



**Title:** *General RNA to cDNA and Real-Time PCR Preparation*

No: RTLP-MOLEC-5

Location:  
*Old CCRC Tripp Lab*

Approval Date:  
11 January 2006

Supersedes Date:

**Materials:**

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- |  |   |   |
|--|---|---|
| • Lab coat   | •Pipetteman                                 | •50 $\mu$ M random hexamer primer         |
| • Gloves   | •Pipetteman tips                            | •50 U/ $\mu$ l MulV reverse transcriptase |
| • Picofuge   | •Thermal cycler                             | •RNase-free water                         |
| • Ice Bucket w/ ice  | •Micro Centrifuge                           | •Taqman Master Mix                        |
| • 1.5 mL RNase free microcentrifuge tubes                    | •25mM magnesium chloride                    | •taq polymerase                           |
| •0.2 mL PCR reaction tube (DNase-free, RNase-free, DNA free) | •10X PCR buffer (no magnesium)              | •primer/probe mix (assay specific)        |
| • Qiagen RNEasy Mini Kit                                     | •25mM dNTPs                                 | •70% ethanol                              |
|  | •20 U/ $\mu$ l RNase Inhibitor              |   |
|  | •Stratagene optical 8X strip tubes and caps |   |

**Procedure:**

**A. To prepare RNA using the RNEasy Mini Kit** (Qiagen, Valencia, CA or equivalent):

1. To  $5 \times 10^6$  cells (amount of RLT buffer will vary based on the number of cells in the sample, check kit insert) add 350  $\mu$ l RLT buffer and vortex until dissolved.
2. Add 1 volume of 70% ethanol and mix by pipetting
3. Apply the sample to a RNEasy mini column and spin at 10,000xg for 15sec.
4. Discard the flow-thru and add 700 $\mu$ l of RW1 buffer. Spin at 10,000xg for 15sec.
5. Discard the flow-thru and add 500 $\mu$ l of RPE buffer. Spin at 10,000xg for 15sec.
6. Repeat step 5, but spin at 10,000xg for 2min.
7. Elute RNA by adding 50 $\mu$ l of RNase-free water and spin at 10,000xg for 1min.
8. Store -80°C until ready for use.

Note: To avoid contamination problems, all preparations should be performed in the PCR hood.

**B. To prepare cDNA:** (Reagents from Applied Biosystems, Foster City, CA, or equivalent)

Each 20 $\mu$ l Reverse transcriptase reaction contains (prepare in 0.2 mL PCR reaction tubes):

1. 4 $\mu$ l of magnesium chloride 25mM
2. 2 $\mu$ l of 10x PCR buffer (No magnesium)

*Protocol for Real-Time PCR Using cDNA*

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3. 0.8ul of 25mM dNTPs
4. 1ul of 20U/ul Rnase Inhibitor
5. 1ul of 50uM random hexamer primer
6. 1ul of 50U/ul MuV reverse transcriptase
7. 7.2 ul distilled sterile water
8. 3ul of RNA

Mix reagents and centrifuge using the picofuge. Incubate 1hr at 37°C

**C. Real-Time PCR:** (Reagents from Applied Biosystems, Foster City, CA, or equivalent)

A. Prepare Taqman Master Mix for 50 reactions:

1. 125.0 ul of 10X PCR Buffer
2. 50.0 ul of 2.5mM dNTPs (prepare by diluting 25 mM dNTPS 1:10 in RNase-free water)
3. 487.5 ul H<sub>2</sub>O

B. Prepare Master Mix: per reaction (use the Stratagene optical tube strip and caps):

1. 13.25 ul of Taqman Master Mix
2. 2.5 ul of 25mM Magnesium chloride
3. 0.25 ul Taq polymerase
4. 1 ul of primer/probe mix
5. 4.75 ul H<sub>2</sub>O
6. 3 ul of cDNA
- 7.

Mix reagents and centrifuge using the picofuge.

**C.** Load samples into machine. Open MX3000P software to appropriate experiment type and label the wells on the screen corresponding to the reactions placed in machine.

**D.** Set cycling parameters according to optimum conditions for primers/probes to be used. Consult the user's manual for specific parameters.