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Hetero-stagger cloning: efficient and rapid cloning of PCR products

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A variety of methods have been developed for cloning PCR products, including blunt-end cloning (1), restriction cut back (2), ligation-independent cloning (3), uracil DNA–glycosylase (UDG) treatment of uracil-containing deoxyoligonucleotide primers (4,5) and TA cloning (6–8). Blunt-end cloning of PCR products often requires treatment of PCR products to polish the ends (9). Even with treatments, blunt end cloning is inefficient. The key element in improving cloning efficiencies has been the creation of cohesive compatible ends. Other than blunt end cloning, various current available PCR cloning methods differ in how the cohesive ends are created. In the restriction cut back strategy, restriction sites are incorporated in the PCR primers and the cohesive ends are created by restriction digestion after PCR. This procedure involves two drawbacks: (i) extra bases for restriction sites increases the cost and leads to primer dimer formation; and (ii) restriction enzymes function poorly at the ends of the molecules (2). In both the ligation-independent cloning and the UDG cloning strategy, a 12 base overhang is created by treatment of the PCR products with T4 DNA polymerase or uracil DNA–glycosylase respectively. These procedures are highly efficient, but the main disadvantages are complications involved in vector preparation and the added expense of 24 extra bases to the PCR primer set (11). The TA cloning strategy utilizes the terminal extendase activity of *Taq* DNA polymerase (9) adding an extra base to the 3′ of the PCR products, which is template-independent, but is sequence-specific. A single G is added if the 3′ nucleotide on the fragment is a G, but an A is added if the 3′ nucleotide is a C, T or A, with very low efficiency of additions when the 3′ nucleotide is an A (10). As a result, TA cloning can only clone molecules with extended A. Most importantly, TA cloning is not useful with the newer proof-reading DNA polymerases for PCR amplifications such as *Pfu* or *Pwo* thermostable DNA polymerases (11).

In this communication, I present a novel method for PCR cloning termed ‘hetero-stagger PCR cloning’ (Fig. 1). The procedure is based on the fact that related PCR products of different lengths can be obtained by preparing related primers of different lengths. These related PCR products are mixed, heat-denatured, and annealed to form heteroduplex thereby molecules with cohesive ends can be generated. These molecules with cohesive ends can be readily cloned into compatible vectors (Fig. 1). PCR products amplified by using *Taq* polymerase as well as by using proof-reading thermostable enzymes can be cloned by this procedure.

Three extra bases, GGG, are added to half of the customized PCR primers at the very 5′ ends. The other half of the primers is made as determined by the target sequences without extra GGG. Two PCR reactions are set up: one PCR reaction with primer pair of GGG-upper primer plus lower primer, and the other PCR reaction with upper primer plus GGG-lower primer. The PCR products of the two reactions are identical except that each one harbors three extra

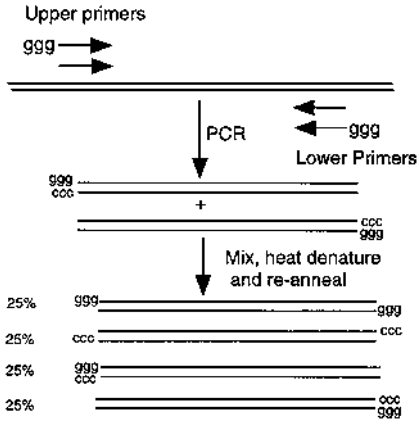

Figure 1. The hetero-stagger cloning strategy. Two PCR reactions were set up: one with GGG-upper primer (GGG + arrow) and lower primer (arrow), and the other with upper primer (arrow) and GGG-lower primer (GGG + arrow). The two PCR products are different only at the ends by 3 bp as indicated. These two PCR products were mixed, heat-denatured, and re-annealed. Four annealing products were expected, each at a 25% probability. Two of the four annealing products are molecules with cohesive ends: one with GGG 5′ overhang and the other with CCC 3′ overhang. These molecules with cohesive ends can be readily cloned into compatible vectors.

bases at different ends. When the two products are mixed, heat-denatured and allowed to re-anneal, four different annealing products are expected: two blunt end types of molecules, same as the PCR products, and two types of molecules with three base overhang, one of which with GGG 5′ overhang, and the other with CCC 3′ overhang. These cohesive molecules can now be readily cloned into compatible vectors (Fig. 1). Because the GGG-upper and upper, and the GGG-lower and lower primers are related primers, they can be made in the same synthesis.

Primers were designed to amplify the cystic fibrosis transmembrane conductance regulator (CFTR) gene exon 10 and exon 11. Primers were also designed for amplification of the λ phage DNA to generate sizes of 2 and 5 kilobase pairs (kb). *Taq* DNA polymerase (Boehringer) and *Pfu* DNA polymerase (Stratagene) were used to compare cloning efficiencies. For cloning each exon, two PCR reactions were set up. The first reaction used GGG-upper plus lower primer; the second used upper primer plus GGG-lower primer. PCR cycling conditions were set for 30 s at 94°C, 1 min at 55°C and 2 min at 72°C, for 30 cycles followed by a 7 min extension at 72°C. In the standard reactions, the PCR products from reactions 1 and 2 are mixed, heated at 95°C for 3 min and then at 37°C for 30 min. Standard ligation period was 4 h. For cloning with the TA cloning, DNA was mixed without heat treatment. The 2 and 5 kb λ DNA was amplified by using the extra

long XL PCR kit (Perkin Elmer) according to manufacturer's instructions. The plasmid vector pBSTFI was created by using synthetic linkers to generate a three base 3′ GGG overhang compatible with ends of heteroduplex (National Biosciences, Plymouth, MN). The hetero-stagger cloning vector was kept at a constant amount of 50 ng. TA cloning was performed according to the manufacturer's instructions (Invitrogen, CA). Transformation was performed by standard transformation as described (12). The transformation results were recorded 15 h after plating. Screening of the transformants were performed by using the mini-plasmid preparations with the alkaline lysis procedure (13). Plasmids were digested with *Sac*I plus *Kpn*I to release the insert. The digestion reactions were analyzed by agarose gel electrophoresis.

The hetero-stagger cloning strategy predicts that 50% of the PCR products are clonable by the cohesive end ligation or 25% of the cohesive-ended molecules are clonable by a single vector. To test this hypothetical efficiency, DNA from human cheek buccal cells were prepared (14) to amplify CFTR exons 10 and 11 of 390 and 301 bp respectively. As summarized in Table 1, the hetero-stagger cloning procedure efficiently cloned both the CFTR fragments. At least 5–10 times more white colonies were generated as compared with the TA cloning. This difference in efficiency of cloning was consistently observed. Ligations for TA cloning was established similarly as the hetero-stagger cloning, except that the mixed DNA was not treated for heteroduplex formation. The percentage of clones that harbor correct inserts were analyzed by restriction analysis and sequencing of representative clones. The hetero-stagger cloning gave 97–100% of white colonies harboring the correct inserts, and TA cloning gave 82–90% of white colonies harboring correct inserts. Similar cloning efficiencies were observed for both exon 10 and exon 11 fragments (Table 1A). To determine whether the incubation periods for heteroduplex formation can affect cloning efficiency, heteroduplex formation was incubated at 37°C for 5 min, 30 min, 2 h and 15 h after 95°C for 3 min. The period of incubation for heteroduplex formation did not significantly affect the cloning efficiencies although a slight decrease in overnight incubations were observed. Similarly, the ligation time did not have any noticeable effects on cloning efficiency (data not shown), indicating that ligations occurred rapidly with the three base GGG/CCC overhangs. To test the cloning efficiency of PCR products generated by using proof-reading thermostable DNA polymerases, CFTR exon 11 was amplified with *Pfu* DNA polymerase (11). The hetero-stagger cloning efficiently cloned the fragment while TA cloning failed to clone the PCR fragments amplified with *Pfu* polymerase, as expected. While 100% of the white colonies from the hetero-stagger cloning harbored correct inserts, only one of a few white colonies from TA cloning harbored the insert (Table 1B); Sequencing of this clone indicated that the insert was cloned by blunt end ligation.

To test the capability of the hetero-stagger cloning procedure for larger DNA fragments, sizes of 2 and 5 kb were amplified from the λ phage DNA and tested with the hetero-stagger cloning procedure. The DNA fragments of 2 and 5 kb were cloned successfully. In two independent experiments, an insert of 2 kb was cloned with 80% of white colonies harboring correct insert; the background seemed to be higher with large insert of 5 kb, with 40% of white colonies harboring the right insert (data not shown).

This paper describes a novel method for cloning PCR fragments. The main strength of the procedure is its high efficiency and abilily to clone faithfully amplified PCR products using proof-reading enzymes such as *Pfu*. Planning before synthesizing primers is required for using this method. Because the ligation involves three bases of strong G/C annealing, the ligation time can be reduced considerably, enabling PCR, ligation and transformation to be finished in <1 day. The hetero-stagger cloning, therefore, offers high speed, efficiency and fidelity for cloning PCR products.

Table 1. Cloning of the CFTR exons using hetero-stagger method and TA cloning method

Vector	Inserts	White colonies[a]	% with insert[b]
A. Cloning of fragments amplified by using *Taq* polymerase			
Hetero-stagger cloning	exon 10/exon 11	118/121	97/100
TA cloning	exon 10/exon l l	14/17	82/90
B. Cloning of exon 11 fragment amplified by using *Pfu* polymerase			
Hetero-stagger cloning	exon 11	195	100
TA cloning	exon 11	11	9

[a]Numbers were averages from three experiments.
[b]As a rule, 10 colonies were picked and plasmids prepared for analysis of inserts from each experiment.

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