Cloning cDNAs into pVALIUM10-roe via Recombination

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1. Oligos design
   - Make sure the 5’ primer has a Kozak DNA sequence (ANNATGGNN).
   - There is no need to add a restriction enzyme site if using the PENTR/D TOPO vector (Invitrogen, K240020).
   - If you are using the TRiP’s modified mENTRY vector, you need add restriction enzyme sites to both at the 5’ end and 3’ end (from the mENTRY protocol: at the 5’ end add either EcoRI or MfeI and at the 3’ end add either XbaI or SpeI).

2. PCR
   - For TOPO cloning the reaction volume is 20ul.
   - For mENTRY vector cloning, the total reaction volume is 40ul.
   - Make sure to use genomic DNA or cDNA as the PCR template.
   - Load 5ul onto an agarose gel to verify the quality and quantity of your PCR product. If multiple bands are seen, we usually cut out the relevant band and purify it.

3. PCR purification
   - See Qiagen manual for details (Qiagen, Cat No. 28104): Elute with 40ul ddH2O.
   - For TOPO cloning, there is no need to purify; i.e., directly use the PCR product for the ligation (For details see Invitrogen manual, Cat No. K240020).

4. Enzyme digestion
   Purification product .......................... 40ul
   Buffer X ................................. 4.8ul
   Enzyme A and/or Enzyme B  ............... 2ul each enzyme
   Mix, incubate at 37°C for 2 hours

5. Purification
   - Same as step 3 (above)
6. **Ligation**
   - Purification product ........................................ 6ul
   - 10X ligation buffer ........................................ 2ul
   - ddH₂O ......................................................... 9ul
   - Backbone (linearized by enzyme digestion) .... 2ul
   - T4 DNA ligase (Roche, Cat No. 13580621) .... 1ul
   Mix, incubate at 16°C for 1 hour

7. **Transformation**
   - Ligation mix ........................................... 10ul
   - Top10 competent cells ................................... 50ul
   Mix, place on ice for 30 min, heat shock, add SOC medium and incubate at 37°C for 50 min, then plate (Kan resistant).

8. **Select clone by PCR**
   - make sure the direction of the insertion is correct if use single enzyme

9. **Miniprep**
   - See Qiagen manual for details (Qiagen, Cat No. 27104)
   - DNA sequencing to confirm the correct DNA sequence

10. **Recombination**
    - 45-85ng/ul Entry vector ...................................... 0.3ul
    - 200ng/ul pVALIUM10-roe .................................... 0.3ul
    - LR clonase (Invitrogen, Cat No. 11791-100) .............. 0.8ul
    - Add ddH₂O (or TE buffer, pH8.0) .......................... 4ul total volume
    Mix, incubate at 25°C for 1 hour

    To stop the recombination, add 1ul proteinase K (from LR clonase kit), mix, incubate at 37°C for 10min.
11. Transformation
Recombination product ........................................ 5ul
TOP10 competent cells .......................................... 50ul
Mix, incubate on ice 30min followed by a 42°C heat shock

Add SOC medium ..................................................... 150ul
Mix, incubate at 37°C for 30min, then plate (Amp resistant)

12. Select correct clone by PCR

13. Miniprep
   - See Qiagen manual for details (Qiagen, Cat No. 27104)

14. Prepare DNA for injection
   - Ethanol precipitate
   - Dissolve in injection buffer (100nM NaPO4, pH 7.2, 5mM KCl)

Questions? Contact Jian-Quan Ni at jni@genetics.med.harvard.edu

Good Luck!

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