RT-PCR from zebrafish embryos

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(also see protocol Grinblat et al, in Methods chapter)

1) Isolate RNA from embryos:
   Use about 50 embryos
   Use a standard method for isolating RNA, such as TRIZOL (Invitrogen) or RNeasy (Qiagen)

2) DNase treat isolated RNA:
   Resuspend the pellet from the isolation step into 100 µL DNase reaction:
   5 µL (5 U) RNase-free DNase
   10 µL 10X reaction buffer
   85 µL RNase-free H_20
   Incubate at 37°C for 30 minutes

   Phenol:$CHCl_3$ extract and precipitate with NaOAc, glycogen and EtOH
   Resuspend pellet in 250 µL H_20 (not sure about this volume – test possibilities)

3) RT reactions:
   Set up two reactions for each RNA sample, for **+/- RT**
   1 µL 25 µM random hexamer
   10 µL RNA
   Incubate at 70°C for 10 min, snap freeze
   Add: 4 µL 5X RT buffer
   2 µL 0.1M DTT
   1 µL 10 mM dNTPs
   Incubate at 37°C for 5 minutes
   Add: 1 µL Superscript RT (or do not include this for **-RT** sample)
   Incubate at 37°C for 2 hr.
   Inactivate at 95°C for 10 minutes

4) PCR amplification of cDNA samples:

Make a common PCR reaction mix with the following for each sample:

37 μL H20
5 μL 10X PCR buffer
1 μL 10mM dNTPs
0.5 μL α[^{32}P]-dCTP (3000 Ci/mmol; 10 mCi/mL; 3.3μM)
0.5 μL HotStarTaq

Add 44 μL of this mix to:

4 μL template (RT reaction from above)
1 μL each 20μM primer

Cycles: 15 min. at 95°C
xx cycles of: (cycle number determined empirically for each primer pair)
1 min. at 94°C
1 min. at xx°C (temp determined by primers - probably 55-60°C)
1 min. at 72°C

Note about determining cycle numbers: for RT-PCR to work, the cycle numbers must place the reaction progress within the linear amplification range. This is usually around 20-30 cycles, and to test this, set 4 reactions as above (all with the same primers and template), then remove a tube after 21, 24, 27, 30 cycles.

Primer design: probably want a product that is 300-600 bp

5) Acrylamide gel analysis of PCR products:

Add standard loading buffer to entire 50 μL PCR reaction

Load 17.5 μL of each sample onto a 5% Polyacrylamide gel:

6.25 mL Accugel (40% 19:1 acrylamide:bis)
42.4 mL H_20
1 mL 50x TAE
0.35 mL 10% APS
17.5 μL TEMED

Run gel at 250V for 1.5-2 hr.

Dry gel at 80°C for 0.5 hr.