Cloning

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- **How can we clone PCR products?**

pGEM®-T Easy Vector System is a convenient system for the cloning of PCR products .

- **How is the vector prepared?**

The vector is prepared by cutting the pGEM®-T Easy Vector with EcoRV and adding a 3' terminal thymidine to both ends (Fig. I). These single 3'T overhangs at the insertion site greatly improve the efficiency of ligation of a PCR product into the plasmids by preventing recircularization of the vector and providing a compatible overhang for PCR products generated by Taq as a thermostable polymerase. Taq polymerase often adds a single deoxyadenosine, in a template-independent fashion to the 3' ends of the significant proportion of amplified fragments as shown in the Figure. Using this method, only one insert will be ligated into the vector as opposed to multiple insertions that can occur with blunt ended cloning. In addition, with T vector cloning there is no need to dephosphorylate the vector, and there is a low background of relegated vector.

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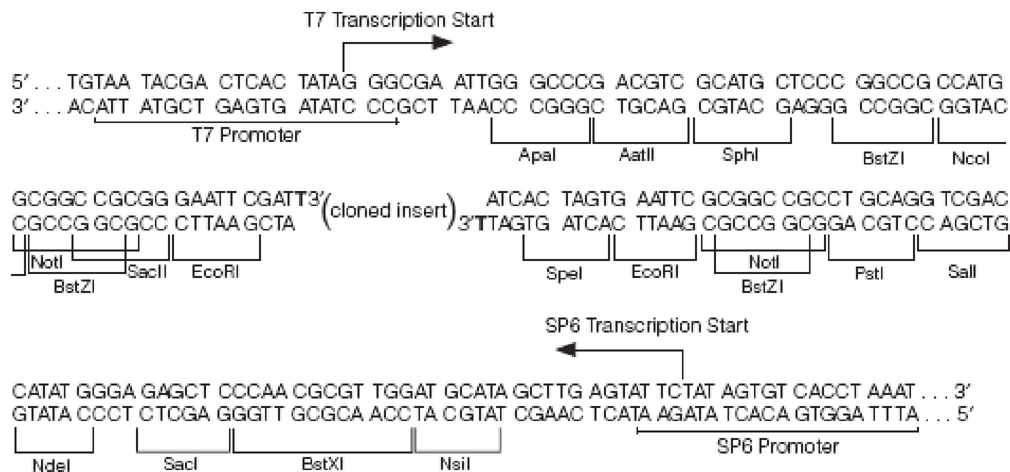

Fig. I. The promoter and multiple cloning sequence of the pGEM®-T Easy Vectors. The top strand of the sequence shown corresponds to the RNA synthesized by T 7 RNA polymerase whereas the bottom strand corresponds to the RNA synthesizes by SP6 RNA polymerase.

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pGEM®-T Easy Vector also contains the origin of the replication of the filamentous phage f1 for the preparation of single stranded DNA. The ssDNA molecule exported corresponds to the bottom strand shown in the Fig.II.

pGEM®-T Easy Vector contains multiple restriction sites within the multiple cloning regions. This region is flanked by recognition sites for the restriction enzymes EcoRI, BstZI and NotI, thus providing three single enzyme digestions for release of the insert.

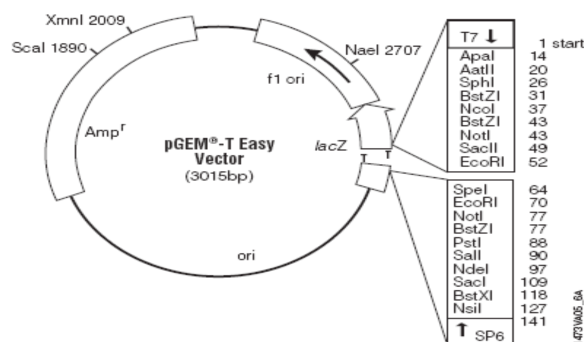

Fig. II. pGEM®-T Easy Vector circle map.

- **How does cloning work?**

PCR products are cloned using an *E.coli* competent cell prior to sequencing in the following three steps:

- Ligation reaction using pGEM®-T Easy Vectors
- Transformation of ligated pGEM®-T Easy Vector
- Blue/white screening

The ligation step will be discussed in this section while the other two will be treated in the next section of cloning.

- **How does ligation occur?**

50ng/µl pGEM®-T Easy Vector (approximately 3kb) is briefly centrifuged to collect the contents at the bottom of the tubes. Rapid Ligation Buffer is also vigorously whirled to a vortex before each use. This buffer contains ATP, which degrades during temperature fluctuations. Multiple freeze-thaw cycles and exposure to frequent temperature changes are avoided by single use aliquots of the buffer.
Collectively, 3-7µl of purified PCR fragments is used in ligation reaction. The ligation reaction is prepared for each sample separately in a 0.7ml tube to have low DNA binding capacity. One µl of the vector and 1µl of the buffer are added to the PCR product and lastly 1µl of the 100U T4 DNA Ligase is mixed with them. The reaction is mixed by pipetting and incubated overnight at 4 °C for maximum number of transformants.

- **When does cloning fail or succeed?**

To optimize cloning efficiency, the amount of DNA in the ligation reaction is adjusted depending on the molar yield of the purified PCR product. When molar concentrations are high due to small fragment size or good amplification, small volumes of the PCR fragment are needed for ligation reaction and therefore 2X Rapid Ligation Buffer is used. However when molar concentration is low due to large fragment size or poor amplification, large volumes of the PCR fragment are needed for the ligation reactions and therefore 10X Rapid Ligation Buffer is used.
The following equation is therefore used to calculate the appropriate amount of PCR product (insert) to include in the ligation reaction:

ng of vector × kb size of insert/ kb size of vector × insert:vector molar ratio = ng of insert

For example: 30ng is needed for 0.6kb PCR product added to a ligation in which 50ng of 3.0kb vector is used in a 3:1 insert:vector molar ratio.

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- **Contact us**

For more information or troubleshooting on your PCR purification, please do not hesitate to contact us at *ijpp@iau-saveh.ac.ir*. You can simply mention your problem by attaching your gel picture. We look forward to hearing from you soon.

- **Read more on**

http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Cloning/PCR-cloning.html

http://www.promega.com/products/pcr/pcr-cloning/

http://www.protocol-online.org/prot/Molecular_Biology/Molecular_Cloning/PCR_Cloning/index.html

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