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Simultaneous randomization of antibody CDRs by a synthetic ligase chain reaction strategy

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We have adapted the ligase chain reaction (LCR), previously used as a DNA diagnostic tool (1, 2), to the covalent linking of synthetic oligonucleotides. The reaction exploits the capacity of a thermostable ligase to ligate two oligonucleotides which are hybridized to a targeted DNA sequence, provided the adjoining ends are perfectly base-paired to the target. Using eight oligonucleotides, instead of two or four as used in diagnostic applications, we have constructed a library of sequences encoding the variable domain (V_H) of an antibody heavy chain in which the three complementarity-determining regions (CDRs) have been randomly and simultaneously mutated by spiking the synthetic fragments (Figure 1). Only oligonucleotides of the correct size and base-pairing at adjoining ends are incorporated into the LCR product. The method provides a highly efficient way of assembling randomized CDR sequences. Introduction of diversity is an essential feature of antibody redesign and *in vitro* affinity maturation by the phage display technology. Semisynthetic approaches to this problem have thus far been limited to a single CDR (3, 4). In this work, the wild-type gene encoding a single-chain antibody specific for *Salmonella* serogroup B O-polysaccharide (5) was randomized in all of the heavy chain CDR regions.

Oligodeoxynucleotides (56mer–85mer) were synthesized using an ABI 384 DNA synthesizer such that the central 15–24 bases encoding the CDRs were randomized by equimolar spiking with A, G, C and T at a level of 10%. The LCR mixture contained 8 μl oligonucleotides (4 pmol each of non-phosphosphorylated 1–56mer and VIII-80mer, 4 pmol each of kinased II-73mer, III-71mer, IV-85mer, VII-69mer, VI-71 mer, V-65mer), 5 μl 10×reaction buffer (200 mM Tris–HCl, pH 7.6, 100 mM KCl, 100 mM $MgCl_2$, 1% Triton X-100, 1 mM ATP, 10 mM DTT), 4 μl *Pfu* DNA ligase (Stratagene), 5 μl (20 ng) plasmid harbouring the wild-type scFv gene and 30 μl water. LCR was performed in a thermocycler (Perkin-Elmer Cetus GeneAmp PCR system 9600) as follows: step 1 = 92°C for 3 min and 60°C for 3 min, 1 cycle; step 2 = 92°C for 45 sec and 60°C for 30 sec, 30 cycles; step 3 = 92°C for 4 min and 60°C for 3 min, 1 cycle. The 300-bp LCR product was purified by electrophoresis (Figure 2), eluted from the agarose gel and phosphorylated.

Synthesis of the full-length 300-bp LCR product was confirmed by PCR using the upstream primer 5′-AGCTGCAAAGCTA-GCGGTTACACCTTCACC-3′ and the downstream primer 5′-CGCCACCAGATCTGGAGGACACGGTCAGGC-

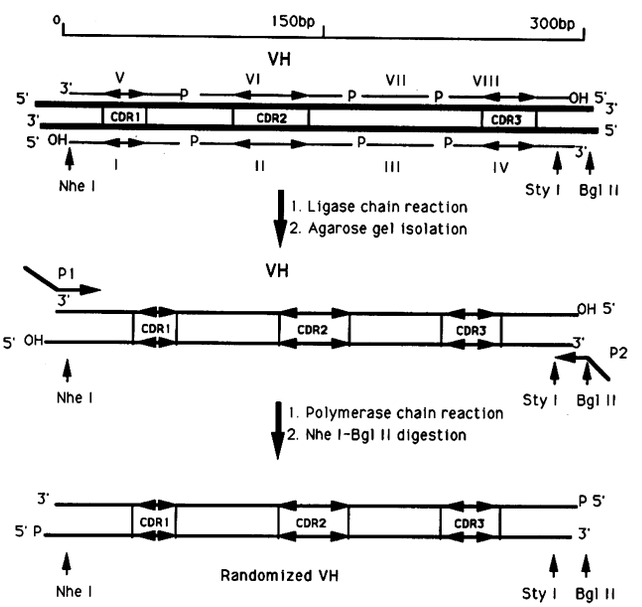

Figure 1. Simultaneous randomization of three CDRs by LCR using eight spiked oligonucleotides. The bold line represents the template DNA for LCR and the double arrows indicate the DNA sequences encoding the five, eight and six amino acid stretches (in CDR1, CDR2 and CDR3, respectively) that were mutated by the spiking procedure. P1 and P2 represent the PCR primers used for product detection and amplification.

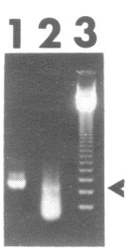

Figure 2. Agarose gel electrophoresis of PCR and LCR products. Lane 1, PCR product from LCR mixture at step 1; lane 2, final LCR product at position indicated by arrowhead; lane 3, BRL 123-bp ladder. The lane 1 PCR product is slightly larger than the lane 2 LCR product because of the restriction site flanking sequences in the P1 and P2 primers (Figure 1).

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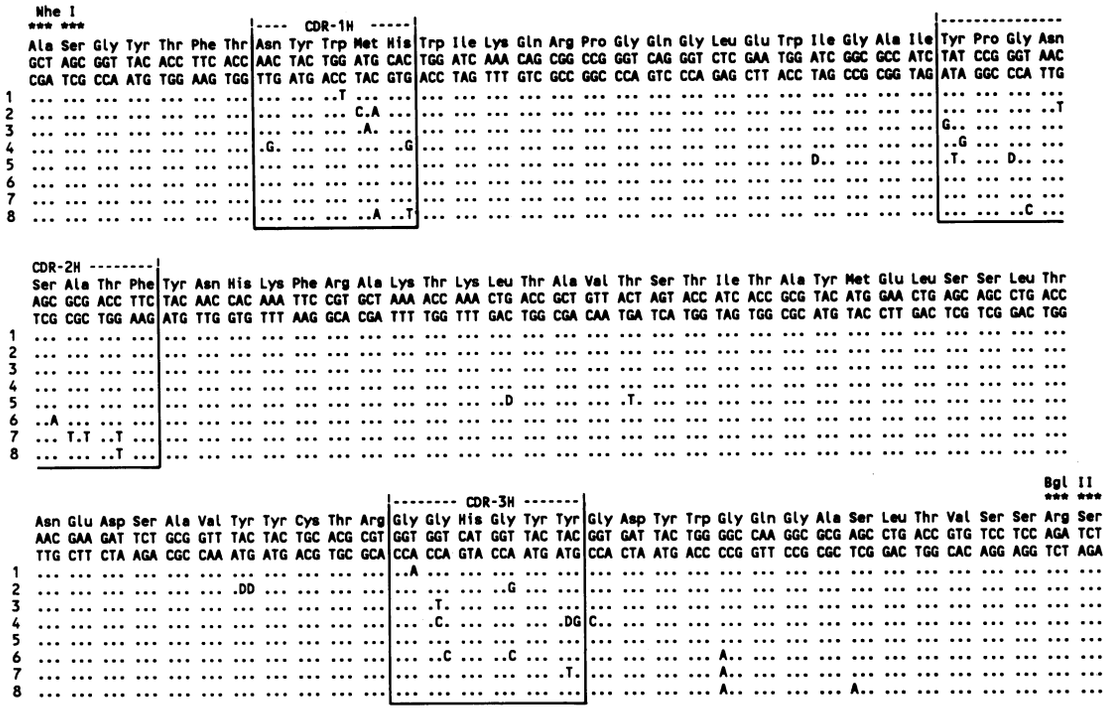

Figure 3. Heavy chain sequences of single chain antibody clones, randomly picked before biopanning, showing mutations clustered in the three CDRs. The sequences subjected to 10% spiking are enclosed in boxes. The eight clones contained all possible base substitutions (i.e. A to T, G, C; G to T, A, C; T to A, C, G; C to G, T, A). Some deletions (indicated by D) and framework mutations, thought to be DNA synthesizer errors, were also observed. Both the sense and anti-sense strands are shown for the wild-type sequence but only the sense strand is given for the mutant sequences with the dots representing unmutated nucleotides.

TCGCGCCTTGG-3′, the latter containing a diagnostic *Sty*I site not present in the wild-type ScFv gene. A range of product sizes were observed throughout the LCR procedure but full-length product was present as early as step I and was a significant component of mixture at step III (Figure 2). Half of the full-length LCR product (mutated VH) was amplified by PCR. The direct kinased LCR product and the PCR-amplified product, following *Nhe*I−*Bgl*II digestion, were pooled and ligated with the large *Nhe*I−*Bgl*II fragment of phagemid vector containing the light chain segment of the ScFv gene; this was done in an effort to give sufficient yield while not compromising the diversity of the library. The resulting mutated phagemid library was electroporated into XLl-Blue cells. The complete heavy chain nucleotide sequences of eight randomly picked clones from the phagemid library were determined (Figure 3). The sequences showed a clustering of mutations in the three heavy chain CDRs with no obvious bias for particular base substitutions. Antibody-gIII fusion phage was prepared and panning against antigen gave clones exhibiting higher antigen binding activity in ELISA screening. DNA sequencing of these clones showed that mutations had been introduced in all three heavy chain CDRs. The main advantage of the LCR approach is that it allows any number of selected regions to be randomized simultaneously and to varying degrees.

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