

Construction and evolution of antibody-phage libraries by DNA shuffling

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Antibody engineering efforts are currently focused on the construction of ever larger naive human antibody-phage (Ab-phage) libraries, from which the best clones are purified over several selection cycles, mostly without significant evolution of the antibody sequence¹.

A different strategy described here is the multistep evolution of human antibody sequences from naive libraries of modest size. Our approach uses *in vitro* homologous recombination, called DNA shuffling^{2,3} or sexual PCR⁴, for the construction of naive human Ab-phage libraries followed by the evolution of antibody sequences specific for human receptors.

Library construction

A stable and well expressed human single-chain Fv framework (V_H251-V_LA25) was obtained from an Ab-phage library constructed from naive human mRNA by selection for binding to diphtheria toxin. This scFv framework was used to construct a naive Ab-phage library containing six synthetically mutated complementarity-determining regions (CDRs), based on the germline sequences of the CDRs of these V-region families. The degree of mutagenesis of each residue was similar to its naturally occurring variability within its V-region family⁵ (Fig. 1a). Four different lengths of CDR H3 were used. A PCR product con-

taining the scFv gene was randomly fragmented by DNaseI digestion and fragments of 50–100 base pairs (bp) were purified. Synthetic oligonucleotides, each containing a mutated CDR flanked by 19 bases of homology to the scFv template, were added to the random fragments at a 10:1 molar ratio (Fig. 1b). A library of full-length, mutated scFv genes was reassembled from the fragments by sexual PCR (refs 3, 6). Cloning into the pIII protein of M13 phage yielded an Ab-phage library of 4×10^7 plaque-forming units. The combinations of mutant and native CDRs were characterized by colony PCR with primers specific for the native CDRs (Fig. 1c). All six mutated CDRs were incorporated with 32–65% efficiency and in a wide variety of combinations. Sequencing of the mutated CDRs showed that the observed mutation rate matched the expected rate (Fig. 2).

Selection and evolution

This small Ab-phage library was panned for two rounds in microtiter plates for binding to ten human protein targets, and seven of these targets yielded at least weakly ELISA-positive clones. We focused on obtaining improved clones against the human granulocyte colony-stimulating factor (G-CSF) receptor. The G-CSF receptor response was only average compared with that of the other targets. One-quarter of the phage clones of this second-round pool were weakly ELISA-positive for G-CSF receptor.

Comparing mutagenesis strategies

This diverse pool was used to test the suitability of three different sequence optimization strategies, namely regular PCR, error-prone PCR and DNA shuffling. Surprisingly, already after a single cycle of each of these alternatives, the DNA shuffling showed a severalfold advantage, both in the percentage of Ab-phage recovered (Fig. 3), and in the G-CSF-receptor-specific ELISA signal.

The panning was continued for six additional cycles, shuffling the pool of scFv

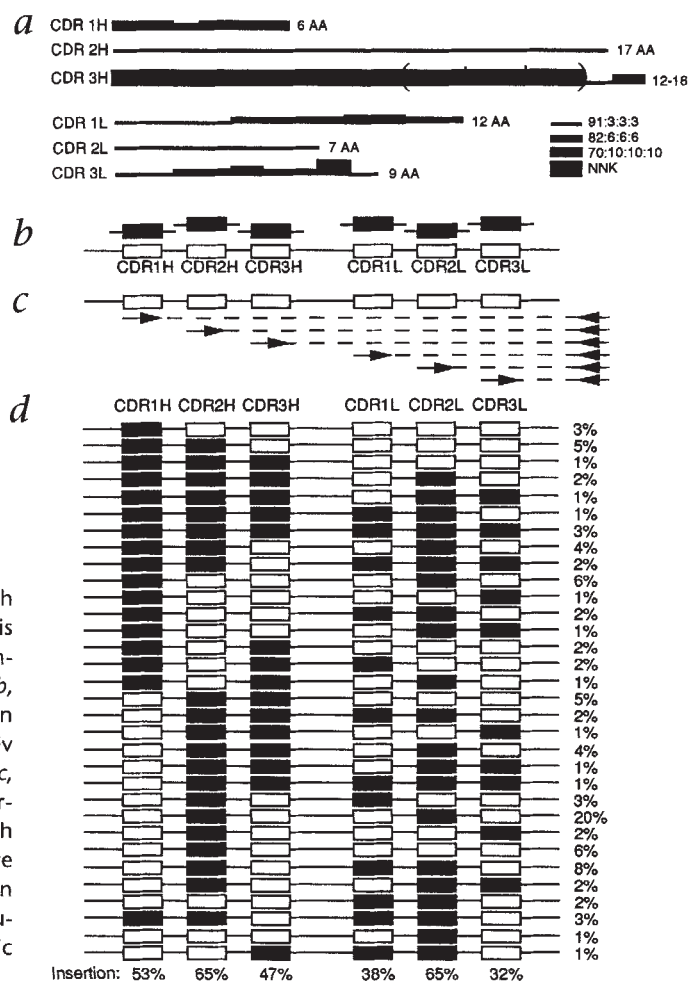


Fig. 1 *a*, Length and mutagenesis rate of all six synthetic CDRs. *b*, Library construction by shuffling scFv with all six CDRs. *c*, CDR insertion determined by PCR with primers for native CDRs. *d*, Insertion rates and distribution of synthetic CDR insertions.

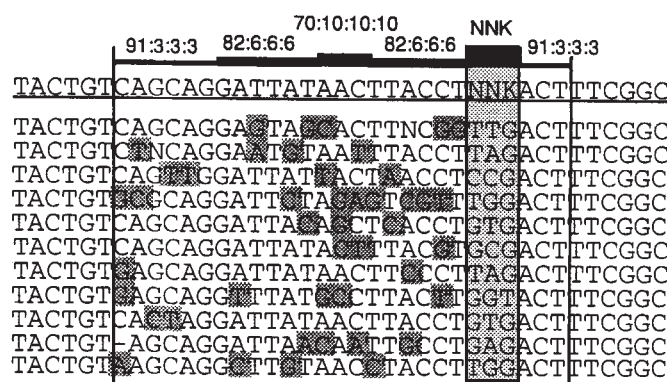


Fig. 2 DNA sequence of mutated CDR3L from library clones.

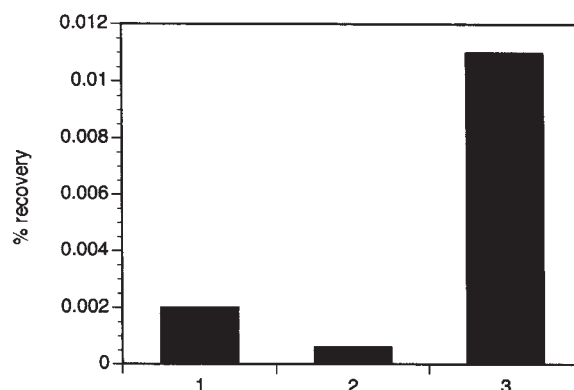


Fig. 3 Ab-phage recovery versus mutagenesis method. PCR (1) versus error-prone PCR (2), versus DNA shuffling (3), all applied once to Ab-phage pool from selection round two.

genes after each round. The stringency of selection was gradually increased to two one-hour washes at 50 °C in PBS-Tween in the presence of excess soluble G-CSF-receptor. In rounds three to eight, nearly 100% of the clones were ELISA-positive. When pools from different cycles were assayed at identical stringency, the percentage of phage bound increased 440-fold from cycle two to cycle eight (Fig. 4). Individual phage clones from each round showed a similar increase in specific ELISA signal (data not shown). Sequencing showed that the scFv contained an average of 34 ($n = 4$) amino acid mutations, of which only four were present in all sequences.

Removing neutral mutations

In order to reduce the potential immunogenicity, we attempted to remove any neutral or weakly functional mutations by two cycles of backcrossing³, with a 40-fold excess of a synthetically constructed germline scFv gene, followed by strin-

gent panning. The average number of amino acid mutations in the backcrossed scFvs was nearly halved to 18 ($n = 3$), of which only four were present in all sequences. These different scFv clones appeared distantly related.

The backcrossed Ab-phage clones were shown to bind strongly and with excellent specificity to the G-CSF receptor (Fig. 5), and western blot analysis showed the scFv in the expected size and amount. However, soluble scFv antibodies obtained from these phage clones had no detectable affinity for the G-CSF receptor by BIAcore (Pharmacia, Piscataway, New Jersey) or ELISA assay. Additional analysis suggests that the tight binding of the Ab-phage to the G-CSF receptor requires high valency. Based on published work⁷, it appears likely that our selection conditions (up to 50 °C) favored the swapping of scFv domains between phage, allowing those phage to achieve very high valency and avidity by crosslinking. This selection

problem has been solved by many others⁸ and should not reduce the usefulness of DNA shuffling as a tool for antibody engineering.

Recursive versus “single pot” libraries

The largest libraries that others have constructed now contain nearly 10^{11} different sequences⁹. From such libraries the best clones can simply be "purified" over several selection cycles, without evolving the sequence. This approach is particularly suitable for therapeutic antibodies because it yields few potentially immunogenic mutations.

In contrast, our goal was stepwise to evolve functional sequences from libraries of modest size that can be generated rapidly. The number of different sequences in our initial library was only 4×10^7 , and the subsequent libraries contained about 10^7 different sequences. Screening any library at very high stringency quickly yields the single best clone, but then the

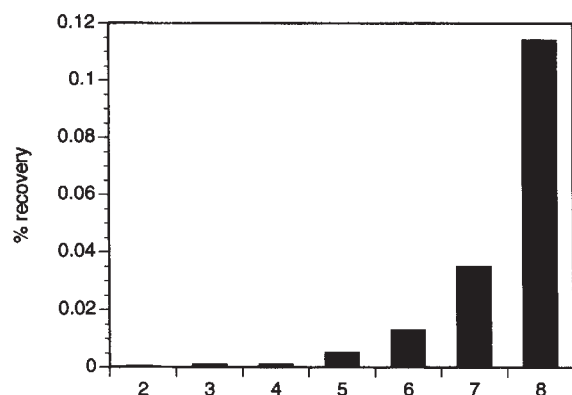


Fig. 4 Ab-phage recovery rate versus selection cycle. Shuffling was applied after selection rounds two to eight. Total increase is 440-fold.

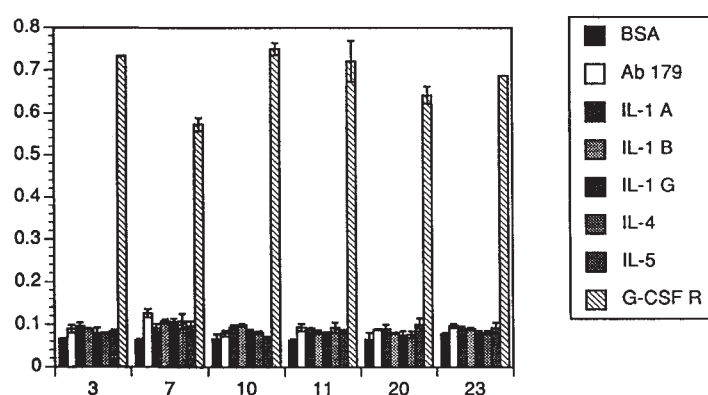


Fig. 5 Binding specificity after ten selection rounds, including two rounds of backcrossing. ELISA signal of different Ab-phage clones for eight human proteins.

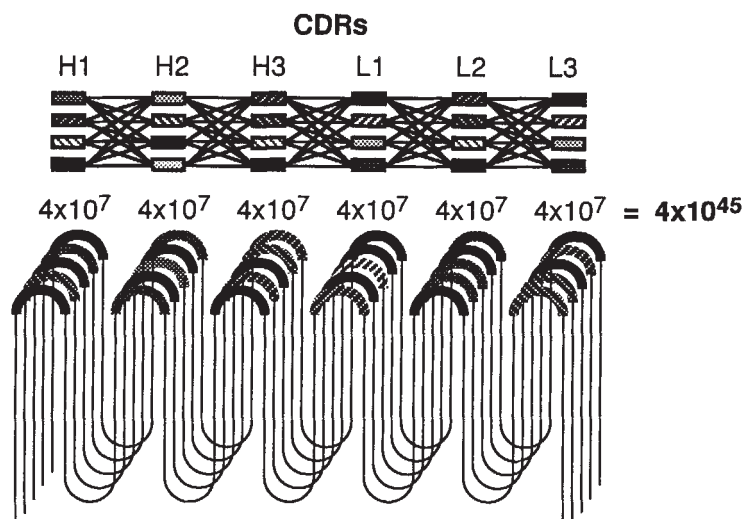


Fig. 6 Potential diversity. Additional diversity can be generated after each selection cycle by permuting the heterologous CDRs by recombination in the homologous scFv framework regions.

potential for further optimization by recombination is lost.

Utilizing potential diversity

Shuffling causes recombination primarily in the homologous framework sequences separating the heterogeneous CDRs, creating new combinations of CDRs. The number of potential CDR combinations is initially about $(4 \times 10^7)^6 = 4 \times 10^{45}$ ("potential diversity," Fig. 6)¹⁰. Even though we only screened a total of 1×10^8 clones, our recursive small-library approach using shuffling and a gradually increasing selection stringency may in theory be as powerful as screening a sin-

gle library of 10^{15-30} different sequences. As in molecular computation¹¹, recursive selection can effectively sample a sequence space that is as high as the product of the sizes of the individual libraries, rather than being merely additive.

For example, the size of a library of random sequence DNA needed to obtain a specific 8-bp restriction site is 64,000 bp. To obtain five different 8-bp restriction sites in specific locations would require a library of $(64,000)^5$, or 10^{24} , clearly too large to be obtained from a single library. But the five sites could efficiently be obtained in five recursive selection cycles from a library of 10^{5-6} bp,

by selecting for a different restriction site at each of five cycles¹¹.

Future prospects

The use of our small library required evolving an average of 18 mutations per scFv to achieve strong binding. For diagnostic applications the number of mutations is irrelevant. However, to obtain high-affinity therapeutic antibodies with fewer mutations, the combination of shuffling with large libraries is particularly promising.

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