



## **Large Construct Isolation Protocol**

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### **Inoculation**

1. In 10 ml culture tube, add 1 ml of LB with Chloramphenicol (12.5 ug/ml)
2. Inoculate a BAC clone with 1-2 ul of stock.
3. Shake for 4-6 hours at 37 C with 220rpms.
4. Inoculate 250 ml LB (12.5 ug/ml of Chloramphenicol) in 500 ml flask with 250 uls of the seed culture.
5. Grow for 18-20 hours in a shaker at 37 C at 220rpms

### **Isolation-modified protocol of the Qiagen Plasmid Large Construct protocol**

1. Prepare solutions.
  - Lysis Buffer : Make "**fresh**" before starting the BAC isolation. To make 50 mls, 35 ml of DDD water + 10 ml of 1N NaOH + 5 ml of 10% SDS.
  - P1 Buffer : Add RNase (100mg/ml) to the P1 buffer solution before starting the large clone isolation procedure. RNase once added to the P1 buffer, needs to be refrigerated at 4 C.
  - P3 Buffer : Make sure P3 buffer is ice cold before starting the large clone isolation procedure.
2. Pour culture into 250 ml centrifuge bottle. Pellet cells at 5,000 rpms for 15 minutes at 4 C.
4. Resuspend cell pellet in 12 mls of P1 (Resuspension) buffer. Add RNase to P1 buffer so that the final concentration of the P1 buffer is 100 ug/ml. Make sure no cell clumps are left over.
5. Add 8 mls of P2 (lysis) buffer to the resuspended cells. As soon as you add the P2 buffer to the very first bottle, start a timer for 5 minutes. Invert the bottles twice VERY GENTLY and HALFWAY (45 degree angle). Stand them on bench. DO NOT shake since this may lead to nicking of the DNA.
6. At the end of 5 minutes, add 8 mls of chilled P3 (Neutralization) buffer to the lysed material. As soon as you add the P3 buffer to the very first bottle, start the 15 minute timer. Invert the bottles 3 - 4 times GENTLY and HALFWAY to make sure

the solution is mixed completely. Keep the bottles on ICE no longer than 15 minutes.

7. At the end of the 15 minute neutralization period, centrifuge the samples at 15,000 rpms for 30 minutes at 4 C.
8. After the 30-minute spin is completed, transfer the supernatant to a clean 250-ml centrifuge bottle and spin at 15,000 rpms for 15 minutes at 4 C. Transfer the supernatant with wide-mouth pipette into 50 ml centrifuge tubes
9. Add 14.4mls of 100% Isopropanol, mix, and centrifuge at 15,000 RPMs for 30 minuets. You should see a white pellet at the bottom of your tube. During this spin, prepare the Exonuclease by dissolving it with 225uls of Exonuclease solvent. Mix and then allow to sit for at least 15 minutes undisturbed. Refrigerate the Exonuclease until it is needed.
10. After the 30 minute spin, pour the supernatant off and then add 5mls of 70% Ethanol. Spin for 15 minutes at 15,000 RPMs. Try and not disturb the pellet when pouring off the supernatant. During this step, pull out the appropriate amount of ATP tubes you will need (1 ATP tube per sample if each tube holds 300 uls).
11. After the 15-minute spin, pour off the supernatant. Should be left with a white pellet at the bottom of the centrifuge tube. Place the tube upside down for 2-3 minutes and allow to air-dry around 30 min.
12. After air-drying, add 9.5mls of Buffer EX (Reaction Buffer) to the sample and make sure the white pellet is completely dissolved before proceeding.
13. Once the pellet is dissolve, add 300uls of ATP solution and 200uls of ATP-dependent Exonuclease. Gently mix a few times and incubate the tubes in a 37 C incubator for one (1) hour.
14. About 45 minutes into the incubation, set up the appropriate amount of Qiagen columns and equilibrate them with 10mls of QBT (Equilibration) buffer and allow the buffer to empty by gravity flow.
15. Once the 1-hour incubation is done, pull the samples out of the incubator and add 10mls of Buffer QS to each sample. Pour the entire sample onto the column and allow to empty by gravity flow.
16. Wash the columns with two (2)-30ml aliquots of QC (Wash) buffer. Allow emptying by gravity flow.
17. Elute the DNA from the columns into a clean 50-ml centrifuge tube with heated (65C) QF (Elution) buffer. Add 8mls of the heated buffer then reheat it while buffer is eluting out the DNA. Add another 7mls of re-heated buffer to finish off the elution. (You can stop here if you need. Keep the eluted DNA in 4C O/N)

18. Precipitate the DNA with 10.5mls of 100% room temperature isopropanol. Mix and centrifuge at 14,000 rpms for AT LEAST 30 minutes at 4 C (usually 60 min).
19. Carefully pour off the supernatant as not to disturb the precipitated DNA pellet.
20. Wash the DNA pellet with 5mls of 70% ethanol by spinning the pellet with the ethanol at 14,000 rpms for 15 minutes at 4 C.
21. Carefully pour off the ethanol without disturbing the DNA pellet.
22. Air dry the pellet in a vent hood for 30 minutes or more until the majority of the ethanol has evaporated. Angle the tubes so that the ethanol is as far away as possible from the DNA pellet as possible.
23. Resuspend in 1ml (1000 uls) of filtered TE overnight in a refrigerator at 4C. Angle the tubes so that the TE completely submerges the DNA pellet and ensures that the whole pellet resuspends back into solution. Anchor your tube rack so that the rack does not tip over at all when resuspending overnight.