately indicates that only

two of the ten DNA mutations in 1E2A are responsible for the increased protein thermostability, a result that has been confirmed by further biochemical analysis. Similar sorted library profiles are very useful for estimating the mutagenic rates associated with error-prone PCR or other random mutagenesis approaches [28,29]. Computerized data acquisition will certainly facilitate exploitation of the enormous amounts of information available in protein libraries as opposed to single individuals.

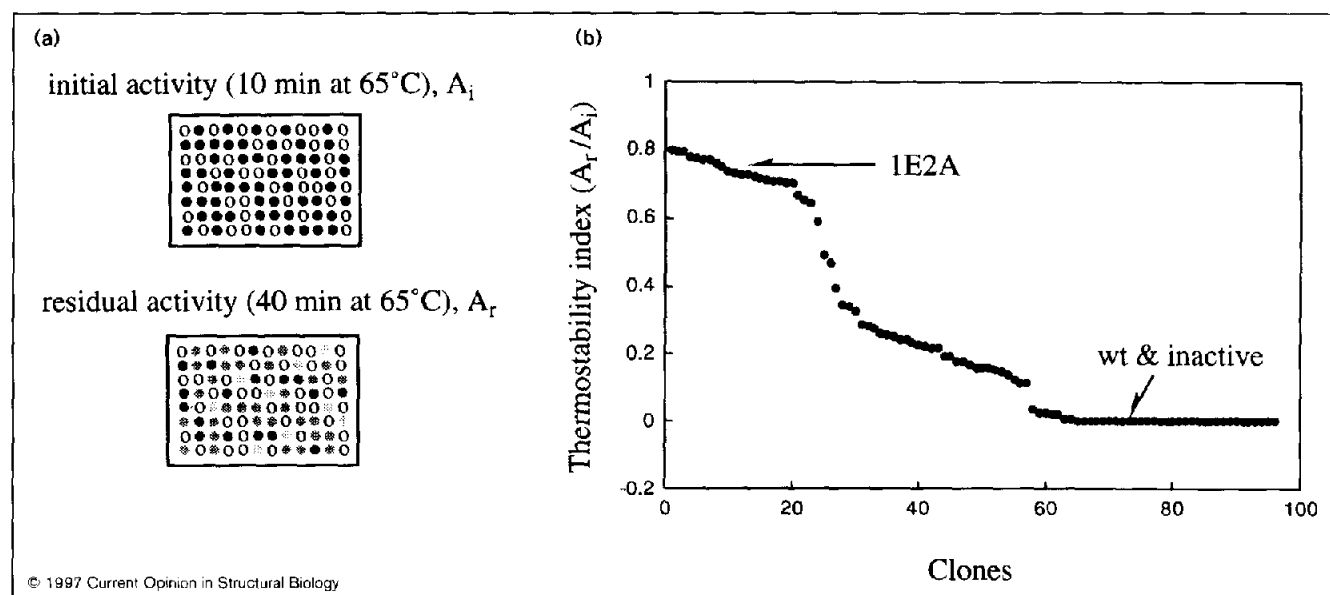
Many conventional assays such as enzyme-linked immunoadsorbent assays (ELISA), radioimmunoassays, and enzyme-substrate assays can be readily converted into automated formats. Two new assays that require no steps to separate free from bound tracer have been described: scintillation proximity assay (SPA) [7*] and fluorescence polarization equilibrium binding assay [30]. Several novel screening approaches for catalytic activity [31–34] and enantioselectivity of catalysis [35*] have also been developed for the 96-well plate format. Tawfik *et al.* [31,32] have devised a sensitive method, catELISA, on the basis of immobilized substrates, and immunodetection of the end product of the catalyzed reaction. This strategy was used by MacBeath *et al.* [33] to screen antibody libraries for catalysis of a bimolecular Diels–Alder reaction. The main limitation of this approach is that it requires a specialized antibody that can strongly discriminate between substrate and product. Fenniri *et al.* [34] have

described an encoded reaction cassette for the detection of chemical bond breakage and formation for biocatalyst screening. The reaction cassette is immobilized on a matrix and encoded by a polynucleotide containing two primers. Thus, when the functionalized matrix is exposed to a library of biocatalysts that are able to selectively cleave the reaction cassette at the substrate juncture, the polynucleotide is released and can be amplified by PCR. For large library screening, the PCR reaction can be carried out in 96-well plates, and the PCR products can be further detected by the addition of a fluorescent probe.

Janes *et al.* [35*] have developed a rapid spectrophotometric method to measure the enantioselectivity of hydrolases, based on the addition of a chromogenic reference compound that introduces competitive binding for both enantiomers. The relative hydrolysis rates of the two enantiomers competing with the reference compound are measured separately; the ratio of the two relative rates gives the enantioselectivity. Compared with an endpoint method, this 'quick E' method has advantages in terms of speed, the amounts of enzyme required, and the higher enantioselectivities that can be measured.

A number of other screens based on 96-well plates have been described during this past year. Moore and Arnold [36*] have utilized a chromogenic substrate (an antibiotic *p*-nitrophenyl ester, pNP) that serves as a surrogate for

Figure 1



A rapid screen for enzyme thermostability based on residual activity after incubation. (a) Catalytic activity is measured before (A_i) and after (A_r) incubation at high temperature. (b) Results from a typical 96-well plate. *Bacillus* transformants of a randomly recombined subtilisin E library are picked and grown in 96-well plates. Initial subtilisin activity (A_i) and residual activity (A_r) after incubation at 65°C of supernatants are measured (towards succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide) using a microtitre plate reader. A_r/A_i values are sorted and plotted in descending order. Residual activities of wild-type (wt) and 1E2A, a thermostable variant of subtilisin E, are indicated by arrows. The probability that any given mutation will appear in the randomly recombined population of two equally mixed genes is 1/2. Thus the probability that N specific (functional) mutations will appear together in a sequence is $(1/2)^N$. In this example, two mutations are responsible for the enhanced thermostability of 1E2A [27*].

the desired substrate of an esterase (the *p*-nitrobenzyl ester, pNB) in a rapid screening assay coupled with sequential random mutagenesis and recombination. While the reaction with the pNB ester must be analyzed by HPLC, the cell growth and pNP screening reactions can all be carried out in 96-well plates and analyzed using a microtitre plate reader. The surrogate assay has been validated by comparing the activities of random mutants on the two substrates, which shows a relatively high correlation. Thus, the positives identified during the rapid screening assay can be verified during a second level screen using HPLC. Panchal *et al.* [37] have described the screening of libraries generated by combinatorial cassette mutagenesis to isolate mutants of the pore-forming toxin α -hemolysin that are rapidly and preferentially activated by a tumor protease, cathepsin B. Mutants exhibiting the desired activation after preliminary screening with clostripain have been reassayed more carefully for their hemolytic activity after treatment with human liver cathepsin B or clostripain. Cells are grown and screened in 96-well plates.

Functional complementation coupled with screening

An attractive approach to screening large libraries is to couple functional complementation with screening [2,3,24,38*,39,40,41*,42,43]. By requiring that at least some of the biological function of the protein is retained, functional complementation can greatly reduce the subsequent screening requirements. A major limitation, however, is finding or constructing an appropriate complementation system. Furthermore, the retention of biological function may preclude acquisition of other functions. Loeb and coworkers [38*] have applied this strategy effectively in several studies whose goals have been to identify residues responsible for protein functions as well as to engineer novel properties. The proteins studied recently include herpes simplex virus type 1 thymidine kinase (HSV-1 TK), human immunodeficiency virus reverse transcriptase (HIV RT) [3] and human DNA alkyltransferase [39]. Six residues adjacent to the putative nucleotide-binding site of HSV-1 TK have been completely randomized in an effort to increase the enzyme's specificity for the phosphorylating nucleoside analogs gancyclovir (GCV) and/or acyclovir (ACV) [38*]. 426 active mutants selected from more than one million mutants by functional complementation have been screened for enhanced sensitivity to GCV and/or ACV. One drug-sensitive mutant produced stable mammalian cell transfectants that are 43-fold more sensitive to GCV and 20-fold more sensitive to ACV.

Warren *et al.* [40] have altered each polar residue within 6 Å of the catalytic center of glycylamide ribonucleotide transformylase using saturation site-directed mutagenesis. Approximately 50 transformants from each mutagenesis have been picked, sequenced and screened for their activity using functional complementation of auxotrophic cells. None of the polar residues close to the catalytic

center of the enzyme has been found to be irreplaceable. Further study of the three key polar active-site residues has revealed that none is irreplaceable, although any change leads to substantially decreased catalytic activity and no more than one mutation can be tolerated [2].

Axe *et al.* [41*] have developed a sensitive biological screen on the basis of the extreme autotoxicity of barnase when produced in *E. coli* in the absence of its natural inhibitor, barstar. Two amber stop codons have been introduced to replace serine codons at positions 28 and 57 of the synthetic barnase gene (*synbar*), which prevents lethal production of barnase in a nonsuppressing (*sup*⁻) *E. coli* strain. When plasmid DNA prepared from the *sup*⁻ strain is used to transform a suppressing (*supD*) strain, these two amber stop codons are read as serine codons, producing wild-type barnase. Only *synbar* mutations yielding variants with dramatically reduced activity allow the *E. coli supD* strain to grow. The activity of these mutants is then qualitatively estimated. A barnase mutant library in which 12 hydrophobic core residues have been randomly replaced by hydrophobic alternatives has been screened. A significant fraction (23%) of these mutants retain activity, which implies that hydrophobicity is a nearly sufficient criterion for the construction of a functional core. A similar screening method has been developed to detect low activity barnase mutants, in which a barnase-barstar plasmid inversion system has been constructed [42].

Sorting single cells or proteins

In order to move toward automated, high-throughput screening, it will be important to increase the level of miniaturization. Silicon wafers with many thousands of compartments housing liquid volumes in the nanoliter and picoliter range have been developed [44]. Liquid handling systems have also been developed to accurately dispense biological samples of ~5 picoliters to a few nanoliters and speeds up to 10,000 drops per second [45]. Innovative approaches that may be applicable to large-scale screening of protein libraries have been described in recent years, including fluorescence correlation spectroscopy coupled with single molecule trapping devices [46–48] and cell surface display libraries (CSDL) coupled with fluorescence activated cell sorting (FACS) [49*]. Fluorescence correlation spectroscopy coupled with devices for trapping single molecules in an electric field may be used in the future to sort single cells (or parts of their surfaces), organelles, viruses, single genes, proteins, or even small molecular entities such as peptide hormones or other oligomeric compounds [46–48]. This approach is capable of monitoring very low concentrations (<10⁻¹⁵ M) without the need for amplification; however, actual applications to library screening have not yet been reported. For protein library screening, phage displayed [6] or bacterial/yeast CSDLs [49*] could be used with the advantage that an isolated sample can be amplified since the genotypic information is linked with the phenotypic information. Because of the relatively large size of cells, CSDLs can be

coupled with FACS for high throughput screening. As with phage display, however, there are difficulties in using these systems for screening for properties other than binding.

Conclusions

Most current screening methods are labor intensive and can be used to search small libraries of only a few thousand members. Automation and miniaturization, which reduce labor and materials requirements and increase reproducibility, can extend screening capabilities by a factor of 10–100. Methods for screening single cells or even single molecules, however, may dramatically increase the numbers of variants that can be screened and will significantly enhance the power of *in vitro* evolution. By screening much larger protein libraries, one can hope to identify rare events such as the acquisition of a novel catalytic activity.

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