SOP: RP002

Establishing Stocks of Low Copy Recombinant Plasmids – Large Scale

Materials and Reagents:

1. Glycerol (frozen) stock or single colonies of plasmid in maintenance E. coli (eg. DH5α)
2. 1 liter LB broth in 4 liter flask (note 1)
3. 1000X ampicillin stock (100 mg ampicillin/ml water – note 2)
4. Ice bucket, ice
5. One sterile Erlenmeyer flask with 250-500 ml volume marks
6. Two sterile 500 ml angle neck centrifuge bottles
   or four sterile angle neck 250 ml centrifuge bottles
7. Eight x 40 ml sterile polypropylene Oakridge centrifuge tubes (4 balanced pairs)
8. Four disposable sterile 50 ml conical centrifuge tubes
9. Four–six disposable sterile 15 ml conical centrifuge tubes.
10. Disposable plastic sterile pipets: 1.0 ml, 10 ml, 25 ml
11. Six sterile microcentrifuge vials, 1.5 ml
12. Pipetman P1000, P200, P20 pipettors and sterile tips
13. Qiagen Plasmid Maxi Kit buffers and purification tips
14. Qiagen tip rack
15. 2-propanol (isopropanol, IPA)
16. 70% EtOH
17. Buffer 10 mM Tris pH 7.5 (Qiagen EB buffer)
18. Sterile low retention microcentrifuge vials for stock storage (note 12)
19. 37^{o}C shaker incubator
20. Sorvall RC-5B centrifuge

Protocol:

1. _____ Scrape a small aliquot (eg 10 μl) of cells from frozen stock or scrape 2-3 colonies from agar surface with a one ml disposable pipet or sterile pipetman tip.

2. _____ Transfer cells into 2.0 ml LB broth supplemented with 100 - 130 μg/ml ampicillin in a 50 ml centrifuge tube.

3. _____ Incubate 4-6 hr at 37^{o}C, 150-300 rpm.

4. _____ Set up a 1 liter overnight culture in late afternoon. Add approximately 0.050 ml of 2.0 ml over-day culture to 1 liter LB broth + 100 μg/ml ampicillin in a 4 liter flask.

5. _____ Incubate overnight at 37^{o}C, 150-300 rpm.

6. _____ Transfer cells into 4 x 250 ml or 2 x 500 ml angle neck centrifuge bottles by pouring cells first into a sterile Erlenmeyer flask to a 250 or 500 ml volume mark.

7. _____ Distribute cells in approximately even volumes, then BALANCE PRECISELY (note 3).

8. _____ Place balanced pairs in Sorvall F16-250 rotor (250 ml bottles) or SLA3000 rotor (500 ml bottles).

9. _____ Spin cells at 5000 rpm 20 min at 4 ^{o}C – 25 ^{o}C.

10. _____ Return broth supernatant to 4.0 liter growth flask for sterilization (note 4).

11. _____ Drain pellets further by inverting bottles on paper toweling. Store pellets in capped bottles at 20^{o}C if later processing is desired.

12. _____ Resuspend pellets in Qiagen Buffer P1 (note 5) (Pellets in 250 ml bottles: add 9.5 ml P1 per pellet. Pellets in 500 ml bottles: add 19.0 ml P1 per pellet.)

13. _____ Resuspend pellets thoroughly by pipeting up and down and vortexing.

14. _____ Transfer volume as 10.0 ml aliquots into 4 × 40 ml Oakridge tubes at room temperature.

15. _____ Add 10 ml Qiagen Buffer P2 (NaOH/SDS) to each tube to lyse cells.

16. _____ Cap and mix by gentle inversion 4X. Avoid shearing genomic DNA.

17. _____ Incubate 5 min at room temperature. Use a timer (note 6).

18. _____ Add 10 ml iced Qiagen Buffer P3 (potassium acetate pH 5.5) per tube to terminate alkaline lysis.

19. _____ Mix lysate by gentle inversion 4-5X.

20. _____ Incubate on ice 30 minutes.

21. _____ Centrifuge 45 min at 4^{o}C, 25,000 x g (14,400 rpm) in Sorvall SS-34 rotor.

22. _____ Pour partially cleared lysates into four clean, iced Oakridge tubes (note 7).

23. _____ Invert transferred lysates gently to mix remaining SDS.

24. _____ Repeat step 20 centrifugation.

25. _____ Discard pellets (cell debris) from both spins.

26. _____ During second centrifugation, place 2 Qia500 tips in Qiagen rack.

27. _____ Add 10 ml Qiagen Buffer QBT to each tip to equilibrate resin.

28. _____ Immediately after 2^{nd} centrifugation, pipet the cleared lysate from two centrifuge tubes onto the two equilibrated tips (one 30-ml cleared lysate to one tip). Drain by gravity flow.

29. _____ Pipet lysate from remaining two tubes into 50 ml conical tubes on ice immediately.

30. _____ Add the remaining iced lysates to the Qia500 tips after first set drains. At end of drain, each tip will have contents of 60 ml cleared lysate.

31. _____ Wash each Qia500 tip with 4 × 30 ml Qiagen QC. Drain by gravity flow

32. _____ Position a 50 ml conical centrifuge tube under each tip (total, 2 x 50 ml tubes).

33. _____ Add 13.0 ml Qiagen QF to each Qia500 tip. Drain by gravity flow

34. _____ Add 9.0 ml isopropanol (2-propanol, IPA) to each eluate to precipitate plasmid.

35. _____ Invert to mix.

36. _____ Transfer eluate as 6 x 1.3 ml aliquots into 6 x 1.5 ml vials (note 8).

37. _____ Centrifuge 5 minutes at 10,000 – 14,000 rpm.

38. _____ Remove supernatant carefully with P1000. Avoid pellet.

39. _____ Add a second round of 1.3 ml eluate to same vials.

40. _____ Repeat steps 37 and 38.

41. _____ Repeat process, ultimately concentrating the eluates into 6 vials.

42. _____ Remove final supernatant carefully with P1000 to ~ 0.1 ml.

43. _____ Add 0.5 ml 70% EtOH. Cap vials and invert to thoroughly rinse vial and pellet.

44. _____ Centrifuge 5 min at 10,000-14,000 rpm.

45. _____ Remove EtOH rinse with P1000 to ~ 0.1 ml.

46. _____ Carefully remove most of remaining EtOH with p200.

47. _____ Quick-spin vials to move residual EtOH to bottom of vial.

48. _____ Remove excess EtOH with P20 and allow pellets to air dry about 5 min. (note 9).

49. _____ Add 25 ul sterile 10 mM Tris pH 7.5 to each pellet. (note10).

50. _____ Store at 4 ^{o}C overnight (note 11).

51. _____ Combine aliquots into one vial.

52. _____ Quantitate plasmid yield and assess quality by measuring A260 UV absorbance of a 1:50 – 1:100 dilution of stock (see SOP SP014).

53. _____ Dilute DNA to 0.1 ug/μl in 10 mM Tris pH 7.5 in 15 ml conical tube.

54. _____ Transfer to 0.5 ml low retention vials (2-5 vials) (note 12).

55. _____ Dispense 10 ul plasmid (1.0 ug) per vial to low retention vials for long term storage of contract shipping stock at -20 ^{o}C.

Notes:

1. LB broth:
20 gm Lennox LB broth powder (Difco 240230) + 990 ml deionized water
in 4.0 liter flask. Solubilize by swirl mixing. Autoclave 20 min on liquid cycle.
2. Ampicillin (sodium salt, Sigma A9518) 1000X stock is 100 mg/ml.
   Preparation of 10.0 ml stock.
   a. Weigh 1.0 gm ampicillin.
   b. Carefully transfer powder to 50 ml centrifuge tube.
   c. Add 9.0 ml deionized water
   d. Solubilize ampicillin by swirl mixing (don't vortex).
   e. Pull solution (carefully) into 10 ml pipet and note volume.
   f. If volume is less than 10.0 ml, bring to 10.0 ml with water.
   g. Sterilize by passing ampicillin through a 0.2 micron syringe filter attached to a 10 ml syringe. Collect solution in a sterile 15 ml centrifuge tube.
   h. Dispense aliquots of 0.1 – 1.0 ml into pre-labeled sterile 0.5 – 1.5 ml microcentrifuge vials.
   Label: 1000X amp + date, initials of preparer. Alternatively, label Amp100.

i. Store at –20°C. Ampicillin tolerates 1-2 thaw/freeze cycles.
j. Supplement LB broth with ampicillin at a 1:1000 dilution (diluting 100 mg/ml to 100 υg/ml final concentration).
eg. 0.002 ml (2.0 µl) stock ampicillin in 2.0 ml broth or
1.1 ml stock ampicillin in 1 liter broth.

3. Place two open bottles on double pan balance, caps also on pans. Transfer cells from heavier bottle to lighter bottle with a sterile pipet until weights are equal (when needle stabilizes in exact center).
4. All glassware, pipets, paper, centrifuge bottles in contact with living bacteria must be autoclaved for 20 minutes on liquid cycle before cleaning or discarding.
5. Qiagen buffers are held at room temperture with exceptions.
a. P1 is supplemented with Rnase A before first use (see Qiagen instructions) and is stored at 4 °C.
b. P2 is stored at room temp but SDS may precipitate. Heat at 37 °C for a few minutes to solubilize any precipitate.
c. Store P3 at 4 °C. Hold on ice before addition to lysate.
6. IMPORTANT: incubate no longer than 5 minutes in Buffer P2 to avoid denaturing plasmid DNA.
7. Transfer as much volume as possible to maintain tube balance. Some supernatants will contain stringy genomic DNA which is removed by the second centrifugation.
8. Qiagen procedures suggest centrifugation of eluted plasmid in Oakridge tubes. However, low Copy plasmid DNA can be extremely difficult to capture. The small, nearly invisible pellet (40 – 300 µg) easily detaches from the tube wall and disperses into the supernatant. Iterative processing in microcentrifuge vials results in visible pellets that are easy to handle without loss.
9. IMPORTANT: don't dehydrate pellets beyond initital stage of air dryness. Do not dry by evaporation in Savant. Desiccated pellets will not completely resuspend, and DNA can denature during extreme drying processes.
10. Use sterile processes and solutions (fresh tips, buffers) in remaining steps to maintain integrity of plasmid during long-term storage.
11. Complete resuspension of plasmid requires at least four hours at room temperature. In general, overnight resuspension at 4 °C is recommended before quantitation.
12. Store plasmid DNA in low retention vials with o-ring screw cap lids at all steps after combining precipitated plasmid (step 52). As an example, it's typically convenient to dilute plasmid to 0.1 µg/µl and store in low retention vials at -20°C before long term storage vials are generated.

Vials: OPTIMUM TUBES from Life Science Products, Inc.
Product # LS-4199LRT-2 500/pack
0.5 ml Low Retention Microtubes with Screw Caps (o rings)
Autoclave tubes and caps in separate containers for sterility.

References:
Qiagen Plasmid Purification Handbook pp 16-20