



Rapid Restriction Enzyme-Free Cloning of PCR Products: A High-Throughput Method Applicable for Library Construction

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Abstract

Herein, we describe a novel cloning strategy for PCR-amplified DNA which employs the type IIs restriction endonuclease Bsal to create a linearized vector with four base-long 5'-overhangs, and T4 DNA polymerase treatment of the insert in presence of a single dNTP to create vector-compatible four base-long overhangs. Notably, the insert preparation does not require any restriction enzyme treatment. The Bsal sites in the vector are oriented in such a manner that upon digestion with Bsal, a stuffer sequence along with both Bsal recognition sequences is removed. The sequence of the four base-long overhangs produced by Bsal cleavage were designed to be non-palindromic, non-compatible to each other. Therefore, only ligation of an insert carrying compatible ends allows directional cloning of the insert to the vector to generate a recombinant without recreating the Bsal sites. We also developed rapid protocols for insert preparation and cloning, by which the entire process from PCR to transformation can be completed in 6–8 h and DNA fragments ranging in size from 200 to 2200 bp can be cloned with equal efficiencies. One protocol uses a single tube for insert preparation if amplification is performed using polymerases with low 3'-exonuclease activity. The other protocol is compatible with any thermostable polymerase, including those with high 3'-exonuclease activity, and does not significantly increase the time required for cloning. The suitability of this method for high-throughput cloning was demonstrated by cloning batches of 24 PCR products with nearly 100% efficiency. The cloning strategy is also suitable for high efficiency cloning and was used to construct large libraries comprising more than 10⁸ clones/μg vector. Additionally, based on this strategy, a variety of vectors were constructed for the expression of proteins in *E. coli*, enabling large number of different clones to be rapidly generated.

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Introduction

The availability of genome sequences from a large number of organisms has created a wide-spread need to clone complete sets of open reading frames (ORFs) followed by the expression and purification of the encoded gene products in a high-throughput manner to examine the functions of genes and proteins in organisms. Using a variety of thermostable polymerases, the target ORFs can be efficiently amplified by polymerase chain reaction (PCR) with virtually no errors. However, high efficiency and high-throughput cloning of amplified products into a suitable expression vector still remains an arduous task.

An ideal high-throughput cloning method should include a minimal number of steps without the need for intermediate purification and preferably be performed in the same tube in which the gene-of-interest was amplified. For such cloning, (i) the reagents used to prepare the vector and insert should not be proprietary and

should be available from multiple sources; (ii) the vector should not self-ligate and the insert should not form concatemers, rather ligate to the vector in the desired orientation; (iii) the primers for amplification should carry only small additional sequences without the need for any modifications, and thereby only add a sequence encoding a few amino acids at the vector/insert junctions to serve as spacers, and finally; (iv) the vector design should be amenable to modifications such as incorporation of different tags to enable purification and/or enhance solubility, or changing the promoter, antibiotic selection marker and even the origin of replication.

Traditional methods such as restriction enzyme-based strategies cannot be considered suitable for the high-throughput cloning of a large set of different ORFs due to the presence of numerous restriction enzyme sites within the ORF sequences [1]. As an alternative, the commonly used TA cloning methodology is simple, but lacks directionality in cloning and requires the inserts to be