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Screening-Based Directed Evolution: Developing Display and Expression Systems for High-Throughput Selective Screening

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With the advent of techniques in genome research and discovery, it is now become routine to find the diverse array of genes of interests to research and bioindustry communities. However, rarely is the case that nature-made enzymes are perfectly fitted to the current needs, defined by industrialized and sophisticated ways of modern life style. A rapid improvement or evolution of biocatalysts is a prerequisite for tailoring nature's repertoire to real world applications.

The techniques mimicking natural's diverse mechanisms of random and combinatorial mutagenesis in laboratory is maturing rapidly to the point where the screening is limited. Then, 'you got what you screen for' becomes the first testament witnessed when the researchers set to try to find out the improved enzymes. Or, this may be better translated into 'You got what you can express and screen for'.

Combinatorial library generation technology is changing the way of library generation away from error-prone PCR to combinatorial shuffling of several parent genes or error prone-PCR generated variant genes. Our data with beta-lactamase will show that our combinatorial library generation technology is quite comparable to that of standard DNA shuffling method. Another approach that we are developing with surface display is that we check and select improved variants along the evolution cycle and make combinatorial mutants between the interim variants. This approach greatly facilitates the combinatorial shuffling of improved variants before reaching the final evolution cycle. Other combinatorial approaches and *in silico* design guide are being presented.

Screening with a critical throughput is of utmost importance because library diversity should be expressed and screened in a reasonable time and manpower limits. This is interlinked with expression system. When the selection strategies such as antibiotic selection and growth selection, only the improved clones will appear and to be analyzed for further activities, thus eliminating the needs for the extensive primary analysis. Thus, any expression systems enabling selective screenings is a plus: 1) sorting out in the order of the better clones, 2) sorting the parent and better ones in one mutant pool, or 3) sorting only the clones showing activity after mutagenesis, thus eliminating the assay of the 'dead' mutants. These three selective cases are addressable with our extensive portfolio of display and expression technologies. When there is no available selective screening strategy, high-throughput assay system should be designed: measuring activities of all the clones with a sortable assay such as flow cytometry or measuring activities of all the individually grown clones with high-throughput and automatable assay should be available. And measuring all the activities of individually grown clones with normal assay will be a last method of choice.

More specifically, the protein display is unique in that the displayed proteins are accessible to the external milieu and thus available for direct measurement of catalytic or binding activity with target substrates. More importantly this enables us to identify the genes responsible for improved activities because the protein is linked to the carrier containing the corresponding genes. Our display technologies include INP (ice-nucleation protein) display, Bacillus spore display, and yeast display. Each display system with its advantage and disadvantage will be discussed in this presentation. Furthermore, we have also developed an alternative microbial display technology for displaying and evolving currently undisplayable proteins such as

multimeric proteins or non-secretory proteins. Also it is now possible to display the target proteins in their native configuration without any display motif.

By displaying enzymes, it is possible to link the improved enzyme activities to the growth rates of the individual clones, thus facilitating greatly the selection procedure. Also possible is to broaden the substrate spectrum because the displayed enzymes can generate the products for the growth and to use in biocatalysis with surface-displayed enzymes.

We have been complementing these display technologies with periplasmic expression, stationary expression systems, flow cytomettry and nano-scale living chip assay.

Table 1. Display and directed evolution: A conceptual throughput. [from D.Wittrup, Curr. Opin. Biotechnol. 10: 117–122(1999)]

Number of individual candidate clones	Assay methods
$>10^7$	FACS, genetic selection, display
10^{4-6}	Solid state assays: colorimetric or fluorescence
10^{2-4}	Microtiter format assays: colorimetric or fluorescence
$1\sim10^2$	Individual high precision assays: GC, HPLC, mass spectrometry

Suggested Readings

1. Chen W. & Georgiou, G. "Cell-Surface Display of Heterologous Proteins: From High Throughput Screening to Environmental Applications", *Biotechnol. Bioeng.*, 79, 469-503, (2002).
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4. Olsen MJ, Stephens D, Griffiths D, Daugherty P, Georgiou G, Iverson BL "Function-based isolation of novel enzymes from a large library" *Nat Biotechnol.* 18:1071-4 (2000).
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