100g Critical Aspects of Oligonucleotide Assembly by Pcr

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In the classic method of gene assembly used by Khorana (1979), synthetic DNA oligonucleotides are 5' phosphorylated using T4 kinase, annealed to form overlapping duplexes, and then enzymatically joined together using T4 DNA ligase. A variation of this method was used recently by Smith et al. (2003) to synthesize a 5,386 bp ÖX174 RFI DNA molecule in two weeks. Over 250 42-mers were 5' phosphorylated, gel-purified, annealed, and ligated together using Taq ligase at 65°C. After overnight high temperature ligation, the DNA reaction product was then amplified using the polymerase chain assembly (PCA) strategy of Stemmer et al. (1995).

All of the DNA assembly processes described to date suffer from three major shortcomings. (1) Gene assembly and PCA have always been carried out manually and therefore relatively slowly. To our knowledge, the fastest kilobase DNA syntheses were performed by Young and Dong (2004) and Smith et al. (2003). Young and Dong (2004) described the construction of a 1,200 bp synthetic DNA fragment in one week; Smith et al. (2003) reported synthesis of a 5,386 bp DNA molecule in two weeks. (2) In all reported experiments using PCA-based synthesis, error rates have been in the range of 2-5 errors/1,000 bp. For example, Smith et al. (2003) observed 11 sequence mistakes per synthetic 5,386 bp ÖX174 RFI molecule. If longer synthetic DNA molecules are to be constructed automatically, then such error rates will be catastrophic. At an error rate of 2 errors/1,000 bp (Smith et al., 2003), a synthetic 48.5 kilobase pair phage ë DNA molecule would contain ~97 errors, a molecule >100 kbp long would contain >200 errors, and a 1 Mbp DNA molecule would contain ~2000 errors. (3) The lack of a mathematical model to support a fundamental and systematic procedure to find the optimum assembly strategy.

The talk will be focused on developments in our laboratory towards resolving these critical aspects of assembly. (1) The two-step PCA method of Stemmer et al. (1995) was adapted to high-speed assembly reaction conditions using our rapid thermocycler, the PCRJet® (Quintanar and Nelson, 2002). Assembly of a synthetic 929 bp â-lactamase gene from component 52 synthetic 5'OH oligonucleotides (Integrated DNA Technologies, Corallville, IA) was completed in 20 minutes. The gene assembly was confirmed by DNA restriction digest analysis; and the fidelity of the 929 bp PCA construct was confirmed by dideoxy sequence analysis. The testing of functionality by cloning and expression in an ampicillin-resistant E.coli recombinant plasmid were also successful. (2) The 929 bp â-lactamase gene was gel-purified using a Quiagen Gel Purification Kit. Standard dideoxy sequencing using both forward and reverse primers was carried out in a CEQ8000 DNA Sequencer (University of Nebraska Core Facility). The DNA sequence obtained was consistent with the genomic sequence (GenBank Accession #L09137) of the pUC19 AmpR gene. Following these results, a brief review of the sources of errors will be given; errors from the phosphoramidite synthesis of the oligodeoxyribonucleotides, editing errors that occur during DNA polymerase-catalyzed enzymatic copying, and errors due to DNA thermal damage. The importance of thermal management and the advantages of rapid synthesis will be stressed. (3) The talk will conclude with a brief review of our modeling of PCA (Viljoen et al. 2005)and some of the key results of the model.