PCR Cloning Considerations

Nature of the Insert

The cloning of PCR-amplified fragments into a linear vector is typically a rapid and efficient process. However, not all PCR fragments will clone with the same efficiency into the same vector. These differences may be due to fragment size, insert toxicity, and the complexity of the insert. Inverted, AT-rich, or GC-rich repeats may contribute to the instability of the fragment as a cloned product in any vector (pCR®II, pCR®2.1, pcDNA™3.1, pUC18).

Insert Size

The size of the fragment being cloned is a primary contributor to the overall cloning efficiency. Large fragments of DNA (≥ 5 kb) are amenable to cloning in high-copy number vectors, yet at a much lower efficiency.

Vector-to-Insert Ratio

Optimization of molar concentration ratios of the vector to insert is critical to ensure efficient cloning. Successful cloning ratios may range from 1:1 to 1:10. One common strategy for determining the optimal ratio is by preparing several vector: insert ratios: 1:1, 1:3, and 1:5. While these ratios may not be ideal for all cloning events, they are useful for most cloning needs. For example, if the vector is 3 kb and the insert is 1 kb, one-third the amount of insert needs to be added to attain a 1:1 molar ratio. When performing TOPO® -TA or Directional TOPO® Cloning, optimal results are achieved most often when using a 1:10 dilution of the PCR product.

Fresh PCR Product

The use of fresh PCR products in TA, TOPO® TA, and Directional TOPO® Cloning is recommended due the potential presence of exonucleases that will, over time, degrade the nucleotide overhangs, reducing the efficiency of the cloning event. While it is not recommended, some PCR products have been successfully cloned after 1 week of storage at +4˚C.

Importance of Positive and Negative Controls

In any cloning experiment, the use of positive and negative controls is important. Without appropriate positive and negative controls for your cloning and transformation reactions, it is very difficult to evaluate the results of a cloning event. These controls are indicators of enzyme activity in DNA preparation and transformation efficiency of competent cells. Troubleshooting is virtually impossible without any controls. To ensure the efficiency of the cloning reaction, each of Invitrogen's kits includes controls.

Compatibility of DNA Ends of Vector and Insert

TA Cloning® technology was designed to clone PCR products produced by *Taq* polymerase. It takes advantage of the terminal transferase activity of this polymerase which adds a single 3'-A overhang to each end of the PCR product. Blunt cloning vectors and directional TOPO® cloning technologies are designed to clone PCR products produced by proofreading polymerases. Successful cloning depends upon using the correct polymerase with your cloning vector.

Addition of 3'-A Overhangs Following PCR Amplification

Direct cloning of DNA amplified by proofreading polymerases into TA Cloning® or TOPO TA Cloning® vectors is often difficult because of very low cloning efficiencies. This is because proofreading polymerases possess 3´→5´ exonuclease activity that removes the 3´-A overhangs necessary for TA Cloning® and TOPO TA Cloning®. A simple procedure to add 3´ adenines to blunt-end fragments is provided below. Other protocols may be suitable.

You will need the following items:

- *Taq* polymerase
- A heat block equilibrated to 72˚C
- Phenol-chloroform
- 3 M sodium acetate
- 100% ethanol
- 80% ethanol
- TE buffer

1. After amplification with a proofreading polymerase, place samples on ice and add 0.7-1 unit of *Taq* polymerase per tube. Mix well. It is not necessary to change the buffer or remove the proofreading polymerase. A sufficient number of PCR products will retain the 3´-A overhangs.
2. Incubate at 72˚C for 8-10 minutes (do not cycle).

3. Place on ice and use immediately in a TA Cloning® or TOPO TA Cloning® reaction. If you wish to store your reaction, continue to Step 4.
4. Extract reaction immediately with an equal volume of phenol-chloroform. This removes all of the polymerases.
5. Precipitate the DNA by adding 1/10 volume of 3 M sodium acetate and 2X volume of 100% ethanol.
6. Centrifuge at maximum speed (14,000 rpm in a microcentrifuge) for 5 minutes at room temperature to pellet the DNA.
7. Remove the ethanol, rinse the pellet with 80% ethanol, and allow to air dry.
8. Resuspend the pellet in TE buffer to the starting volume of the PCR amplification reaction. The PCR amplification product is now ready for ligation into the TA Cloning® or TOPO TA Cloning® vector.

Note: If you have more than one PCR product, you may wish to gel-purify your fragment using the S.N.A.P.™ MiniPrep Kit. After purification, add *Taq* polymerase buffer, dATP, and 0.5 unit of *Taq* polymerase, and incubate 10-15 minutes at 72˚C. Proceed directly to the cloning reaction.

Designing the Forward PCR Primer for Directional TOPO® Cloning

Successful directional TOPO® Cloning depends on the design of the forward PCR primer and, to a lesser extent, on the design of the reverse PCR primer. To clone directionally, the forward PCR primer must contain a simple Kozak sequence (CACC**ATG**) at the 5´ end of the primer. The four nucleotides, CACC, base pair with the overhang sequence, GTGG. The bold ATG is the initiation codon of your protein of interest.

Designing the Reverse PCR Primer

To ensure that your ORF clones directionally with high efficiency, the reverse PCR primer **must not** be complementary to the overhang sequence GTGG at the 5´ end. A one base pair mismatch will reduce the directional cloning efficiency to 75%, and may result in your ORF being cloned in the opposite orientation. We have not observed any evidence of PCR products cloning in the opposite orientation because of a two base pair mismatch, but this has not been tested directly. Other options to consider are listed in Table 1.

Table 1 - Options to consider when designing the reverse PCR primer

Option	Action
Include the C-terminal tag encoded by the vector	Design your reverse PCR primer so that your ORF is in frame with the C-terminal tag and does not contain a stop codon
Omit the C-terminal tag encoded by the vector	Design your reverse PCR primer to include a stop codon or design it to anneal downstream of the native stop codon
Use another C-terminal tag	Design your reverse PCR primer to contain the tag of interest and include a stop codon to prevent inclusion of the C-terminal tag
Secrete your PCR product using mammalian or insect Gateway® native expression vectors	Use the pENTR™/D-TOPO® vector and include the appropriate secretion signal

In addition to the major considerations above, you may have other options to consider depending on the directional cloning vector you are using. Please refer to the respective manuals for detailed information.