Cloning using Gateway® Technology

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1. Materials

1.1 Generating an entry clone

1. Gene of interest in some vector
2. dNTP 10mM (Invitrogen). Store at –20°C.
3. Pfx platinum polymerase Kit (Invitrogen). Store at –20°C.
4. PCR purification kit (Qiagen)
5. PENTR D/TOPO vector (Invitrogen). Store at –20°C.
6. Salt solution (1.2 M NaCl, 0.06 M $MgCl_2$) store at –20°C
7. Sterile water
8. One Shot Top10 competent *E.coli* cells
9. S.O.C medium
10. LB agar plates with kanamycin (50 mg/l)
11. LB liquid medium
12. Kanamycin (Invitrogen)

1.2 Performing the LR recombination reaction

1. Destination vector of choice
2. Gene of interest in entry clone
3. Clonase enzyme mix (Invitrogen). Store at –80°C
4. 5X LR clonase reaction buffer. Store at –80°C

5. TE buffer pH 8.0
6. 2 µg/µl Proteinase K solution
7. One Shot Top10 competent *E.coli* cells
8. S.O.C medium
9. LB agar plates with kanamycin and hygromycin (50 mg/l)

2. Methods

2.1 Generating an entry clone

1. PCR primers are designed to amplify the gene of interest. The forward primer contains a CACC sequence at 5' end of the primer for enabling directional cloning.
2. The gene of interest is amplified by using these primers in a thermocycler using appropriate conditions.
3. The PCR product is purified using the PCR purification kit (Qiagen) and quantified.
4. Mix the following in an eppendorf tube: the PCR product (gene of interest) 5-10 ng depending on the size of the gene of interest, 1µl salt solution, sterile water to make a final volume of 5 µl and 1µl of TOPO vector.
5. Leave the mix at room temperature for 5-30 min.
6. Place the reaction on ice.
7. Thaw the One Shot Top10 competent *E.coli* cells on ice. Add 3 µl of TOPO cloning reaction (From step 4). Mix gently. Do not mix by pipetting up and down.
8. Incubate on ice for 30 minutes.

9. Heat shock the cells for 1 min. at 42°C.
10. Transfer the cells to ice and add 250 µl S.O.C medium.
11. Shake the eppendorf tube at 37°C for 1hr.
12. Plate 100 µl on prewarmed LB+ Kanamycin plate and keep at 37°C overnight.
13. Pick up 5 colonies and do a colony PCR to check the presence and right orientation of the insert.
14. Grow putative clones in 3 ml LB + Kanamycin (50 µg/µl) overnight at 37°C with constant shaking and isolate the DNA.
15. Check putative clones by sequencing for the presence of CACC at 5' end and for any mutation introduced during PCR.

2.2 Performing the LR recombination reaction

1. Add the following in an eppendorf tube at room temperature: Entry clone with the gene of interest (100-300 ng/µl) 2-4 ul, destination vector of choice (150 ng/µl) 2 µl, 5X LR Clonase reaction mix 2 µl, TE buffer, pH 8.0 to make volume to 8 µl. Mix well.
2. Transfer LR Clonase enzyme mix from -80°C to ice to thaw it for 2 minutes. Vortex it twice for 2 seconds each time.
3. Add 2 µl of LR Clonase enzyme mix. Mix by votexing two times for 2 second each.
4. Incubate the reaction at room temperature for 1 hour.
5. Add 1 µl of Proteinase K solution and incubate at 37°C for 10 min.

6. Transform 5 µl of the LR mix in One Shot competent *E.coli* cells as done before in 2.1 (steps 8-11).
7. Plate 100 µl of *E.coli* culture on prewarmed LB + Kanamycin (50 mg/l) + Hygromycin (50 mg/l).
8. Pick up 10 colonies and do a colony PCR to check the presence of the insert.
9. Culture putative clones in 3 ml LB + Kanamycin (50 µg/µl) + Hygromycin (50 mg/l) overnight at 37°C with constant shaking and isolate the DNA.
10. Check putative clones by sequencing for the right frame between gene of interest and the tag.

Note

1. While making the reverse primer, be careful that the primer is not complementary to the overhang GTGG sequence at the 5'end. If it is so, the chances of the gene of interest cloning in opposite orientation are very high.
2. If you are making a fusion of the PCR product to C-terminal tag (after recombination of entry clone with the Gateway destination vector), then remove the stop codon while making the reverse primer for the gene of interest.
3. If you are making a fusion of the PCR product to N-terminal tag (after recombination of entry clone with the Gateway destination vector), then make sure that the stop codon is intact while making the reverse primer for the gene of interest.
4. For generating entry clones, a 0.5:1 to 2:1 molar ratio of PCR product and TOPO vector gives a large no. of colonies.
5. After using the Clonase enzyme mix, immediately return it to –80°C.

6. Always use ccdB survival cells for propagating destination vector. But use One Shot competent cells for propagating entry vectors and recombination vectors.