Screening for Protein Catalysts

Development of a High-Throughput Screen for Protein Catalysts: Application to the Directed Evolution of Antibody Aldolases**

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High-throughput screens can be powerful methods for identifying and evolving biological catalysts.[1–3] To date, nearly all high-throughput screens (e.g. blue/white colony screens, plate lifts, cell sorting, etc.) assay catalytic activity in live cells or crude cell lysates. As a result, there are significant limitations on the substrates, reactions, and conditions that can be used. In addition, detection of products in the presence of many proteins, lipids, nucleic acids, and other cellular components can be problematic. To circumvent these limitations, we have developed a high-throughput system to express, purify, and assay the catalytic activity of proteins. By carrying out assays on purified proteins, a much wider range of substrates and reactions can be explored, including those involving cell-impermeable substrates, endogenous background activities, and nonchromogenic products. To illustrate the potential of this system, we have applied it to the directed evolution of catalytic antibodies with aldolase activity.

The first step involved the development of a general system for expressing and purifying large numbers of antibodies in parallel.[4] The system should be amenable to optimization of expression levels and catalytic activity as both are important properties for antibody development. Our approach was to grow high-density 1-mL cultures of *E. coli* in 96-well plates, lyse the cells, and then capture the His-tagged antibodies with Ni-NTA beads. To maximize the utility of the system, a single set of expression and purification conditions was required that would accommodate many different antibodies. In addition, sufficient quantities of purified antibodies must be produced to obtain good signal-to-noise ratios in catalytic assays. The expression levels and purity of protein must also be reproducible so that mutants with incremental changes in activity and expression levels can be detected with confidence. Finally, protocols should be compatible with automation and high-throughput formats; steps requiring extensive manipulation of plates such as centrifugation and sonication were avoided.

After extensive optimization, we developed a semiautomated system capable of routinely screening 6000 clones/run in a parallel 96-well format. Yields of protein range from 0.5–3 μg/well (1 mL culture/well) and purities are greater than 90%. In addition, a wide range of antibodies have been successfully expressed and purified using the system without modifying the protocol.[5] A typical screen starts by transforming bacteria (Top10) with a library of plasmids encoding mutant antibodies (mouse–human chimeric Fabs) fused to His6 tags.[6] The expression vector is a modified pBAD plasmid with expression controlled by the arabinose promoter. Single colonies are picked into individual wells of shallow, 96-well plates containing 2xYT/amp using a robotic colony picker. These starter cultures are grown overnight and then 10 μL is transferred from each well to a corresponding 1 mL expression culture in a 2-mL, deep, 96-well plate.[7] To expedite the process, the 96 liquid transfers required for each plate are done in parallel using a robotic liquid handler fitted with a 96-needle head. The starter cultures are grown to saturation to ensure that each expression culture is inoculated at about the same density. In addition, the starter cultures serve as a source of plasmid DNA for hits identified during the screen (starter cultures are stored at 4 °C until completion of the screen). The cultures are then grown for 7 h at 37 °C and 250 rpm, and protein production is induced by parallel addition of arabinose (4%, 50 μL/well). Cultures are shaken at 25 °C and 250 rpm for an additional 12 h, and then 100 μL/well of 10x PBS that contains 10units DNase mL^{-1} is added. Cells are lysed by addition of 100 μL/well of deoxycholic acid (10 mg mL^{-1}), and His6-tagged proteins are captured by addition of 50 μL/well of Ni-NTA resin. Each of these additions is carried out with the robotic liquid handler. After shaking for an additional 4 h, the plates are allowed to sit for 5 min to allow the Ni-NTA resin to settle to the bottom of the wells. The needles of the liquid handler are inserted into the wells just above the resin, and the lysate is removed. The resin is then washed once with 500 μL/well of PBS that contains 5 mM imidazole and then three times with 500 μL/well of PBS to afford the immobilized, purified antibodies.

To test the performance of the system, we conducted a series of model studies using catalytic antibody 38C2, an aldolase developed by Wagner, Lerner, and Barbas.[8] This antibody efficiently catalyzes aldol reactions with a broad range of substrates; it has also been extensively characterized both mechanistically and structurally.[9,10] We found that 38C2 retains activity while immobilized on Ni-NTA resin (~50% relative to the antibody in solution) and can be assayed directly without elution. In a representative assay, 38C2 was expressed and purified in 80 wells distributed over 20 plates and then assayed for activity by addition of 200 μL/well of substrate **1** (20 μM in PBS).[11] The product of the retro-aldol reaction, **2** (Scheme 1), can be detected easily using a fluorescent plate reader ($\lambda_{ex} = 330$ nm, $\lambda_{em} = 440$ nm). After incubating the plates for 1 h at 25 °C and 250 rpm, the reaction was transferred to white 96-well plates, and fluorescent signals were measured. The average signal was 1100 with a CV of 30% (signal variation from well to well and plate to plate).

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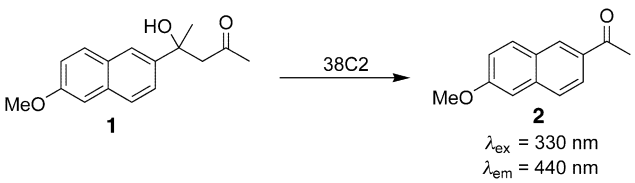

Scheme 1. 38C2-Catalyzed conversion of substrate **1** into **2**.

With this level of variation, a two- to threefold change in activity is detectable. For comparison, the initial signal for substrate alone was about 220, the background signal after incubation of substrate for 1 h in wells containing a catalytically inactive antibody was about 250, and the fluorescent signal of cell cultures and crude cell lysates lacking substrate was about 3000. The products were also analyzed by mass spectrometry using an LC–TOF instrument equipped with a MUX eight-channel unit, albeit with lower overall throughput (~ 500 samples day^{-1}). Moreover, because the assays are performed on resin-bound catalyst, up to three catalytic assays can be conducted on the same sample of protein without significantly affecting the signal-to-noise ratio and variability by simply washing the resin twice with PBS between assays. Furthermore, the antibodies can then be eluted from the resin and analyzed by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) or ELISA (enzyme-linked immunosorbent assay) to probe expression levels and hapten binding. By conducting multiple assays, we can simultaneously screen for mutants with improved activity, selectivity, and/or expression levels.

To test the high-throughput screen as a tool for directed evolution, we constructed two antibody aldolase libraries in which key amino acid residues in the active site were randomized to all 20 possible amino acid. One library had residues 96–98 on the heavy chain randomized and the other had residues 27B-D on the light chain randomized. From these libraries, 17 unique mutants were identified that produced signals two- to threefold higher than the 38C2 controls. The four heavy-chain mutants and 13 light-chain mutants were then crossed and rescreened (with 20 μM **1**) in an effort to find combinations of mutations with additive effects. Two clones, 2G7 and 3D6, resulted in yields 15-fold higher than those for 38C2 in the screen. These mutants were expressed in 1-L cultures, purified, and characterized in detail (see Table 1). Both mutants have a 2.4-fold higher k_{cat} value and express at levels five- to sixfold higher levels than those for the wild-type antibody, 38C2, demonstrating that improved antibodies can be reliably identified using the high-throughput screen.

Table 1: Amino acid sequences, catalytic properties, and expression levels for selected antibodies.

Antibody	Residues				k_{cat}[a,b]	K_M[a,c]	Expression[d]
	L27B-D	H96-98	L92	H34	[min^{-1}]	[μM]	[mg L^{-1}]
38C2	L-H-T	F-Y-S	T	M	0.63	15	0.25
2G7	D-V-L	G-Q-K	T	M	1.55	30	1.2
3D6	L-V-S	G-Q-K	T	M	1.51	21	1.5
1A4	L-V-S	G-Q-K	N	V	2.35	9	6.0

[a] k_{cat} and K_M were measured using racemic substrate **1**. [b] ± 0.04 min^{-1}. [c] ± 1 μM. [d] ± 0.05 mg L^{-1}.

To further improve the activity and expression levels of the aldolase antibodies, 26 single amino acid positions in close proximity to the active site of 3D6 were randomized. These residues included 31–36 (CDR1) and 89–97 (CDR3) on the light chain and 33–37 (CDR1), and 94, 95, and 99–103 (CDR3) on the heavy chain. The 26 single amino acid libraries were combined and then screened (with 10 μM **1**).[12] Two unique mutants, Thr92 LAsn and Met34HVal, were identified that resulted in yields threefold higher than those for 3D6 in the high-throughput screen. The double mutant (1A4) was generated, expressed, and characterized. Antibody 1A4 was found to have a sixfold improvement in catalytic efficiency (k_{cat}/K_M) and a 25-fold improvement in expression level over 38C2, corresponding to an overall 150-fold increase in product yield. As with 38C2, 1A4 was found to be highly selective for the *S* enantiomer of substrate **1**. The generation of antibody 1A4 from high-throughput screens of two iterative focused libraries demonstrates that this is a reliable and effective tool for directed evolution.

In conclusion, we have developed a high-throughput screen capable of rapidly analyzing thousands of protein mutants. The screen can be used to evolve activity, selectivity, and expression levels of proteins directly or in combination with selections. In addition to identifying mutants with improved properties, one can use the screen to rapidly compare protein library design strategies, evaluate the quality of protein libraries, and determine the effectiveness of rounds of selections.

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[6] Detailed procedures for a typical high-throughput screen, the construction of the expression vector, the construction of the antibody libraries, and characterization of the antibodies can be found in the Supporting Information.
[7] A control antibody (typically 38C2) is expressed in 2–4 wells/plate to serve as an internal control.

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[12] The 26 libraries were constructed in four groups, based on the location of the randomized residue: light CDR1, light CDR3, heavy CDR1, and heavy CDR3 (see Supporting Information). The four groups were transformed separately and screened as described in the supporting information with a unique clone in each well. Plasmid DNA from each hit identified in the screen was obtained by isolating the DNA from the corresponding starter culture well.

