TRIzol RNA prep

Parinaz Fozouni
King Lab – University of California, Berkeley
Visit Dates: June 19, 2011 – August 19, 2011
Rationale and background: To isolate the total RNA of various choanoflagellate species.

Collect cells

1. Scrape attached cells off 150mm plates, combine in SDS-free flask to ensure sample consistency
2. Pour into 50 ml conicals
3. Spin down 20 minutes, 3220g at 4C
4. Pour off supernatant slowly, continue tilting tube, remove residual supernatant by aspiration

Resuspend pellets in TRIzol, spin out unlysed material

1. Add 5 ml TRIzol per pellet, on ice
2. Pipette up and down to mix/resuspend pellet, on ice
3. Transfer 1 ml aliquot per original pellet into 1.5 ml Eppendorf tube
4. Immediately spin 1.5 ml tubes at max speed at 4C for 10 minutes to pellet debris and unlysed material
    1. Store remaining 50 ml conicals with cells in TRIzol at -20C
5. Transfer supernatant to new tubes
6. Incubate 5 minutes in RT dry bath to dissociate nucleoprotein complex

Phase separation

1. Add 1:5 ratio of chloroform to TRIzol (200 ul per 1.5 ml tube)
2. Shake vigorously inside fume hood for 15 seconds
3. Incubate standing at RT for 2 mins
4. Spin at max speed at 4 C for 15 minutes
5. Remove 500ul of aqueous layer containing RNA (being very careful not to remove interphase), transfer to new 1.5 ml Eppendorf tubes
6. Discard remainder in chemical waste

RNA precipitation / wash

1. Add 1.5 ul of GlycoBlue (final amount ~25 ug, working stock 15 ug/ul)
2. Add 1:1 ratio of isopropanol to aqueous phase (500 ul)
3. Mix gently by inverting
4. Incubate at RT for 10 minutes
5. Spin at max speed for 10 minutes at 4C to pellet RNA
6. Carefully remove supernatant
7. Wash pellet with 1 ml 75% ethanol, vortex briefly
8. Spin at 7,500g (9,591 rpm on Eppendorf 5415C) for 5 minutes at 4 C
9. Carefully remove supernatant

DNase treatment

1. Resuspend pellets in 44 ul water + 5 ul SUPERase-In, final concentration 2U/ul (stock 20U/ul)
2. Take 5 ul aliquots of each sample for Nanodrop + Bioanalyzer, place at -80C
3. Add Roche DNase mix: 5 ul 10x buffer, 1 ul DNase (10 U)
4. Incubate 15 minutes at 25C in dry bath
5. Add 650 ul water to bring final volume to 700 ul

Phenol:chloroform extraction

(perform all steps on ice)

1. Add an equal volume (700 ul) of phenol:chloroform:IAA (125:24:1) pH ~4.5
2. Mix by inverting
3. Centrifuge at max speed for 15 minutes at 4C
4. Transfer aqueous phase to new tube
5. Repeat phenol:chloroform extraction (steps 35-38) at least twice, until no protein remains at interphase
6. To remove residual phenol, add an equal volume (roughly 500ul or less) of chloroform:isoamyl alcohol (without phenol)
7. Mix by inverting
8. Centrifuge at max speed for 15 minutes at 4C
9. Transfer aqueous phase (roughly 400ul or less) to new tube
10. Add 1.5 ul of GlycoBlue (final amount ~25 ug, working stock 15 ug/ul)
11. Precipitate aqueous phase with 1/20 volume 10M ammonium acetate and 2.5 volumes 100% EtOH
12. Incubate at -20C for several hours or overnight
13. Centrifuge at max speed for 15 minutes at 4C
14. Remove supernatant
15. Wash pellet with 1 ml 75% ethanol, vortex
16. Centrifuge at max speed for 10 minutes at 4C
17. Remove supernatant
18. Repeat 75% ethanol wash (steps 15-17)
19. Air dry pellet 5-10 minutes in laminar flow hood, do not over dry
20. Resuspend pellet in 20 ul water
21. Spec on Nanodrop, make dilutions for Bioanalyzer (aim for 1 ng/ul, so dilute in volume of water equal to concentration in ng/ul)

Phenol/chloroform genomic DNA extraction – 1.5 ml Eppendorf tube

Parinaz Fozouni
King Lab – University of California, Berkeley
Visit Dates: June 19, 2011 – August 19, 2011
Rationale and background: To isolate DNA of various choanoflagellate species.

20 ml Lysis Buffer:

200ul 1M Tris-Cl (pH 8.0)
4ul 0.5 M EDTA (pH 8.0)
0.1g SDS

1. Spin cells down in 50ml conicals at max speed (3220 g) for 20 mins at 4C
2. Add multiple of 700 ul of lysis buffer per cell pellet (e.g. 1.4 ml, 2.1 ml, etc.)
3. Pass through 1 or 5ml syringe fitted with 21 gauge needle twenty times
4. Spin 20 minutes at maximum speed (3220 g) at 4 C to pellet unlysed cell material
5. Split supernatant from each conical into 700ul aliquots in 1.5 ml Eppendorf tubes (if desired, retain largely bacterial unlysed pellet for later use)
6. Add 3.5 ul RNAse A per tube (@ 4 mg/ml, final concentration 20 ng/ul)
7. Incubate 5 min at RT on nutator
8. Add 7 ul Proteinase K (@ 10 mg/ml, final concentration 100 ng/ul)
9. Incubate 3 h at 50 C, swirling occasionally
10. Cool to RT
11. Add 700 ul per tube phenol:chloroform:isoamyl alcohol pH 8.0 (all phenol:chloroform steps should be performed in the chemical hood)
12. Shake vigorously inside hood to mix
13. Spin 10 minutes at maximum speed at RT
14. Transfer aqueous phase (upper) to new tube
15. Repeat phenol:chloroform:isoamyl extraction (steps 11-15) until no protein remains at interphase (sometimes up to 3-4x)
16. Add an equal volume of chloroform:isoamyl alcohol (with no phenol)
17. Shake vigorously to mix
18. Spin 10 minutes at maximum speed at RT
19. Remove aqueous phase (upper), combining into a single tube with room for 3x volume of sample (can be 1.5 ml Eppendorf tube or 15 ml conical)
20. Add 1/20 volume 10M ammonium acetate (final concentration 0.5 M)
21. Add 1/3 ul of GlycoBlue per 100 ul of aqueous phase (@ 15 mg/ml, final concentration 50 ng/ul)
22. Add 2 volumes 100% EtOH
23. Invert 5-10x to precipitate DNA
24. Precipitate several hours or overnight at -20 C
25. Spin at maximum speed at RT for 20 min
26. Pour off supernatant
27. Add 1ml or 10 ml 100% EtOH to wash pellet
28. Wash on nutator for 5 minutes at RT
29. Spin 5-20 minutes at max speed
30. Repeat 100% EtOH wash a total of 2-3x
31. Pour off supernatant
32. Add 1ml or 10 ml 70% EtOH to wash pellet
33. Wash on nutator for 5 minutes at RT
34. Spin 5-20 minutes at max speed

35. Repeat 70% EtOH wash a total of 2-3x (4-6 total washes)
36. Remove supernatant, dry pellet, 5-10 min, do not over dry
37. Resuspend overnight in a small volume (~ 50-500 ul) of DNAse-free water