Cloning vectors for expression-PCR products

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Traditionally, the production of a recombinant protein requires a preliminary cloning step of the target gene into an expression vector before evaluating its cellular expression. However, efficient expression of some genes requires further optimization steps (exchange of codons for example) that are time-consuming and carried out at relatively late stages in the cloning-expression process. An alternative cloning-expression strategy, where expression is tested first from a PCR product before cloning the target gene, is more versatile and amenable to automation for high-throughput studies. Recent progress in cell-free expression systems allows efficient protein production using a linear DNA template (1), which contains the target gene with all the regulatory elements necessary for transcription and translation, and extends the application of the expression-PCR technology (2). By combining a mutagenic PCR technique with cell-free protein synthesis, proper expression can be optimized in short periods of time. Once this preliminary step is achieved, the PCR product that gives satisfying expression results is selected and then cloned in a vector for its maintenance to produce the target protein, either in vitro in a large-scale exchange cell-free system (3) or in vivo in bacterial cells.

Several strategies for cloning blunt-ended PCR products have been developed to reduce the background caused by self-ligated or uncut vectors. Here, two such independent techniques: (i) a positive selection based on the cytotoxic gene ccdB (4) and (ii) the use of a restriction endonuclease in the ligation buffer (5,6) are combined to further increase the cloning efficiency of PCR products. This combination results from the serendipitous discovery of a natural SrfI site, an octanucleotide sequence recognized by the rare-cutting SrfI enzyme (7), inside the ccdB gene at the center of the β-sheet in the corresponding protein structure (8). Thus, any insertion into the SrfI site disrupts the lethal ccdB gene. In order to facilitate the final stage of this alternative expression-cloning scheme, plasmids without any other promoter than those of the ccdB and antibiotic resistance genes were designed, and a PCR product carrying a gene coding for the green fluorescent protein (GFP) under the control of the T7 promoter was cloned for testing its Escherichia coli expression from these plasmids.

The two first plasmids pCRamp and pCRcam were constructed in two steps from plasmid pDESTM17 (Invitrogen, Carlsbad, CA, USA) by deleting either a 2934-bp Tth1111/BssHII fragment (pCRamp) or a 2119-bp Tth1111/NcoI fragment (pCRcam) and then by deleting from the two preceding constructs either a 614-bp SalI/HindIII fragment (pCRamp) or a 1638-bp SalI/AhdI fragment (pCRcam). Both resulting plasmids carry the complete ccdB gene and a pBR322 origin of replication with an ampicillin- or chloramphenicol-resistance marker. A third plasmid, pCRkan, was constructed from pCRamp by replacement with the antibiotic-resistance genes. The substitution of the bla gene by the aph gene from pSU38 (9) into pCRamp was performed by a three-step PCR protocol (10). The PCR fragment, containing the gene coding for GFP under the control of the T7 promoter, was amplified from the Rapid Translation System (RTS) control vector (Roche Diagnostics, Indianapolis, IN, USA) using the proofreading Platinum PfX DNA polymerase (Invitrogen) and the T7 primers provided in the RTS E. coli Linear Template Generation Set kit (Roche Diagnostics). Correct expression of GFP from the linear DNA template was verified in vitro using the RTS100 E. coli HY kit (Roche Diagnostics).

The ligation reactions were performed in 15 μL of ligation buffer [50 mM Tris-HCl, 10 mM MgCl2, 1 mM ATP, 1 mM dithiothreitol (DTT), pH 7.6] containing 600 ng purified PCR product, 50 ng circular plasmid (pCRamp, pCRcam, or pCRkan), 10 U of SrfI (Stratagene, La Jolla, CA, USA), and 5 U of T4 DNA ligase (Roche Diagnostics). If the PCR product has no SmaI site, the alternative blunt-end restriction SmaI enzyme can be used instead of SrfI. This cloning occurs in a reaction similar to that described for the pCR-Script vector from Stratagene (11), except that the actual protocol used circular plasmid DNA instead of a predigested linear plasmid. The best cloning efficiencies were obtained with a final molar ratio insert:vector of 20:1. The reactions were incubated at room temperature for 15 min, and then each mixture was used to transform competent E. coli strain DH5α (Invitrogen). After transformation, the cells were spread on LB plates containing either 100 μg/mL ampicillin, 25 μg/mL chloramphenicol, or 30 μg/mL kanamycin and incubated overnight at 37°C. Twenty-five transformants were analyzed by colony PCR using the primers CCDB5, 5’-CTGTGGTTGTGAGATG-TACAGAG-3’, and CCDB3, 5’-CTG-GCCAGGGGATCATC-3’, flanking the SrfI site. In all cases, the DNA fragment (1200 bp) was correctly inserted into the ccdB gene of the three different plasmids (Figure 1A). These vectors are routinely used in my laboratory for cloning blunt-ended PCR products, and the number of false-positive colonies, depending on the quality of the DNA fragment, varied from 0% to 5%. Since blunt-end cloning results in bidirectional insertion, both orientations of the PCR product were expected. Restriction mapping on purified plasmid DNA showed that both orientations are effectively found with the same probability among the twenty-five recombinants (Figure 1B).

For protein production, bidirectional cloning is not a drawback if the plasmid backbones do not have any influence on the expression of the gene cloned into the PCR-based cassette. To test this hypothesis, expression of GFP in the E. coli BL21(DE3) strain (12), transformed with each of the plasmids containing the PCR fragment in both orientations, was compared. Cells were grown at 30°C in LB medium supplemented with the appropriate antibiotic, and expression was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After 3 h, cells were harvested by centrifugation at 20,000×g for 10 min and lysed,
Figure 1. Analysis of recombinants by colony PCR, restriction, and expression. (A) Using the primers CCDB5 and CCDB3, a colony PCR screen was conducted from 25 recombinants producing products that discern the presence of the cloned insert in pCRamp. (B) Plasmid DNAs from 25 recombinants as shown in panel A were purified, and EcoRI restriction endonuclease was used to determine orientation of the cloned insert. In one orientation, 1→, EcoRI digestion will yield fragments of 3491 and 372 bp. In the other orientation, 2←, the fragments will be 2740 and 1123 bp. (C) Expression of green fluorescent protein (GFP) in the BL21(DE3) strain. Proteins from whole cell extracts were separated on a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel and stained by Coomassie blue. Lanes: 1, BL21(DE3) without plasmid; 2, pCRampGFP.1→; 3, pCRampGFP.2←; 4, pCRcamGFP.1→; 5, pCRcamGFP.2←; 6, pCRkanGFP.1→; 7, pCRkanGFP.2←; and M, Prestained Protein Markers (New England Biolabs, Beverly, MA, USA).

and proteins from whole cell extracts were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) following Coomassie® blue staining (Figure 1C). In all cases, whatever the orientation of the PCR fragment, the expression level of GFP, encoded by the six plasmids, was similar. Thus, the cloning of an expression PCR product into these new vectors is rapid, highly efficient, and suitable for recombinant protein production in E. coli.

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The author declares no conflicts of interest.

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