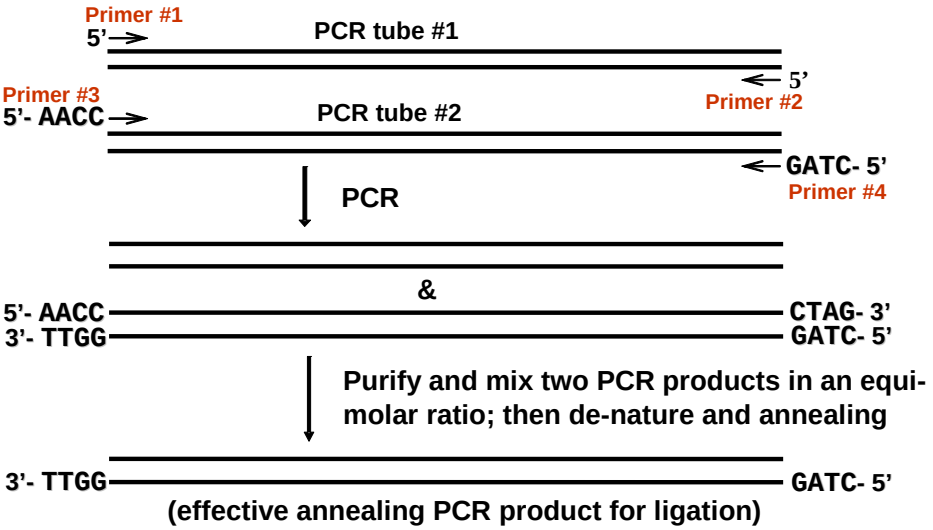
Protocol for Cloning Insert into AS2 Series Plasmids (Sticky End PCR method)

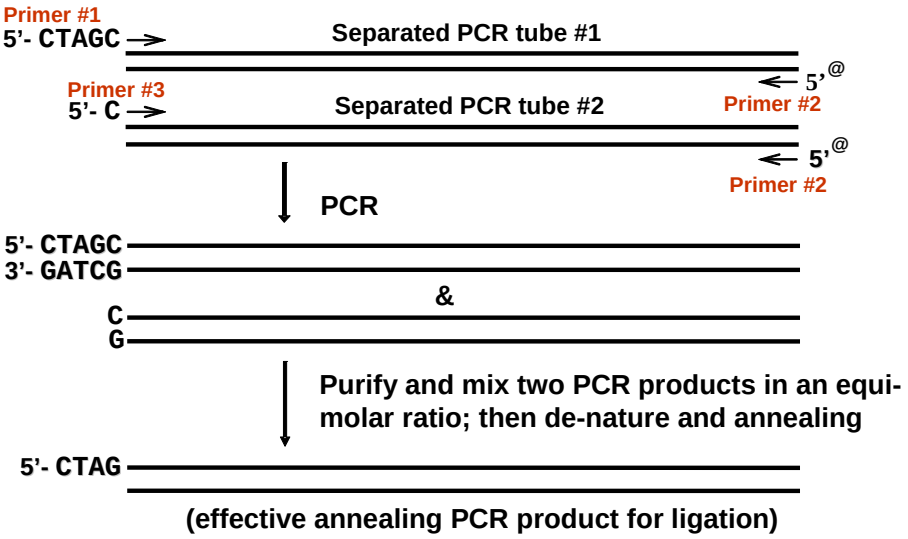
Introduction

96/12/27

A PCR cloning strategy called **Sticky End PCR Cloning** (Zeng, 1998) that allows one to generate sticky end by using standard PCR method is described below. In this method, two pairs of PCR primers are designed and are amplified in two different reactions. Both PCR products are mixed in an equimolar ratio and purified using Roche PCR Cleaning Kit or equivalent products. The purified PCR products are then denatured and re-natured at 95^0C for 5-min and 65^0C for 10-min, respectively; approximately 25% of the final product carries desirable protruding ends and is ready for ligation (Option: The kinase reaction could be performed to phosphorylate 5'-ends of the PCR products to enhance ligation efficiency.). A schematic diagram outlining the procedure is shown below (The pLKO_AS2 in the destination cloning sites downstream of EMCV IRES is used here as an example. The protruding sequences were restricted to end with 5'-AACC-3' (*Bst*XI; 3'end protruding) at one arm and with 5'-CTAG-3' (*Xba*I; 5'end protruding) at the other arm, arrows indicate the primers):



Alternatively, annealed PCR products with protruding sequences at one end and with blunt sequences at the other end can also be prepared by this approach. (The pLKO_AS2 in the destination cloning sites downstream of CMV immediate early promoter (CMViep) is used here as an example. The protruding sequences were restricted to end with 5'-CTAG-3' (*Nhe*I) at one arm and with blunt end' (*Pme*I) at the other arm, arrows indicate the primers.)



@ **Both reactions use the same reverse primer for PCR amplification.**

Procedures

(a) Preparation of insert:

1. Follow standard rules to design two pairs of PCR primers containing desirable protruding sequences at the 5'end according to the requirement as described in aforementioned examples.
2. Perform two sets of PCR reaction by using above two primer pairs.

   PCR mixture:

5X PCR buffer	10 μl
2 mM dNTPs	5 μl
#1 primer (100 μM)	0.5 μl
#2 primer (100 μM)	0.5 μl
10-20 ng/ μl DNA template	1 μl
EHF PCR polymerase (Roche)	1 μl
d.3H_2O to	50 μl

   (EHF= Roche's extend high fidelity enzyme)

   PCR parameter:

   95^0C 2' ---------- 1X
   95^0C 15''
   50^0C 30'' } 20X-25X
   72^0C x'' (follow the rule: 1kb/1 min)
   72^0C 5' ---------- 1X
   Hold at 20^0C

3. Take 1 μl PCR products to check the quality and quantity by using agarose gel electrophoresis.
4. Mix PCR products in equimolar ratio from two tubes based on the result of agarose gel electrophoresis.
5. Purify PCR products by using Roche's PCR Cleaning Kit according to the instructions provided by manufacturer.
6. Elute PCR products with 65 μl autoclaved 0.1X TE buffer.
7. Add 10 μl of 5X EHF PCR buffer, mix well and transfer DNA mixture to PCR tube.
8. Denature and re-nature mixed PCR products using PCR machine under following parameter:

   95^OC 5-min
   65^OC 10-min
   Hold at 4^OC

9. Add 9 μl 10X PNK (polynucleotide kinase) buffer, 2 μl of 2 mM ATP, and 10 U PNK.
10. Mix well; and incubate at 37^0C for 1 hr.
11. Purify PCR products by using 0.8% agarose gel (to get rid off plasmid template).
12. Excise the gel containing desired DNA band with minimum possible amount of agarose.
13. Purify the DNA by using Roche PCR Cleaning Kit or equivalent.
14. Elute the DNA with 60 μl autoclaved 0.1X TE; the eluent is ready for ligation (check the DNA before ligation, if necessary.).
15. **Add 25ng-50ng of vector (1-2 μl) and 6 μl of re-natured insert for ligation.**

16. Take 1-5 μl (dependent on transformation efficiency of competent cells) of ligation mixture to perform transformation.

(b) Preparation of vector:

Use corresponding protruding end and dephosphorylated vector DNA to perform ligation (vector DNA should be purified by agarose gel electrophoresis after RE digestion and dephosphorylation).

Sequencing Primer

1. Forward primer for sequencing downstream of CMV promoter: 5'-ccaaaatgtcgtaacaactc-3'
2. Reverse primer for sequencing upstream of IRES: 5'-attccaagcggcttcggc-3'
3. Forward primer for sequencing downstream of IRES: 5'-acatgtgtttagtcgagg-3'
4. Reverse primer for sequencing upstream of 3'-LTR: 5'-gagagacccagtacaagc-3'
5. Forward primer for sequencing downstream of CAG promoter: 5'-ctggttattg tgctgtctc-3'
6. Forward primer for sequencing downstream of U6 promoter: 5'-caccattatcgtttcacac-3'

Advantages

1. Avoid RE digestion of the PCR products.
2. This method is of greater advantage in uses if the insert sequences contain the same RE sites as in vector that use for cloning, as there is no need to restrict the inserts.
3. Easy and quick method.
4. Higher efficiency of cloning.

Reference

Zeng G., 1998. Sticky-end PCR: new method for subcloning. Biotechniques 25:206-8.