Sample Processing for microarrays (MicroTOOLs)

Irina Shilova
Updated March 10, 2013

Outline

1. RNA Extraction
2. RNA Quality Control
3. DNA Extraction from Organic Phase
4. DNA Quality Control
5. ds cDNA synthesis and amplification
6. DNA extraction Zehr lab protocol
7. Appendix: Sample Collection

1. RNA Extraction

Supplies

1. RiboPure Kit Ambion (AM1924)
2. Sterile 2.0 mL screw cap centrifuge tubes with 200 µL glass beads (for Sterivex)
3. BD Falcon conical tubes (polypropylene) 15 mL or 3mL tubes, two per sample (For Sterivex or if large amount of RNA is expected)
4. Plyers or other ways of cutting the cartridge (PVC cutter) (For Sterivex)
5. Scalpel to cut the filter from the cartridge (For Sterivex)
6. Petri dishes (For Sterivex)
7. 100% ethanol, ACS grade or higher quality
8. Chloroform without added isoamyl alcohol
9. Turbo DNA-free kit
10. TRI reagent (if many samples)

Equipment:

1. Bead beater
2. Microcentrifuge
3. Microcentrifuge for Eppendorf tubes capable of keeping 4°C
4. Vortex mixer

Preparations:

1. RNase precautions
2. Add 1.0 mL of TRI reagent to the centrifuge tubes with beads (two tubes per sample) (For Sterivex)
3. Add 48 mL of ACS grade 100% ethanol to the Wash Solution Concentrate and mix well.

Note: If biomass was collected on cartridge-free filters like Durapore membrane filters, proceed to step 3 in Cell lysis

From Sterivex

Cell lysis

1. Open Sterivex cartridges with plyers by slightly pressuring along and then twisting at a seal line of the cartridge (or use PVC cutter). Withdraw the holder with filter and place it onto a sterile petri dish.
2. Use scalpel (RNase-free, MQ water washed) cut the filter on the holder: first at the top and bottom of the holder, then from both sides of the seal longitudinal line and in half. Using forceps, place each half of the filter into the prepared Eppendorfs with beads and the TRI reagent.
3. For non-Sterivex filters, add 1.0 mL of TRI reagent to the cryotubes with beads.
4. Bead beat the tubes for 2 min at X speed. Repeate.
5. Centrifuge at 12,000 g for 10 min at 4ºC. Transfer the supernatant into a clean Eppendorf tube.
6. Optional, for large amount of RNA. Repeat lysis: add 1000 µL of TRI reagent into the tubes with filter, beat beat, centrifuge, transfer supernatant into a new Epp tube.

RNA extraction

7. To the Epp tubes from step 5 or 6, add chloroform (without isoamyl alcohol) 1/5 of supernatant volume. Vortex for 15 sec. Incubate at room temperature for 5-10 min.
8. Centrifuge at 12,000 x g for 10 min at 4ºC. RNA remains in the aqueous phase, DNA and proteins are in the interphase and organic phase.
9. Transfer aqueous phase (~60% of TRI volume used in the lysis) to a new Falcon tube.
10. Remove any aqueous phase remaining over the interphase and save the tubes with organic phase for DNA recovery. If there is a precipitate, transfer the organic liquid phase into a new tube. The organic phase can be stored at 4ºC for a few days or at -70ºC for a few months.

Final RNA purification

11. Add 100% ethanol to the aqueous phase from step 8, ½ volume of the aqueous phase. Vortex immediately at maximum speed for 5 seconds to avoid RNA precipitation.
12. For each sample, place a Filter Cartridge in one of the Collection Tubes supplied with RiboPure kit. Transfer 800 µL of the sample to a Filter Cartridge-Collection Tube assembly and close the lid.
    - Centrifuge the assembly at 12,000 x g for 30 seconds at room temperature or until all of the liquid is through the filter.
    - Discard the flow-through and return the Filter Cartridge to the same Collection Tube. The RNA is now bound to the filter in the Filter Cartridge.
    - Repeat for the rest of the sample volume (using the same filter-cartridge or additional if more than 100ug of RNA is expected).
13. Apply 500 µL of Wash Solution to the Filter Cartridge-Collection Tube assembly, and close the lid.
    - Centrifuge for 30 seconds at room temperature or until all of the liquid is through the filter.
    - Discard the flow-through and return the Filter Cartridge to the same Collection Tube.
    - Repeat steps above for a second wash.

Centrifuge for 30 seconds at room temperature to remove residual Wash Solution.

14. Transfer the Filter Cartridge to a new Collection Tube.
    - Add 50 μL of Elution Buffer to the filter column.
    - Incubate at room temperature for 2 min.
    - Centrifuge for 30 seconds to elute the RNA from the filter. The RNA will be in the eluate, in the Collection Tube.

DNase treatment

Using Ambion Turbo DNase kit (or use other DNase)

Preparations:

1. Water bath at 37°C
2. Thaw DNAse (on ice), DNase buffer and DNase inactivation reagent.

Procedure:

1. Add 0.1 volume of DNase buffer, mix

2. Add 1 uL of Turbo-DNase (this amount is enough for removing up to 2 ug of DNA from up to 10 ug of RNA in 50 uL according to the manifacture)

3. Incubate at 37°C for 45 min.

4. Add 0.1 volume of DNase inactivation reagent, mix well (vortex the DNase inactivation reagent before adding!). Use either 2uL of 0.1 volume of DNase inactivation reagent whichever is greater.

Alternatively, clean up RNA using Qiagen RNA kit. Using this kit improves the removal of DNase and salts (which is important for following cDNA synthesis), but about 30% of RNA will be lost.

5. Incubate at room temperature (not less than 22°C) for 5 min mixing (by flicking) 2-3 time during the incubation time.

6. Centrifuge at 10,000g for 1.5 min and transfer RNA to a clean tube.

Check RNA samples for DNA contamination by running PCR with primers for a positive genes (rbcL). A second DNase treatment may be needed.

2. RNA Quality Control

Step 1. Spectrophotometric QC of RNA

Prior to synthesizing cDNA, verify that the RNA samples are of sufficient purity to proceed with cDNA synthesis.

1. Quantitate each RNA sample according to the following formula:

RNA Concentration (ug/ml) = A260 x 40 x Dilution Factor

RNA samples must have a concentration >= 50 ng/ul (if less, concentrate with ethanol precipitation, see below)

2. Verify all samples meet the following requirements:

• A260/A280 >= 1.8

• A260/A230 >= 1.8. If low (less than 1.0), it indicates phenol contamination, do chloroform extraction again.

Step 2. Bioanalyzer/Gel QC of RNA

After concentrating the sample

Verify RNA samples are of sufficient molecular weight. Roche NimbleGen recommends the use of an Agilent 2100 Bioanalyzer, because the required sample size is very small. If a Bioanalyzer is not available, use a denaturing agarose gel.

1. Transfer 50ng total RNA to a sterile microcentrifuge tube. Store the remainder of your sample on ice or at -80°C.

2. Analyze samples using the Agilent Bioanalyzer and RNA 6000 Nano Assay Reagent Kit.

3. Compare the Bioanalyzer traces to those traces in Figures 2 and 3. Degraded samples appear as significantly lower intensity traces with the main peak area shifted to the left and typically exhibit much more noise in the trace.

Note: If using a denaturing agarose gel, one reagent source is Ambion NorthernMax® reagents for northern blotting. This kit's first section includes reagents and instructions for using denaturing agarose gels. You can also purchase reagents individually. Compare gel images to the Bioanalyzer electropherogram images.

Samples exhibiting degradation should not be carried through labeling and hybridization because there is an unacceptable risk of poor results.

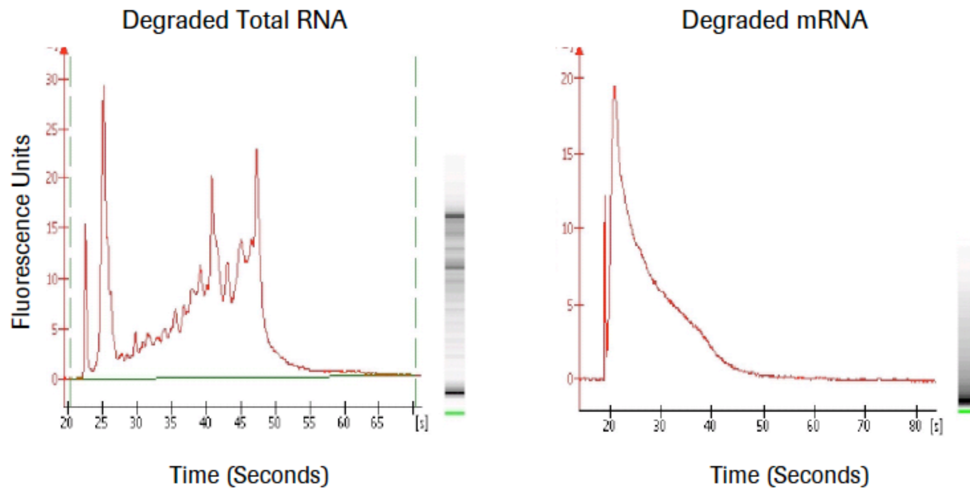

Figure 2: Example Traces for Degraded RNA Samples from a Eukaryotic Organism

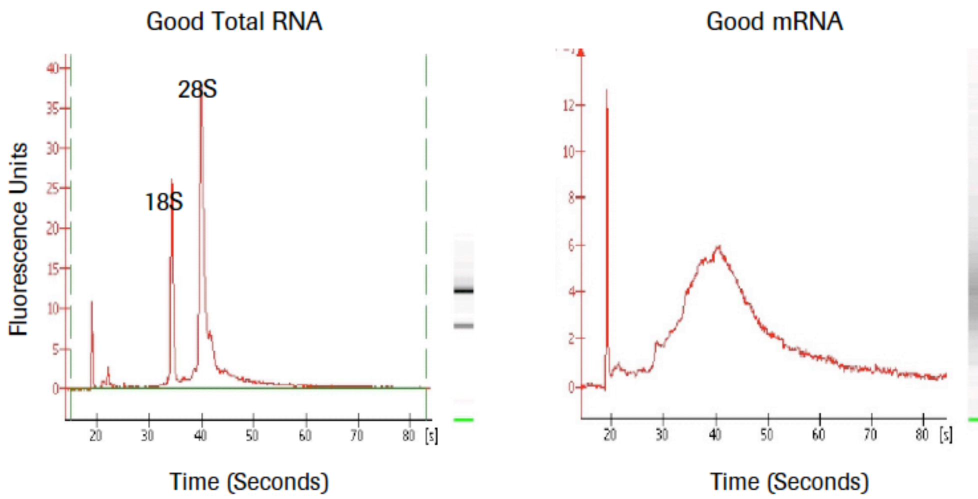

Figure 3: Example Traces for Non-degraded RNA Samples from a Eukaryotic Organism

Save an aliquote of total RNA for NoRT-qPCR.

Concentrating RNA

Prepare:

3M NaOAc

Glycogen 5 ug/uL

Ethanol 100%

Ethanol 75% (optional)

RNase-free water

Microcentrifuge at 4ºC

Procedure:

1. Add 7 µl of glycogen 5ug/mL, mix (usually, ~20 ug of glycogen is used per 1 mL of RNA solution), mix.

2. Add 0.1 volume of 3M NaOAc (a final concentration of 0.3M), mix.

3. Add 2.5 volumes of cold 95-100% ethanol, mix by vortexing.

4. Freeze overnight at -20ºC or do three cycles of freeze-thaw on dry ice

5. Centrifuge at 10,000g at 4ºC for 30 min (note the orientation of tubes for subsequent pellet identification)

6. After centrifugation, immediately remove the tubes from the centrifuge so that the pellet does not dislodge. Carefully decant the ethanol into a new tube not disturbing the pellet.

7. Remove the residues of ethanol with very narrow-end 20P tip.

It is not recommended to wash small amount of RNA with 75% ethanol; however, if washing with 70% ethanol is necessary to remove all salts, use cold (-20°C) 75% ethanol (2 volumes) and then chill and centrifuge the tubes for 5 min at 4°C. Carefully remove the supernatant after the centrifugation.

8. Resuspend the pellet in RNase-free water to the desired concentration. Take 1.5 uL of concentrated RNA sample for QC. Dilute with 1.5 uL of water. Use 1.5 uL to measure concentration with NanoDrop and 1uL to run gel in Bioanalyzer.

3. DNA extraction

DNA extracted from the same sample as RNA, cannot be used for quantitative analysis; instead, use the Zehrlab protocol for DNA extraction (described below) on a separate sample.

From Organic phase with TRI reagent (Ambion)

DNA is precipitated from the phenol phase and interphase of samples that have been homogenized (or lysed) in 1 ml of TRI Reagent (step 5 in the RNA Isolation Protocol). After a series of washes to remove residual phenol, the DNA pellet is solubilized in a mild alkaline solution, and the pH is adjusted.

For samples with less than 10ug of DNA:

Required Materials

- 100% and 75% ethanol, ACS grade or better
- Nuclease-free water
- 8mM NaOH
- 1M HEPES (free acid)
- EDTA 100 mM
- Glycogen
- Appropriately sized nuclease-free centrifuge tubes with secure closures, compatible with phenol/chloroform, and capable of withstanding centrifugal forces of 12,000 x g.

For samples with more than 10 µg of DNA:

Required Materials

- 100% ethanol, ACS grade or better
- Nuclease-free water
- 0.1 M trisodium citrate in 10% ethanol (no pH adjustment required), 2–3 ml per 1 ml of TRI Reagent used in the initial homogenization
- 8 mM NaOH
- 1M HEPES (free acid)
- EDTA 100 mM
- Glycogen
- Appropriately sized nuclease-free centrifuge tubes with secure closures, compatible with phenol/chloroform, and capable of withstanding centrifugal forces of 12,000 x g.

A. DNA Precipitation

1. Remove any aqueous phase remaining over the interphase. Make sure there is no pellets (filter remains and so), if present, transfer the liquid phase into a new tube. Note: *Careful removal of any residual aqueous phase is critical for the quality of the isolated DNA.*
2. If your sample contains <10 µg DNA, add 7 µl of glycogen 5ug/mL.
3. Add 300 µl of 100% ethanol per 1 ml of TRI Reagent used for the initial homogenization and mix samples by inversion.
4. Incubate at room temp for 2–3 min.

5. Centrifuge at 2,000 x g for 30 min at 4°C, and remove the supernatant.
Remove the supernatant and store it at 4°C for subsequent protein isolation if needed (see *TRI Reagent Protein Isolation Protocol* starting on page 5 of the manual).

B. DNA Wash

For DNA < 10 ugL

1. Wash the pellet with 75% cold ethanol (1 mL per 1mL of TRI reagent used)

2. Incubate for 10 min at room temperature

3. Centrifuge at 2,000 x g for 10 min at 4°C.

4. Carefully remove the supernatant.

5. Repeat steps 1-3.

Continue with DNA solubilization

For DNA > 10 ug:

Note: North Pacific oligotrophic waters: 8L yielded 20-30 ug of DNA, but still use glycogen to see the DNA pellet well.

1. Wash the pellet with 1 ml DNA Wash Solution (1 ml of DNA Wash Solution per 1 ml of TRI Reagent used for the initial homogenization). Be careful not to disturb the pellet. If disturbed, centrifuge at higher g for longer time.

2. Incubate the DNA pellet in DNA Wash Solution for 30 min at room temperature with periodic mixing.

3. Centrifuge at 2,000 x g for 10 min at 4–25°C.

4. Carefully remove the supernatant.

5. Repeat steps 1-4 to wash the DNA pellet a second time.

6. Wash the pellet with 1.5–2 ml of 75% ethanol (1.5–2 ml of 75% ethanol per 1 ml TRI Reagent used). This ethanol wash removes pinkish color from the DNA pellet.

7. Incubate for 10–20 min at room temperature with periodic mixing.

8. Centrifuge at 2,000 x g for 5 min at 4–25°C.

STOPPING POINT

Samples suspended in 75% ethanol can be stored at 4°C for a long period of time (months).
8. Remove the ethanol wash. Remove all residual ethanol by centrifuging again briefly and removing the ethanol that is collected.

C. DNA Solubilization

1. Briefly air dry the DNA pellet by keeping the tube open for 3–5 min at room temperature. Or remove the residues of ethanol with very narrow-end 20P tip

2. Dissolve the DNA pellet in 8 mM NaOH. (Use 50 uL if expected yield is low).

3. Pipette up and down slowly to dissolve the DNA pellet. Samples solubilized in 8 mM NaOH can be stored overnight at 4°C. If the pellet does not dissolve, add more 8mM

NaOH, heat at 55°C for 1h, leave samples at 4°C overnight.

3. Centrifuge at 12,000 x g for 10 min to pellet any insoluble material (fragments of membranes, etc) remaining at this stage and transfer the supernatant to a new tube.

4. Transfer the DNA-containing supernatant to a new tube. High viscosity of the supernatant indicates the presence of high molecular weight DNA.

5. Adjust the DNA solution to the desired pH using 0.1 M or 1 M HEPES (free acid). See Table 1.

6. Add EDTA to a concentration of 1 mM.

Table 1. Adjustment of pH in DNA samples

Use the following amounts of 0.1 M or 1 M HEPES (free acid) per 1 ml 8 mM NaOH					
Final pH	**0.1 M HEPES**	**Final pH**	**0.1 M HEPES**	**Final pH**	**1 M HEPES**
8.4	86 µl	7.8	117 µl	7.2	23 µl
8.2	93 µl	7.5	159 µl	7.0	32 µl
8.0	101 µl	-	-	-	-

For pH=8.4, I used 4.3 uL of 1M HEPES and 0.5 uL of 100 mM EDTA per 50 uL of DNA solution.

Back extraction protocol

This alternative procedure replaces steps of the DNA Isolation Protocol.

1. Prepare a back extraction buffer: 4 M guanidine thiocyanate, 50 mM sodium citrate and 1 M Tris (free base).

2. Remove any remaining aqueous phase overlying the interphase.

3. Add back extraction buffer to the interphase-organic phase mixture. Use 500 µl of back extraction buffer per 1 ml of TRI Reagent used for the initial homogenization.

4. Vigorously mix the sample by inversion for 15 sec and incubate for 10 min at room temperature.

5. Centrifuge at 12,000 x g for 15 min at 4°C to separate the phases.

6. Transfer the upper, aqueous phase containing DNA to a clean tube and save the interphase and organic phase at 4°C for subsequent protein isolation. Do chloroform extraction to clean from residues of phenol.

7. If the expected DNA yield is less than 20 µg, add 5–10 µl of glycogen 5ug/mL to the aqueous phase and mix.

8. Add 400 µl of isopropanol per 1.0 ml of TRI Reagent used for the initial homogenization to precipitate DNA from the aqueous phase. Mix the sample by inversion and incubate for 5 min at room temperature.

9. Sediment the DNA by centrifugation at 12,000 x g for 30 min at 4°C and remove the supernatant.

10. Wash the DNA pellet with 1 ml of 75% ethanol. Proceed with Step *C. DNA Solubilization*.

4. DNA Quality Control

1. Quantitate each DNA sample according to the following formula:

DNA Concentration (ug/ml) = A260 x 50 x Dilution Factor

2. Verify all samples meet the following requirements:

- A260/A280 >= 1.8
- A260/A230 >= 1.8

Note: phenol contamination will give a peak at A270, if it is the case, clean with chloroform extraction (twice), then precipitate DNA again.

5. ds cDNA synthesis and amplification

Note: This is for NimbelGen technology. If using others, follow the appropriate protocol for library preparation.

The WTA process involves two steps. In the first step, sample RNA is reverse transcribed with non-self-complementary primers composed of a quasi-random 3' end and a universal 5' end. As polymerization proceeds, displaced single strands serve as new templates for primer annealing and extension. The resultant Omniplex® cDNA library, composed of random, overlapping 100–1000 base fragments flanked by universal end sequence, is then amplified by PCR with the universal primer to produce WTA product.

Suitable for the most variety of samples, including prokaryotic RNA because primers for cDNA are random.

Supplies:

- TransPlex Whole Transcriptome Amplification Kit (SIGMA-Aldrich)
- ERCC mix 1 (Ambion) (prepare 1:100 dilution)
- Antibody inactivated hot-start Taq DNA Polymerase
- Thermal cycler
- 0.2 mL or 0.5 mL Thin-Walled PCR Tubes or PCR multiwell plate
- PCR cleanup kit similar to GenElute PCR cleanup kit (SIGMA-Aldrich)

Procedure (with modifications)

Library Preparation (cDNA synthesis)

1. Thaw WTA Library Synthesis Buffer and WTA Library Stabilization Solution on ice and mix thoroughly. Dissolve any precipitate in these solutions by briefly heating at 37 °C and mixing thoroughly.

2. To 300 ng (5-300 ng range; 50ng is recommended by NimbleGen, but it is for cultures) of total RNA, add, individually or premixed, the following

    0.5 uL of ERCC mix 1 1:100 dilution

    2.5 uL WTA Library Synthesis Buffer

    2.5 uL WTA Library Stabilization Solution

    Nuclease-free water for a total volume of 24 uL

3. Mix by pipetting and incubate at 70 °C for 5 minutes.

4. Cool reaction immediately on ice and spin down any condensation by centrifugation.

5. Add 1 mL of WTA Library Synthesis Enzyme and mix by pipetting.

6. Incubate in thermal cycler using the following parameters:

    24 °C for 15 minutes

    42 °C for 2 hours

    95 °C for 5 minutes

7. Chill reaction immediately on ice. Spin down any condensation by centrifugation.

! Save an aliquot for QC (5 uL)

Amplification

8. Thaw WTA Amplification Master Mix and dNTP Mix on ice and mix thoroughly.

9. Prepare the following WTA Amplification Mix (*For real-time PCR, include a reference dye as necessary and 3.75 uL of a 1:1000 dilution of SYBRÒ Green stain). This is for 25 uL of cDNA, adjust for other volumes.

  300 uL Water, Molecular Biology Reagent

  37.5 uL WTA Amplification Master Mix

  7.5 uL dNTP Mix

  12.5 units of antibody inactivated hot-start Taq DNA Polymerase

10. Divide the library, placing 5 uL aliquots in individual tubes or wells.

11. Add 70 uL of WTA Amplification Mix to each aliquot and mix well. Incubate in thermal cycler using the following parameters:

  95 °C for 3 minutes.

  17 cycles* x (94 °C for 20 seconds, 65 °C for 5 minutes)

*Optimal cycle number varies with template amount and quality. 17 cycles is recommended for 5 ng of high quality RNA. Optimal cycle number is achieved by proceeding 2–3 cycles into the amplification "plateau".

12. After cycling is complete, maintain the reactions at 4°C or store at –20°C until ready for analysis or purification. The stability of WTA DNA is equivalent to genomic DNA stored under the same conditions.

13. Purify the amplified cDNA using the GenElute PCR cleanup kit (SIGMA-Aldrich) or other PCR purification kits. Elute with sterile RNase-free water.

14. Follow with the NimbleGen protocol from step 7.

Step 7. Spectrophotometric QC of cDNA

1. Quantitate each cDNA sample according to the following formula:

cDNA Concentration (ug/ml) = A260 x 50 x Dilution Factor

2. Verify that all samples meet the following requirements:

  • Concentration >= 100ng/ul

  • A260/A280 >= 1.8

  • A260/A230 >= 1.8

One ug of cDNA is required for labeling and hybridizing to a microarray. If concentration is lower 100 ng/uL, concentrate cDNA sample with ethanol (or isopropanol) precipitation.

Step 8. Bioanalyzer/Gel QC of cDNA

1. Transfer 250ng cDNA to a sterile microcentrifuge tube. Store the remainder of your sample on ice or at -20°C.
2. Analyze the samples using the Agilent Bioanalyzer.
3. Compare the Bioanalyzer traces to the traces displayed below. Verify that all samples meet the following requirement for acceptance:

- Median size >= 400bp when compared to a DNA ladder.
- Looks similar to the examples of good cDNA sample traces displayed below.

Note: If using an agarose gel, compare the gel images to the Bioanalyzer's electropherogram images.

Samples exhibiting degradation should not be carried through labeling and hybridization because there is an unacceptable risk of poor results.

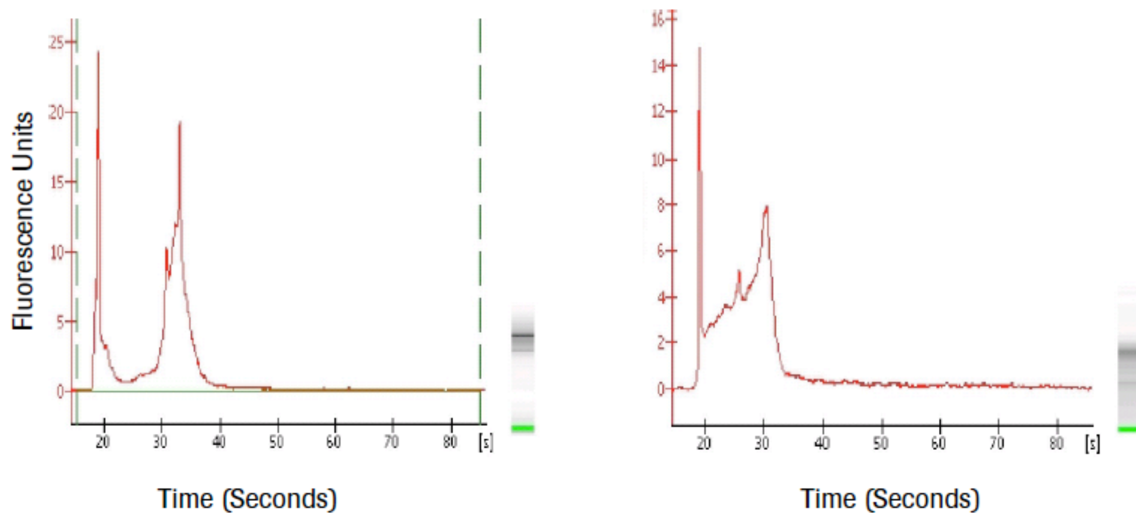

Figure 4: Example Traces Showing Good cDNA from a Eukaryotic Organism
Note: Your traces could look different than these examples. Compare your traces to reference traces for the organism being researched.

6. Zehr Lab DNA Extraction

Kendra Turk-Kubo
2011

Please default to this protocol as a standard method for extracting DNA from filters.

Preparation

- Turn on water bath or heat block to 65°C. To turn heat block to 65°C, turn on High & set high temp setting to just below 3. If using water bath, check water level & add DI water if necessary.
- Turn on Shake' N' Bake incubator in D446 to 55°C.
- Add liquid nitrogen from D446 to benchtop dewar.
- Thaw samples.
- Samples should be in sterile bead beater tubes.
- Heat AE buffer to 65°C before elution step.

Note: Aliquots of most reagents used in protocol can be found in upperware container on top of fridge.

Step 1. FREEZE FRACTURE

- Add 400ul AP1 buffer to each tube.
- Submerge tubes in liquid nitrogen until completely frozen (~30 sec).
- Immediately transfer tubes to water bath or heat block until completely thawed (2-3 min).
- Repeat freeze-thaw for a total of 3 cycles.
- Leave water bath or heat block at 65°C for RNase incubation and heating elution buffer.

Step 2. BEAD BEAT

- Bead beat samples for 2 minutes. (If using new bead beater: Use highest speed setting. Longest time setting is 60 sec, so do two consecutive runs. Note: the 5-min cooling period can be overridden by pressing RUN.)
- Pulse-centrifuge tubes to reduce foam.

Step 3. PROTEINASE-K TREATMENT

- Add 45ul Proteinase-K (in fridge) solution to each tube. Invert to mix.
- Put tubes in whirlpack or ziplock bag, with enough room to lay tubes flat in incubator.
- Incubate in Shake' N' Bake oven (D446) at 55°C for one hour with moderate shaking.

Step 4. MODIFIED QIAGEN PLANT KIT PROTOCOL

- Return tubes to Core. Add 4ul RNase A to each tube and vortex vigorously. **Take care to keep RNase A away from RNA bench!**
- Incubate tubes at 65°C for 10 min. Vortex tubes 2-3 times during incubation.
- Carefully remove filters with sterile needles. (Dispose of filters in solid waste and needles in sharps container.)
- Add 130ul AP2 buffer to each tube. Vortex and incubate on ice for 10 min.
- Centrifuge at 14,000 RPM for 5 min to pellet precipitates and beads.

- Transfer up to 650ul supernatant to purple QIAshredder column. Centrifuge at 14,000 RPM for 2 min.
- Transfer flow-through to new 2-ml tube. If debris pellet has formed, avoid it while transferring.
  If there is more than 650ul supernatant, repeat previous two steps with same columns and 2-ml tubes.
- Add 1.5 volumes AP3/E buffer to cleared lysate. Mix by pipetting.
- Transfer 650ul mixture to white Mini Spin Column. Centrifuge 1 min at 8000rpm. Discard flow-through. Repeat with remaining sample until entire sample has been run through column.
- Place spin column in new collection tube.
- Add 500ul AW buffer to column. Centrifuge 1 min at 8000rpm. Discard flow-through and reuse collection tube.
- Add 500ul AW buffer to column. Centrifuge 2 min at 14,000rpm to dry membrane.
- Transfer spin column to sterile 1.5 microcentrifuge tube, taking care to avoid allowing liquid in collection tube to come into contact with column.

Step 5. ELUTION

- Add 100ul pre-heated AE buffer directly to spin column membrane.
- Incubate at room temp, 5 min.
- Centrifuge 5 min at 8000rpm to elute.
- Store extracts at -20°C.

7. Appendix. Collecting Nucleic Acid Samples for analysis with MicroTOOLs microarray

Supplies and Equipment

1. Peristaltic pump with tubing that can be acid-washed (Masterflex PharMed, Viton)
2. Two pore size 25 mm filters placed in Swinnex holders in line: filters, 0.2 um (supor) and 5 um (or 10um). Two sizes are only needed to increase the filtration speed or for special purposes.
3. Swinnex filter holders, acid-washed after each use
4. Sterile 2.0 mL cryotubes with 0.2 uL of beads.
5. Or can use Sterivex 0.22 um cartridges if need to filter faster. However, extraction of RNA/DNA from each Sterivex takes longer, and also cells like SAR11 tend to pass through.
6. Any other way of filtering, as long as it is relatively fast, clean and not too disruptive.

Notes

- Volume depends on biomass: generally , for surface open ocean 1.5-3L for RNA and 1-1.5L for DNA.
- Time: try to keep filtration time not longer that 20-30 min for RNA samples.
- Biological replicates for both DNA and RNA samples.
- RNA and DNA sample from same incubation/station. (At least one DNA sample every 24h)
- Nucleic acid extracted with the protocol for microarray, can be used in any other molecular analysis (PCR, sequencing), and so samples can be combined if volume is an issue.

Procedure

Filter seawater using the peristaltic pump onto two size filters placed in line or onto Sterivex filter. After biomass is collected on a filter, if not Sterivex, place the filter in a cryotube with beads and flash freeze in liquid nitrogen. The two size filters can be

placed in one tube or in two separate tubes depending on the goal. The microarray targets eukaryotic and prokaryotic microorganisms, and nucleic acid extraction can be done from filters combined. Store cryotubes at -80°C until processing. We do not use RNAlater for RNA preservation, and freezing the samples immediately after filtration is critical.