

Real Time RT-PCR Protocol

Real time RT-PCR can be performed using a number of commercially available kits. We currently use the QIAGEN QuantiTect Probe RT-PCR kit (#204443). The setup shown below is for the QIAGEN kit. Note also that the volume of RNA added per reaction below is 10 ul but can be increased or decreased with the appropriate adjustment of total volume with water.

<u>COMPONENT</u>	<u>VOL PER REACTION</u>	<u>10 REACTIONS</u>
RNase free water	13.2 ul	132 ul
2X Ready mix	25 ul	250 ul
primer 1 (100 uM stock)	0.5 ul	5 ul
primer 2 (100 uM stock)	0.5 ul	5 ul
FAM/probe(25 uM stock)	0.30 ul	3.0 ul
enzyme	0.5 ul	5 ul

Prepare a reagent "master mix" according to the number of reactions desired. The master mix should be prepared in a "clean room" physically separated from all other laboratory activities with dedicated reagents and equipment (i.e. pipette's). For 10 samples make a 10X master mix (see above) by multiplying the volumes of all individual reagents by 10. Combine the reagents in the above order in an RNase free centrifuge tube **on ice**. Divide the master mix into 10 portions of 40ul each into either 0.2 ml optical (specifically for TaqMan assays; emission fluorescence is read through the cap) PCR tubes or a 96 well optical PCR plate. Finally add 10 ul of the individual RNA sample to each tube/well. All samples are tested in duplicate wells. **Include several "NO RNA" negative controls (NTC)** by adding water instead of any RNA. Include a positive control or a dilution series of known quantities of positive control RNA if setting up a quantitative assay.

Cycling conditions (QIAGEN conditions for Real Time RT-PCR):

1 cycle:	45 cycles:
50° for 30 min (RT reaction)	95° 15 sec
95° for 15 min (enzyme activation)	60° 1 min

Interpretation

We use the following algorithm to evaluate the TaqMan results.

Positive:	Ct value # 38 in duplicate wells.
Equivocal:	Ct value # 38 in one of two wells.
Negative:	Ct values >38 in duplicate wells.

All positive and equivocal samples are repeated with a second set of primer/probes for confirmation. A positive result in any of the negative controls invalidates the entire run. Failure of the positive control to generate a positive result also invalidates the entire run.