RT-PCR from total RNA

We have found that that low-level DNA contamination from Trizol isolated RNA is quite common. This interferes with qRT-PCR analysis, especially of low-abundance transcripts (ie miRNA primary transcripts). Therefore, double DNAse treatment of RNA, in addition to -RT controls, are important steps for valid qRT-PCR analyses.

Start with **8-15ug total RNA**--you may lose approximately 5ug in two phenol-chloroform reactions (you loose up to 20% per phenol-chloroform extraction) leaving enough to do both the + and - RT reactions.

DNase treatment: In 0.5ml eppie tube:

15 ul RQ1 DNase (Promega)
15 ul RQ1 DNase Buffer 10X
120 ul total RNA (15ug) + DEPC H2O
____
150 ul total, incubate 37C 30min.
Add 2ul DNase STOP

Extract DNase treated RNA:
1. Add 1ul 20mg/ml glycogen carrier (or 2ul of 10mg/ml)
2. Add 15ul 3M NaOAc
3. Add 165ul phenol:chloroform:isoamylalcohol (25:24:1) - briefly vortex and centrifuge 5min
4. Extract and add 165ul isopropanol; vortex; ice 5min (**NOTE** – if this same treated RNA is to be used for small RNA analyses (PAGE Northerns), precipitate at -20C >30min)
5. Centrifuge 12,000rcf 10min 4C
6. Remove supernatant (you should see a tiny, whitish pellet)
7. Wash with 200ul 70% ethanol; cent. ~5-10min
8. Carefully remove sup., and do a quick spin to collect remaining ethanol; pipet off
9. Air dry pellet 5 min on benchtop or at 50C(cover with kimwipe to keep dust out)
10. Resuspend in 120ul DEPC H2O, and repeat DNAse as above. Repeat steps 1-9.
11. Resuspend in 20ul. Use 1ul for quantification and check levels and quality of treated RNA on a denaturing agarose gel (1ug). Use remaining to split equally for +RT and –RT reverse transcription reactions.

Reverse transcription reaction (Invitrogen reagents):
2.5-5ug of total RNA can be used in a "full size" reaction. Cut in half all reagents for 1-2ug total RNA samples. For example total volume for the oligo anneal step is 12ul for full and 6ul for half.

Full size reaction:

1. In .5ml tube mix:
   3ul 10uM oligo (A95 = oligo dT) (**for random primers** use 1ul of 250ng/ul)
   1ul 10mM dNTP mixture
   RNA
   ____
   total volume 12ul
   65C 5min
   ice ~1min

2. Combine:
   4ul 5X Buffer
   2ul .1M DTT
   1ul water (or RNase inhibitor)
   ____
   mix this with step one tube to get total volume of 19ul
   42C 2min
   Add 1ul RT enzyme to "+RT" samples or 1ul H2O to "-RT" samples and incubate at 25C 10min, 42C 50min, 70C 15min (Program "RT" on PCR # 4)

3. Add 1ul RNaseH, 37C 30', store at -20C

Semi-quantitative PCR (GoTaq Promega reagents) to check RT before sending for qRT-PCR:

1. In 96 well PCR tray (or individual PCR wall tubes), add 1ul from RT reaction (typically dilute an aliquot 1:10 or 1:5 to test with control oligo set – actin or eft-2) and include control templates (genomic DNA, water)

2. Make PCR mix: 1X---> 1.0ul 25mM MgCl2
   5.0ul 5X Buffer
   0.5ul 10mM dNTP
   0.5ul 10uM forward primer
   0.5ul 10uM reverse primer
   0.25ul Taq
   16.25ul water
   ____
   Add 24ul to 1ul template and mix with careful pipeting
   Add 1 drop paraffin oil to top of reactions
   Seal tray with cap strips

General PCR program (vary anneal temp, elongation time and cycles depending on primers and product):

AEP4:
1) 94C 2'
2) 94C 10"
3) 58C 30"
4) 72C 1'
5) GOTO (2) 15-40X
6) Hold 4C

Typically, add 5ul 6X glycerol loading buffer + dye (exclude one of the dyes if it runs at same position as your PCR product) directly to PCR sample and run entire sample on agarose gel to detect PCR products.