Preparation of cDNA from Zebrafish Embryos

Reagents

E3 medium

	quantities for 5 L of 60X stock
5 mM NaCl	86 g
0.17 mM KCl	3.8 g
0.33 mM $CaCl_2$	14.5 g $CaCl_2 \cdot 2H_2O$
0.33 mM $MgSO_4$	24.5 g $MgSO_4 \cdot 7H_2O$
0.00001% (w/v) Methylene Blue	to be added to 1X solution

1X PBS
Trizol
Chloroform
Isopropanol
75% Ethanol/25% RNase-free H_2O
RNase-free H_2O
Invitrogen Micro-Midi spin column
Invitrogen Micro-Midi Lysis Buffer
Invitrogen Micro-Midi Wash Buffer 2
1X Tris-EDTA buffer

Procedure

RNA isolation

1) Mate zebrafish and grow embryos to desired developmental stage in E3 medium.
1) Dechorionate 50 embryos (this step may improve mRNA yield and purity).
1) Transfer embryos to a 1.5 mL centrifuge tube, wash quickly in E3 buffer, and then PBS. At no time allow the embryos to interact with the air/water interface**.**
1) Remove as much of the PBS as possible. A gel-loading pipet tip works well for this.
1) Flash freeze the embryos in liquid nitrogen (embryos may be stored at –80 °C for several months).
1) Add 500 μL Trizol to frozen, dechorionated embryos. Homogenize embryos with a microfuge tube pestle. Further homogenization with a small gauge needle and syringe can be conducted if desired. The homogenate should be dark grey-purple and viscous.
1) Incubate the homogenate for 2-3 minutes at room temperature.
1) Add 150 μL chloroform to the microfuge tube. Shake vigorously for 30 seconds, and incubate at room temperature for 3 minutes.
1) Centrifuge at 10,000 rpm (10,500 *g*) for 15 minutes at 4 °C.
1) Transfer the RNA-containing upper aqueous phase to a new 1.5 mL centrifuge tube and precipitate the RNA by adding 0.8 volumes of isopropanol. Mix well and incubate for 10 minutes at room temperature.
1) Centrifuge at 10,000 rpm (10,500 *g*) for 15 minutes at 4 °C.

2) Carefully remove the supernatant and wash the RNA pellet with 750 μL of 75% RNase-free ethanol (MilliQ H_2O is sufficient here) by centrifuging at 8,000 rpm (7,500 *g*) for 15 minutes at 4 °C.
3) Remove the entire supernatant without disturbing the RNA pellet and air dry for 10 minutes.
4) Resuspend RNA in 50 μL RNase-free H_2O (you can quantitate here if desired; see below).

DNase digestion

RNA purified by the above method is often quite clean and mostly free of contaminating genomic DNA. However, if the RNA is intended for use with sensitive applications, users may want to treat RNA with RNase-free DNase. The DNase-treated RNA can then be purified using the procedure below, or alternatively by phenol/chloroform extraction, ethanol precipitation, etc. (see the *in situ* hybridization protocol for DNA linearization, steps 3-7).

1) Add 1-2 μL of DNase to your RNA solution and the appropriate buffer (PCR or restriction enzyme buffer will suffice if none is supplied). Incubate for 15-30 minutes at 37 °C.
2) Add Midi-Micro Lysis Buffer to the solution for a total volume of 250 μL. Add 175 μL isopropanol and mix thoroughly.
3) Apply the mixture to a spin column matrix and centrifuge at 5,000 rpm for 30 seconds.
4) Remove flow-through and apply 500 μL Wash Buffer 2. Centrifuge at 5,000 rpm for 30 seconds and remove flow-through. Repeat if desired.
5) Centrifuge spin column at 10,000 rpm for 2 minutes to remove remaining wash buffer.
6) Transfer washed spin column to a new RNase-free 1.5 mL microfuge tube. Add 50-75 μL of RNase-free H_2O to spin column membrane matrix and incubate 1-5 minutes at room temperature.
7) Centrifuge spin column at maximum rpm for 2 minutes to recover RNA.
8) Quantitate RNA by making 1:50 and 1:100 dilutions into Tris-EDTA buffer. Measure absorbances of these dilutions at 260 nm, 280 nm, and 320 nm wavelengths versus a Tris-EDTA buffer blank. The A_{260}/A_{280} ratio should be 2.0-2.2. The concentration of RNA in ng/μL is equal to $(A_{260} - A_{320})$ x 40 x dilution factor.
9) Proceed with cDNA production according to the manufacturer's instructions (e.g. RETROscript).