Overview

Ligation-independent cloning makes constructs without use of ligase. LIC-compatible vectors contain the LIC cassette, and these vectors are linearized with 14bp single-strand overhangs when cut with BsaI and treated with T4-polymerase and one appropriate dNTP. These overhangs are complementary to overhangs generated in inserts amplified with the appropriate primers and subsequently T4-polymerase-treated.

Details can be found at: https://www.addgene.org/plasmid-protocols/lic/

1. Make vector LIC-compatible by removing BsaI sites by Site-directed Mutagenesis
2. Construct LIC vector by double digest followed by blunt-end ligation
3. Midiprep vector
4. Treat with BsaI and T4 polymerase

QuikChange II XL Site-Directed Mutagenesis

Mutant strand synthesis reaction

1. Prepare the sample reaction as indicated below:

5 µl of 10× reaction buffer
1 µl (50 ng) of dsDNA template
1.25 µl (125 ng) of 10mM oligonucleotide primer #1
1.25 µl (125 ng) of 10mM oligonucleotide primer #2
1 µl of 5mM dNTP mix
3 µl of QuikSolution
36.5 dH2O to a final volume of 50 µl
Then add 1 µl of PfuUltra HF DNA polymerase (2.5 U/µl)

2. Cycle each reaction using the following cycling parameters (Extension time 1.5min/kb) :
   HOTLID 105, 30 || VOLUME 50 || TEMP 95.0, 60 || TEMP 95.0, 60 || TEMP 60, 50 || TEMP 68.0, 900 || GOTO: 2, 17 || TEMP 68.0, 420 || TEMP 4.0, 0 || END

3. Place the reaction tubes on ice for 2 minutes to cool the reactions to ≤37°C.

DpnI Digestion

1. Add 1 µl of the Dpn I restriction enzyme (10 U/µl) directly to each amplification reaction.
2. Gently and thoroughly mix each reaction mixture by pipetting the solution up and down several times. Spin down the reaction mixtures in a microcentrifuge for 1 minute, then immediately incubate the reactions at 37°C for 1 hour to digest the parental (i.e., the nonmutated) supercoiled dsDNA.

Transformation of XL10-Gold Ultracompetent Cells

1. Gently thaw the XL10-Gold ultracompetent cells on ice. For each control and sample reaction to be transformed, aliquot 45 µl of the ultracompetent cells to a prechilled 14-ml BD Falcon polypropylene round-bottom tube.
2. Add 2 µl of the β-ME mix provided with the kit to the 45 µl of cells.
3. Swirl the contents of the tube gently. Incubate the cells on ice for 10 minutes, swirling gently every 2 minutes.
4. Transfer 2 µl of the Dpn I-treated DNA from each control and sample reaction to separate aliquots of the ultracompetent cells. Swirl the transformation reactions gently to mix and incubate the reactions on ice for 30 minutes. 5. Preheat SOC media at 37°C water bath for use in step 8.
6. Heat-pulse the tubes in a 42°C water bath for 30 seconds.
7. Incubate the tubes on ice for 2 minutes.
8. Add 0.5 ml of preheated (37°C) SOC media to each tube, then incubate the tubes at 37°C for 1 hour with shaking at 225–250 rpm.
9. Plate 250 µl on LB+Kan agar plates.
10. Incubate the transformation plates at 37°C for >16 hours.

<u>Construct LIC vector by double digest followed by blunt-end ligation</u>

1. Mix 500ng plasmid (bsaI-site-removed pKT230) DNA, 5µl CutSmart buffer, 1µl EcoRI-HF, 1µl BamHI-HF, water to 50µl
2. Incubate for 1hr at 37°C
3. Add 4µl 5mM dNTPs, 1µl Klenow polymerase
4. Mix, spin down, incubate 30min at 37°C
5. Clean DNA and Concentrate DNA
    a. To reaction mixture (55µl) add 55µl of magnetic beads, mix by pipetting
    b. Sit at RT for 5 mins, then sit on magnet for 10mins. Beads will be drawn to the side of magnet.
    c. Remove all solution. Add 200µl ethanol gently on side, remove after 30s.
    d. Repeat ethanol wash.
    e. Remove tubes from magnet, quickly spin in nanofuge for 2s.
    f. Place tubes back on magnet. Remove ethanol, let dry for 2s.
    g. Add 20µl of 10mM TRIS on side without beads. Remove from magnet, mix by tapping, sit at RT for 5min.
    h. Put strip back on magnet, allow solution to clear. Pipette supernatant into microtube.
6. To 3µl of vector, add 5µl of insert (cleaned PCR fragment of LIC cassette + T7 terminator), 1µl T4 ligase buffer, 1µl T4 ligase.
7. Incubate overnight at 16°C
8. Add 1µl reaction mix to 20µl DH10B electrocompetent cells, electroporate at 2.5kV in 2mm cuvette.
9. Recover at 37°C for 1hr, then plate 100µl on LB+Kan agar.

<u>Midiprep vector</u>

1. Start 3ml LB+Kan culture using transformed colony (PCR-verified). Culture at 37°C overnight.

2. Transfer culture into 500ml LB+Kan culture. Culture at 37°C until OD ~0.8.
3. Transfer culture into 2x 500ml autoclaved centrifuge bottle.
4. Centrifuge in Beckman Centrifuge (JA-1 rotor) at 5,000 rpm for 30min at 4°C.
5. Decant supernatant and resuspend pellets by vortexing in small amount of remaining media.
6. Add 10ml cold Qiagen Buffer P1. Transfer to 50ml falcon tube.
7. Add 10ml of P2 Buffer at RT. Mix by inversion, incubate at RT for 5min (not longer).
8. Add 10ml of chilled Buffer P3 (4°C). Mix by inversion.
9. Centrifuge in table top centrifuge at 3000rpm for 15mins.
10. While sample is being centrifuged, equilibrate Qiagen-tip 100 with 5mls of Buffer QBT.
11. Decant supernatant into QIA cartridge filter, insert plunger and pass samples through filter into Qiagen-tip 100. Repeat if cartridge and Qiagen-tip 100 filter cannot hold all of supernatant at once. Allow solution to flow through Qiagen-tip by gravity.
12. Wash Qiagen-tip 100 3x with 10ml of Buffer QC.
13. Elute with 5ml Buffer QF (10mM Tris pH 8.0) and collect flowthrough in a Sarsdedt 13ml screw cap tube.
14. Precipitate plasmid DNA by adding 5ml isopropanol to each sample. Mix well by inversion, store overnight at 4°C.
15. Spin samples in Beckman JA-21 centrifuge (JA-17 rotor with fitting sleeves) for 45 min at 10,000rpm at 4°C
16. Carefully remove tube, decant supernatant without losing pellet.
17. Air dry at room temperature, and resuspend in 30µl 1x TE pH8.0
18. Measure OD with ultrospect spectrophotometer.

<u>Treat with BsaI and T4 polymerase</u>

Vector Treatment

1. Mix 10µl CutSmart Buffer, 10µl plasmid (~5µg), 3µl BsaI enzyme and 77µl water.
2. Incubate 2 hours at 37°C, then heat inactivate at 65°C for 20min.
3. Run 5µl of mixture in gel to check for complete digestion.
4. Mix 50µl Bsa-I digested vector, 10µl NEB 2 buffer, 10µl 25mM dGTP, 1µl BSA, 0.5µl 1M DTT, 5µl T4 DNA polymerase, 23.5 water to final volume of 100µl.
5. Incubate mixture at 22°C for 30min.
6. Heat inactivate T4 polymerase at 70°C for 20min.
7. Store at -20°C.

Insert treatment

1. Mix 5µl insert, 1.5µl water, 1µl NEB 2 Buffer, 1µl 25mM dCTP, 0.5µl 100mM DTT, 0.5µl BSA, 0.5µl T4.
2. Incubate mixture at 22°C for 30min.
3. Heat inactivate T4 polymerase at 70°C for 20min.
4. Store at -20°C.

<u>Ligation and Transformation</u>

1. Mix 1µl Bsa1-T4 treated vector, 2µl treated insert.

2. Incubate for 10min at 22°C, then transfer to ice.
3. Add 50µl competent DH10B cells in culture tube.
4. Incubate on ice for 15min.
5. Heat shock at 42°C for 45s.
6. Chill for 2min on ice.
7. Add 100µl prewarmed (37C) SOC, recover for 60min at 37°C.
8. Plate 100µl onto LB-Kan with 5% Sucrose.