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Design and construction of small perturbation mutagenesis libraries for antibody affinity maturation using massive microchip-synthesized oligonucleotides

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ABSTRACT

We report a rational strategy to design and construct multiple small perturbation mutagenesis (SPM) libraries using massively parallel synthesis of oligonucleotides on a microchip for affinity maturation of an engineered anti-ErbB2 antibody chA21. On the basis of a comprehensive analysis of the sequence and structural relationships of six complementary determination regions (CDRs) in the Kabatman database, a computational algorithm was developed to introduce single-site and double-site mutations into variable CDR positions using ambiguous nucleotides. The six SPM libraries were composed of 419 degenerate oligonucleotides that can be expanded into 161,832 unique CDR sequences with a high coverage ratio of 95% natural amino acid diversity. We used Illumina next-generation sequencing to demonstrate that the synthetic CDR library sequences, as well as relative quantities per sequence, can be controlled precisely by adjusting reaction chamber assignment and input nucleoside composition. The microchip-synthesized oligonucleotides were used for construction of single-chain antibody fragment (scFv) phage libraries through one-step mutagenic PCR of double-stranded plasmids with >10^6 *E. coli* transformants. A variant with combinatorial mutations from four individual CDRs achieved more than 19-fold affinity increase. The strategy described herein should be broadly applicable to affinity and selectivity studies of antibodies and other proteins.

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1. Introduction

The high binding affinity of monoclonal antibody is of great importance for clinical applications. In vitro display and screening over natural or, more often, artificial antibody libraries with phages or other systems have led to the discovery of many valuable antibodies (Bradbury et al., 2011). For antibody affinity maturation, one popular strategy used to generate a synthetic library is targeted mutagenesis of the complementarity determining regions (CDRs) at random or hotspot positions (Barderas et al., 2008; Chowdhury and Pastan, 1999; Laffly et al., 2008; Muller et al., 2011; Rajpal et al., 2005; Wark and Hudson, 2006). In many cases, libraries have been constructed by incorporating degenerate nucleotides at selected positions through solid-phase chemical synthesis of DNA oligonucleotides.

Multiple CDRs may be diversified simultaneously with blanket mutagenesis of multiple positions within each CDR. However, it would lead to a library dominated by too many destructive mutants, and successful selection would require a library whose size could easily exceed the capacity of current display systems. In addition, larger libraries are more prone to selection biases toward mutants that are better displayed or allow their host cells to grow faster (Derda et al., 2011; Luck and Trave, 2011). Thus, approaches that can reduce the theoretical size of a library without diminishing the chances of obtaining positive mutants have been actively sought. One method that satisfies this aim uses the rich information available from the increasing number of public sequences and structures of naturally occurring or engineered antibodies. Analysis of amino acid preferences in the CDRs of

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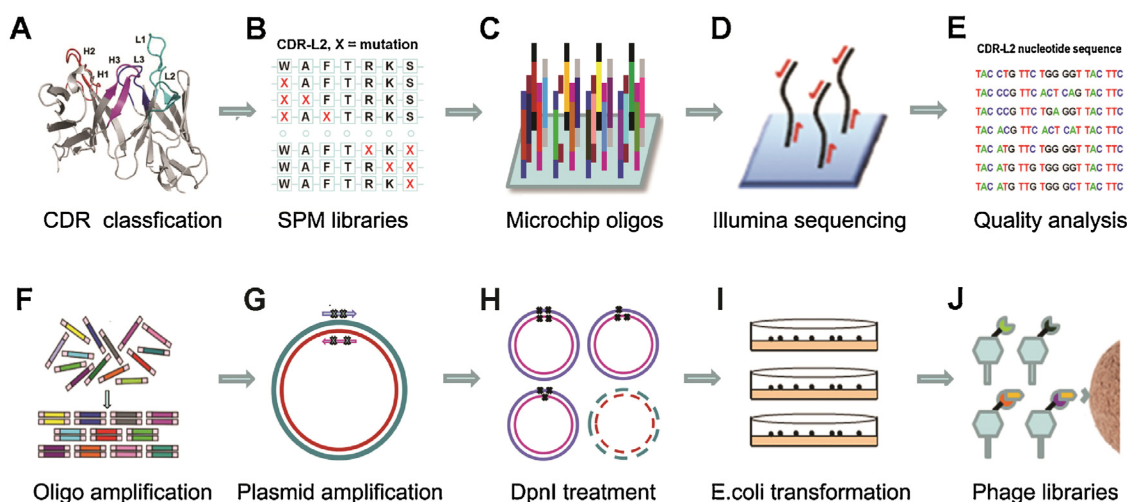

Fig. 1. Strategy for designing, constructing and validating SPM libraries. (A) The amino acid preferences of six CDRs were calculated after canonical structure prediction and sequence alignment. (B) Diversification was introduced at one or two variable CDR positions using degenerate codons. (C) The degenerate oligonucleotides were synthesized on a PicoArray chip. (D) The Oligomix was subjected to Illumina sequencing with single-end 35 bp reads. (E) The sequencing data were analyzed to evaluate the quality of the synthetic library. (F) The double-stranded CDR fragments were PCR-amplified using framework-specific primers from the OligoMix. (G–I) The mutant scFv plasmids were amplified using the CDR fragments as primers, treated with DpnI to remove the wild-type plasmid, and transformed into XL-10 *E. coli* cells. (J) The scFv phage libraries were screened for high affinity mutants on soluble antigen.

these antibodies may guide the design of artificial libraries to focus on mutants that are more likely to affect affinity with fewer negative side effects (Collis et al., 2003; Gonzalez-Munoz et al., 2012).

High-throughput gene synthesis technology has been driven by recent advances in DNA microchips that can produce massive numbers of non-degenerate oligonucleotides for cost-efficient gene assembly (Borovkov et al., 2010; Cleary et al., 2004; Quan et al., 2011; Tian et al., 2004). In the current study, we developed a rational strategy to design and construct small perturbation mutagenesis (SPM) libraries using massive microchip-synthesized oligonucleotides for improving antibody affinity (Fig. 1A–C). Antibody CDRs are first classified into distinct groups on the basis of canonical structure or sequence similarities (Martin, 1996; Al-Lazikani et al., 1997; Morea et al., 1998). In one SPM library, systematical residue mutations are randomly introduced to a limited number of the mutation-preferring CDR positions using tailored degenerate codons. The entire SPM libraries comprising up to tens of thousands of unique nucleotide sequences can be easily synthesized from a few hundred degenerate oligonucleotides on a programmable microchip.

To monitor the quality of the synthetic SPM libraries, we employed Illumina next-generation sequencing (NGS) technology (Fig. 1D–E), which has been successfully used for extensive analyses of antibody libraries (Ge et al., 2010; Larmana et al., 2012; Ravn et al., 2010, 2013). Based on a thorough analysis of the sequencing data, a number of parameters for the microchip synthesis of degenerate oligonucleotides have been optimized to produce balanced libraries that cover the desired amino acid sequences evenly. We further propose a simple approach for constructing single-chain antibody fragment (scFv) phage libraries through a modified methodology for one-step mutagenesis PCR of double-stranded plasmids from the microchip-synthesized oligonucleotides (Fig. 1F–J). With this approach, multiple antibody libraries with few sequence errors and biases can be easily produced with transformation efficiencies greater than 10^6 in bacterial cells.

As an example, the strategy described above was applied to chA21, an engineered tumor-inhibitory anti-ErbB2 antibody, for in vitro affinity maturation (Cheng et al., 2003). We created six SPM libraries, each comprising the single and dual residue mutations within one of the chA21 CDRs. After panning of phage libraries, a number of single CDR mutants with enhanced binding affinity were identified. A variant with combinatorial mutations from four CDRs achieved more than 19-fold increase in affinity.

2. Materials and methods

2.1. Assigning CDRs to canonical classes

The amino acid sequences of chA21 heavy and light chains were reported in Cheng et al. (2003). CDR residues were determined with the Kabat or Chothia definition schemes. Each CDR loop was submitted to canonical structure prediction in the Abysis antibody database server (http://www.bioinf.org.uk/abs/chothia.html). Sequences that shared the same canonical classes as L1, L2, L3, H1, and H2 CDRs of chA21 were collected from the database. We were not able to classify CDR H3 using a canonical clustering method, thus all H3 sequences with the same length as the chA21 H3 loop were collected. The collected sequences, together with the chA21 loop sequences, were aligned accordingly. From the sequence alignments, amino acid types and frequencies were calculated for every position of each CDR.

2.2. Designing degenerate oligonucleotides with ambiguous nucleotides

Ambiguous nucleotides were represented by the IUB convention. For each amino acid position to be mutated, all possible triplet codons composed from normal and ambiguous nucleotides were expanded into non-ambiguous codons. The codons were then ranked according to a computational scoring algorithm as follows:

$$\mathrm{Score}_{DEC} = \frac{1}{\sqrt{\sum (p_{aa}^{syn} - p_{aa}^{site})^2}} - f_1 \cdot \sum miss_{aa} - f_2 \cdot \sum ext_{aa} - f_3 \cdot s,$$

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