Preparation of T-Overhang Vectors with High PCR Product Cloning Efficiency

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Cloning of polymerase chain reaction (PCR) products can be a valuable research technique, but in practice the technical problems associated with the methodology may limit its usefulness. A variety of methods have been developed that facilitate PCR product cloning. These include blunt-end ligation cloning (5), ligation-independent cloning (1,6), introduction of restriction sites into PCR primers (3,5) and introduction of 3'-terminal thymidine nucleotide overhangs (T-overhang) in the plasmid (2,4) (available commercially as pGEM®-T vector from Promega [Madison, WI, USA] or TA Cloning™ Vector from Invitrogen [San Diego, CA, USA]). The first two of these PCR product cloning strategies characteristically have a low efficiency of cloning (i.e., in terms of the percentage of colonies that contain an insert) and also yield many false positives. Designing restriction sites into PCR oligonucleotide primers increases their cost, and many terminal restriction sites are not good substrates for their restriction enzymes. The commercially available T-overhang kits are rather expensive, and (in our hands) the efficiency of cloning is quite variable from lot to lot. For these reasons, we have sought to modify one of the aforementioned cloning strategies so as to develop an inexpensive, efficient and highly reproducible method of generating clones of PCR products.

We routinely use pBluescript® II SK (Stratagene, La Jolla, CA, USA) plasmid digested with EcoRV to clone single PCR fragments. We use a vector:fragment ratio of 1:3 in a 10-µL reaction containing ligase buffer in a 0.5-mL microcentrifuge tube using 1 U T4 Ligase (Life Technologies, Gaithersburg, MD, USA). The efficiency of PCR product insertion is quite low (~1%), but is adequate when the insert is abundant or easily obtainable. However, this level of cloning efficiency may not be acceptable for PCR products that are rare or difficult to generate. Use of the other blunt-end-forming restriction site in this vector (SmaI), and others, yielded even lower cloning efficiencies in our hands. The efficiency of blunt-end cloning is increased (to ca. 60%) when the EcoRV-cut plasmid is dephosphorylated. It is germane to note, however, that the number of clones obtained is quite low. A typical ligation (under the aforementioned conditions) may produce 15–20 white colonies, of which 10–15 are positives.

The plasmid dephosphorylation step, then, increases the cloning efficiency only because the background (i.e., number of blue colonies) is reduced. The typically low efficiency of blunt-end ligation cloning is due, in part, to the template-independent terminal transferase activity of Taq DNA polymerase, which results in the addition of a single adenosine residue to the 3' ends of most of the PCR-generated DNA fragments.

Since most of the PCR-generated DNA fragments have an A-overhang, they can be more efficiently cloned by ligation into a vector prepared with a T-overhang. This is the rationale underlying the T-overhang strategy of PCR product cloning. We prepare T-overhang vector as follows: 10 µg of EcoRV-digested vector are incubated (100 µL total volume) in PCR buffer (Life Technologies) with 2 mM dTTP and 5 U Taq DNA Polymerase (Life Technologies) at 72°C for 2 h. We find that the efficiency of PCR product cloning using a T-overhang vector prepared in this manner is typically about 5%. Of the white (i.e., putative positive) colonies thus obtained, about 60% actually have a PCR-generated insert.

In this report, we present a strategy to further optimize PCR product cloning by modifying the T-overhang cloning protocol. We have found that, by first eliminating the vectors that do not end up with a T-overhang from the total plasmid pool, cloning efficiency is dramatically improved. This particular modification of the T-overhang cloning strategy may be especially useful when cloning a heterogenous population of PCR products, as is the case when generating or amplifying cDNA libraries.

Our modified T-overhang protocol involves phenol/chloroform extraction and ethanol precipitation of the T-overhang vector prepared as described above. After precipitation and drying, the pellet is resuspended in distilled, sterile water and ligated in a 50-µL reaction containing ligation buffer (Life Technologies) for 16 h using 2 U of T4 ligase (Life Technologies). Vectors without the T-overhang will self-ligate (and undergo varying degrees of concatemerization). These vectors will appear on an agarose gel as a broad band and resolve away from the vectors (with a T-overhang), which remain

Figure 1. Agarose (1% wt/vol) gel electrophoresis of pBluescript vector. Lanes (from left to right) show migration of marker DNA (λHindIII), pBluescript plasmid linearized with EcoRV, T-tailed pBluescript subjected to self-ligation and T-tailed pBluescript subjected to self-ligation with subsequent GeneClean II purification of the plasmid fraction that remained linearized. An arrow highlights the band of linearized pBluescript plasmid in each preparation. Note that in the lane farthest to the right, the purified plasmid preparation contains only linearized (i.e., those that are T-tailed) vectors.
linearized (Figure 1). T-overhang vectors resolve as a sharp band that can be excised from the gel and purified. We use Geneclean® II (Bio 101, La Jolla, CA, USA) to purify the linearized plasmid and routinely rerun a sample of the purified vector on a gel to quantify the amount of plasmid that is obtained after purification (Figure 1). Use of vectors prepared in this manner increase the efficiency of PCR product cloning up to 80%. In our hands, 8 out of 10 white colonies obtained after cloning are found to contain an insert.

A comparison of the standard T-overhang protocol and our modified version is shown in schematic fashion in Figure 2. Blunt-end cloning with EcoRV-digested vector can have an efficiency of less than 1%. Dephosphorylation of the EcoRV-digested plasmid increases cloning efficiency to 60%. However, dephosphorylation does not yield more white colonies, just less blue ones. Using Taq DNA polymerase and dTTP yields a plasmid preparation that is a mixed population of blunt-ended and T-overhang vectors. Cloning efficiency, however, is still low (5%). Removing vectors that do not have T-overhangs prior to ligation of PCR products substantially increases cloning efficiency up to 80%. We believe our modified T-overhang cloning protocol may be a worthwhile and useful technique for improved cloning of PCR products.

REFERENCES

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