Qiagen PCR purification for TOPO cloning with MinElute spin column

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Visit Dates: June 19, 2011 – August 19, 2011
Rationale and background: To purify the PCR product or gel extraction product of various choanoflagellate species for 18S sequencing.

No gel extraction

1. Add 5 volumes of Buffer PB to PCR and mix
2. Place 800 ul of sample in a QIAquick column within a collection tube, spin at max speed 1 min
3. Discard flow-through
4. If any sample remains, continue spinning through column and discarding flow-through
5. Wash with 750 ul buffer PE, centrifuge 1 minute
6. Discard flow-through
7. Clean top edge and bottom edge of collection tube by pipetting out remaining liquid with P20
8. Place column with hinge facing **inwards** and spin for 1 min at max speed
9. Place column with hinge facing **outwards** and spin for 1 min at max speed
10. Place column in new 1.5 ml Eppendorf tube
11. Add 10.3 ul water
12. Let stand for 1 min
13. Centrifuge at max speed for 1 min to elute

With gel extraction

1. Add 3 volumes of Buffer QG to gel slice (e.g. 300 ul per 0.1 g of gel)
2. Incubate at 50 C for 10 minutes, or until gel slice is dissolved; mix every 2-3 minutes
3. Add 1 gel volume of isopropanol and mix by inverting
4. Place 800 ul of sample in a QIAquick column within a collection tube, spin at max speed 1 min
5. Discard flow-through
6. If any sample remains, continue spinning through column and discarding flow-through
7. Add 500 ul buffer QG, spin 1 minute, discard flow-through
8. Wash with 750 ul buffer PE, let stand 2-5 minutes, spin 1 minute, discard flow-through
9. Clean top edge and bottom edge of collection tube by pipetting out remaining liquid with P20
10. Place column with hinge facing **inwards** and spin for 1 min at max speed
11. Place column with hinge facing **outwards** and spin for 1 min at max speed
12. Place column in new 1.5 ml Eppendorf tube
13. Add 10.3 ul water
14. Let stand for 1 min
15. Centrifuge at max speed for 1 min to elute

TOPO TA Cloning

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Rationale and background: For 18S isolation of various choanoflagellate species following QIAqick-purified PCR or gel extraction product. Cloning was deemed necessary for our purposes when a species had never had any 18S sequence previously submitted to GenBank.

Add A-tail

Reaction mix:

- QIAquick-purified PCR product 100ng
- dNTPs 1 ul
- 10x PCR buffer 1 ul
- NEB Taq polymerase 0.2 ul
- water to 10 ul

1. Mix PCR product, dNTPs and PCR buffer, then add Taq
2. Incubate at 72 C for 30 minutes
3. Place on ice

Ligate into TOPO vector and transform into DH5a cells

Reaction mix:

- PCR product 0.5 – 4 ul
- Salt solution 1 ul
- water to 5 ul

- TOPO vector 1 ul

1. Add PCR product, salt solution, water and mix
2. Add TOPO vector (keep in enzyme cooler during pipetting), mix by swirling with pipet tip
3. Incubate at RT for 5-30 minutes
4. When incubation starts, retrieve DH5a cells from -80 freezer and place on ice
5. Add 2 ul of reaction mix to DH5a cells, mix by swirling while on ice
6. Heat shock at 42 C for 30 seconds
7. Place on ice for 2 minutes
8. Add 250 ul SOC and tape to shaker at 37 C for 1 hour
9. Plate using glass beads on LB + Kan plates (3 plates, 50-150 ul each) + 40 ul of X-gal
10. Grow at 37 C overnight

Store remainder of PCR product and ligation product at -20 C. Store remainder of transformed cells at 4 C (will go bad after 24h).

Grow up colonies in liquid medium, test cloning by PCR and Miniprep

1. Pick colonies into 3 ml LB + Kan (50 ug/ml) by swiping with a pipette tip and dropping the tip in a yellow-cap tube
2. Grow overnight at 37 C shaking
3. Aliquot 1.5 ml cells from yellow-cap tube into Eppendorf tube and use 0.3 ul for clone PCR

   If clone PCR is successful, continue to Miniprep:

4. Spin 1.5 ml cells in Eppendorf tube at 2000 x g for 4 minutes
5. Discard supernatant
6. Aliquot remaining 1.5 ml cells into tube and spin at 2000 x g for 4 minutes
7. Discard supernatant, remove residual liquid using a P200
8. Resuspend bacterial pellet in 250 ul Buffer P1
9. Add 250 ul Buffer P2 and mix thoroughly by inverting 4-6 times (do not allow lysis reaction to proceed for more than 5 min)
10. Add 350 ul Buffer N3 and mix immediately and thoroughly by inverting 4-6 times
11. Centrifuge for 10 min at max speed
12. Apply the supernatant from step 11 to the QIAprep spin column by decanting
13. Centrifuge for 30 s, discard the flow-through
14. Wash the column with 0.5 ml Buffer PB, centrifuge for 30 s, discard flow-through
15. Wash the column with 0.75 ml Buffer PE, centrifuge for 30 s, discard flow-through
16. Remove residual Buffer PE from the column using a P20
17. Centrifuge for 1 min
18. Rotate column 180 degrees, centrifuge for 1 min
19. Transfer column to a new 1.5 ml tube, add 50 ul Buffer EB, let stand for 1 min
20. Centrifuge 1 min to elute