

liter each of these suspensions was used as a template for PCR. Sequencing of the plasmid DNA can now be performed using two PCR primers flanking the MCS of the vector (we used M13 universal forward primer and M13 universal reverse primer; Figure 1).

If the unique primer is set 100–150 bp from the end of the known sequence, sufficient overlapping sequence is generated to align the sequences unambiguously. This method was used in our laboratory for several cycles of sequencing. In the overlapping sequence, all bases were identical, suggesting that this method is not only fast and inexpensive but also accurate.

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## Scaling Up the Ligase Chain Reaction-Based Approach to Gene Synthesis

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In vitro gene synthesis is increasingly being applied to the extensive manipulation of DNA, RNA, and protein sequences. In particular, codon-optimized synthetic genes are often required for high-level expression of recombinant proteins in heterologous hosts to facilitate structural studies by X-ray crystallography and NMR (3). There are a variety of in vitro methods for synthesizing genes de novo from overlapping oligonucleotides. They rely on either ligation or numerous cycles of PCR to assemble the oligonucleotides into full-length genes. Successful attempts have been limited to shorter genes, generally of 500 bp or less (2,3, 5–7). A rapid method that is both cost effective and can be applied to the synthesis of average-sized proteins will become increasingly useful as the genome project reaches completion.

Design of the oligonucleotides that will be assembled into the full-length gene is the first step of gene synthesis, with the initial decision about the number and lengths of the oligonucleotides to be used being a principal concern. Because of the nature of chemical oligonucleotide synthesis, the need for oligonucleotide purification increases with length due to the corresponding increase in the proportion of incompletely synthesized product. By using larger numbers of shorter oligonucleotides, the need for purification is minimized. However, for successful gene synthesis from so many pieces, a highly efficient and stringent method is required. We investigated the application of a high-temperature ligation method for gene synthesis, developed by Au et al. (1), with oligonucleotides a maximum of 40 nucleotides in length (8). This ligase chain reaction (LCR) method (1) was originally applied to the synthesis of a 440-bp gene and uses a thermostable ligase for the cyclic, high-temperature annealing and ligation of numerous oligonucleotides to

form the gene in segments of 240 bp. Subsequent PCR assembly and amplification of these segments produced the full-length gene. Here, we have scaled up this high stringency method to synthesize a 1.5-kb gene from 84 nonpurified oligonucleotides (Figure 1).

The gene was first synthesized in segments approximately 260-bp in length, each having a 20-bp overlap with the next segment, and consisting of one set of 12 overlapping 40-mers and a pair of 20-mers to fill in the 5' overhangs. We note that the LCR became inefficient with segments composed of more than 14 oligonucleotides. Oligonucleotides were synthesized at standard purity (99% coupling efficiency) (Life Technologies, Melbourne, Victoria, Australia) and resuspended to 250  $\mu$ M in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA (TE).

### 1. Phosphorylation of Oligonucleotides in Sets of 14

Equal volumes of 14 oligonucleotides were premixed. A 15- $\mu$ L aliquot of each of the six premixed sets was diluted twofold by the addition of 15  $\mu$ L 2 $\times$  T4 polynucleotide kinase buffer (Promega, Melbourne, Victoria, Australia), 100  $\mu$ g/mL BSA, 2 mM rATP, 2 mM MgCl<sub>2</sub>, and 20 U T4 polynucleotide kinase (Promega). After incubation at 37°C for 2 h, the reactions were stopped by heating at 75°C for 10 min.

### 2. LCR

The six phosphorylation reactions were diluted to 40  $\mu$ L with 1 $\times$  *Pfu* DNA ligase buffer (Stratagene, La Jolla, CA, USA) containing 8 U *Pfu* DNA ligase (Stratagene) and overlaid with 30  $\mu$ L mineral oil. The LCR conditions consisted of an initial denaturing incubation of 95°C for 1 min, 40 cycles of 55°C for 1 min 30 s, 70°C for 1 min 30 s, 95°C for 30 s, with an additional incubation at 55°C for 2 min and 70°C for 2 min on the final cycle.

### 3. Gel Purification of Approximately 260-bp Segments

One microliter of the complete LCR was electrophoresed through a 2.5% agarose gel (Figure 2A), and the upper-

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most band for each set, corresponding to approximately 260 bp, was excised. Gel slices of three sets were combined for DNA purification by QIAquick™ gel extraction (Qiagen, Melbourne, Victoria, Australia) with elution in 30  $\mu$ L 1 mM Tris-HCl, pH 8.0, each. The eluate of both extracts was combined and dried down under vacuum in a SpeedVac® (Savant Instruments, Sydney, Australia).

#### 4. Assembly of Six Approximately 260-bp Segments into a 1476-bp Gene

The six combined approximately 260-bp segments were redissolved in

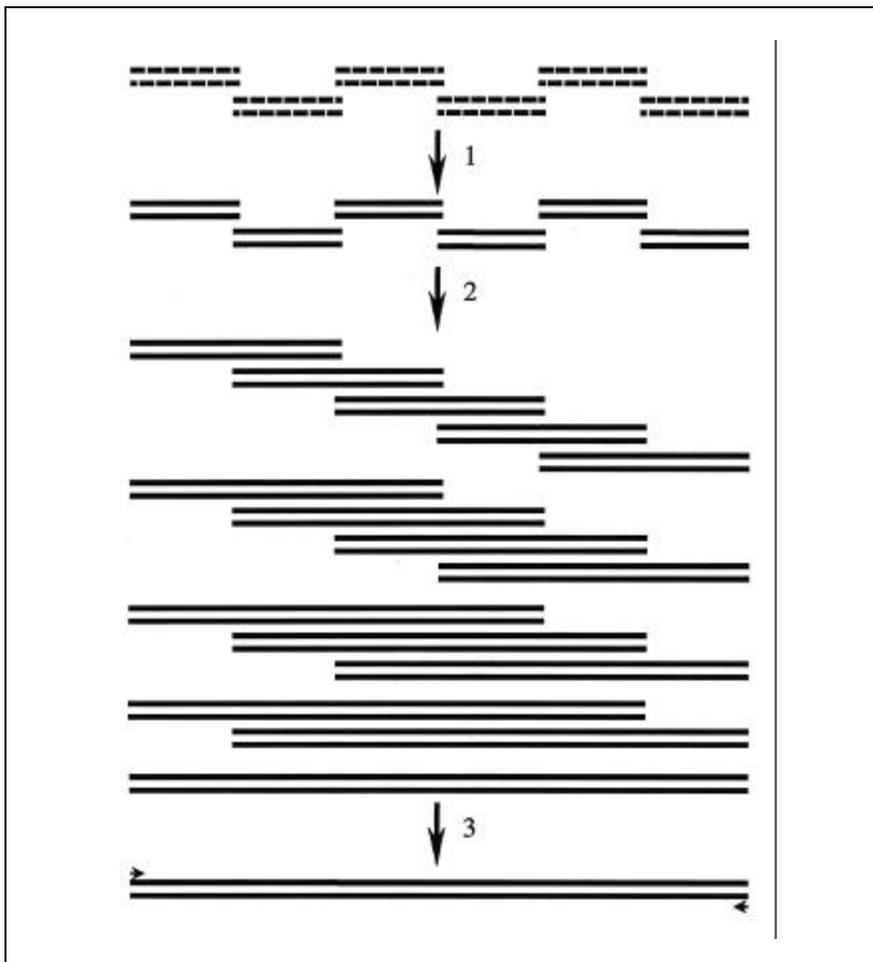
40  $\mu$ L PCR solution consisting of 1 $\times$  *Pfu* DNA polymerase buffer, 0.2 mM each dNTP, 1.5 U *Pfu* DNA polymerase (Promega), and overlaid with 30  $\mu$ L mineral oil. After denaturing at 95°C for 2 min, the PCR conditions were 10 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 2 min 30 s.

#### 5. Amplification of an Assembled 1476-bp Gene

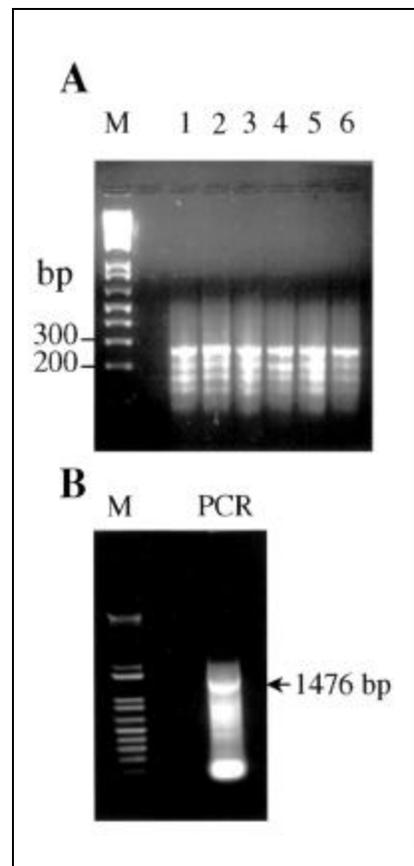
The 40- $\mu$ L assembly reaction was diluted to 50  $\mu$ L so that it contained in addition 1 $\times$  *Pfu* DNA polymerase reaction buffer, 0.2 mM each dNTP, and 1  $\mu$ M of the two primers (40-mers) located at the ends of the assembled se-

quence. The PCR parameters were 95°C for 2 min, then 20 cycles of 95°C for 30 s, 65°C for 30 s, and 72°C for 3 min. The entire reaction was electrophoresed through a 1% agarose gel (Figure 2B), and the band corresponding to 1476 bp was excised and purified with the QIAquick gel extraction. The PCR product was cloned into a bacterial expression vector with restriction sites introduced into the oligonucleotides at either end of the synthetic gene and transformed into *E. coli* DH5 $\alpha$ .

Of the clones that were sequenced, no PCR induced mutations (substitutions) were encountered, despite performing an additional five cycles of PCR compared with the original method of Au et al. (1). Au et al. found one clone of four contained a substitution



**Figure 1. Schematic representation of the method for scaling up the LCR-based approach to gene synthesis.** (Arrow 1) Oligonucleotides are combined in sets of 14, phosphorylated, and then ligated (LCR) to form doubled-stranded segments approximately 260 bp in length. (Arrow 2) The full-length segments are purified from an agarose gel (Figure 2A), combined, and then assembled into a full-length gene by self-priming PCR. (Arrow 3) Oligonucleotides corresponding to end sequences (represented by arrows) are subsequently added to the self-primed reaction to PCR amplify the full-length gene before gel purification (Figure 2B), restriction digestion, and subcloning.



**Figure 2. Ethidium bromide-stained agarose gels showing yields and quality of DNA at various stages of LCR-based gene synthesis.** Molecular weight markers (M) are the 1-kb plus DNA ladder™ (Life Technologies) (A) Six different LCR products (lanes 1–6) before purification of upper approximately 260-bp bands for PCR assembly. (B) Amplification of assembled gene by PCR with “end” primers.

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when synthesized with *Vent* DNA polymerase. Our decreased substitution rate presumably reflects the use of the high-fidelity *Pfu* DNA polymerase (4).

Less satisfying was the finding of small point deletions in all clones sequenced. Different clones had different deletions, but most (60%) had only a single base missing. These deletions are presumably the result of incorporation of a non-full-length oligonucleotide, amongst the 84, during LCR. No deletions were detected in the clones produced by Au et al. (1) with gel-purified oligonucleotides, suggesting that oligonucleotide purification is an important consideration.

Several options are therefore open to the investigator working to scale up the LCR-based approach to gene synthesis at minimum expense. Costs will rise steeply if gel purified oligonucleotides are purchased, as not only does purification incur additional charges but the synthesis must also be on a larger scale. In house gel purification of oligonucleotides is possible but increases the time spent on gene synthesis. A higher annealing temperature could be tested for LCR to minimize incorporation of non-full-length oligonucleotides, and/or more clones could be sequenced, at additional expense, to identify those without deletions. Considering all these factors, and our experience, the most cost- and time-effective strategy is to simply assemble the gene from non-purified oligonucleotides and perform site-directed mutagenesis to correct a single base pair deletion.

As anticipated by Au et al. (1), the LCR-based method for gene synthesis can be applied to larger genes by increasing the number of segments. In addition, this study shows that scaling up the LCR method with non-purified oligonucleotides is a cost- and time-effective approach to *in vitro* gene synthesis.

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## Improvement of the pBRINT-T<sub>s</sub> Plasmid Family to Obtain Marker-Free Chromosomal Insertion of Cloned DNA in *E. coli*

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The expression of heterologous genes by recombinant microorganisms usually depends on the use of extra-chromosomal replicating plasmid vectors that carry the gene(s) of interest. Although the usefulness of this type of vehicle has been well established in many cases, several problems related with their use have been identified, the most important being inefficient segregation and/or structural instability, as well as undesired copy number effects. An alternative approach for increasing the stabilization and controlling the copy number of heterologous genes is the chromosomal insertion of the gene(s) of interest.