Real Time PCR at the FSU Molecular Cloning Facility
We provide SYBR Green qPCR using an Applied Biosystems 7500 Fast Real-Time PCR System.

The following covers RT-qPCR of RNA from eukaryotes. Some of the information may not be relevant for DNA or other genomes (e.g., bacterial or viral). It is a draft set of guidelines based on my experience and is not meant to be comprehensive or official in any way. Comments are welcome!

A. Materials provided by the user:

1. **Real time PCR work order**
   Be sure to include budget to be charged and PI signature.

2. **DNA-free RNA samples**
   TRIzol, and Qiagen RNeasy (often with the addition of various physical disruption steps) are two popular methods. Best isolation method will be determined by the investigator, but we are happy to help with the evaluation. See also "extra controls" below*.

3. **RNA quantitation summary**
   Nanodrop reading with full report (concentration and absorbance ratios) is recommended.

   How much RNA do you need? The amount of RNA that is needed depends on the biological system and how many reactions you want done. For a 20 ul reverse transcription reaction, ~0.5-1 ug RNA (in a volume of 8 ul) typically gives a robust reaction. Even more RNA (up to 2ug per RT rx) would be OK. Amounts <0.5 ug per RT rx may work fine in many cases, but as the concentration gets lower the variability/failure rate will eventually increase. There is no absolute threshold, but typicallu we don't usually use less than 0.5 ug per RT rx.

   Typically 1 ul of an RT reaction will be used for each PCR reaction, so a 20ul RT rx would provide enough to test expression of up to 9 genes (assuming duplicate PCRs). See also "extra controls" below*

4. **RNA quality verification**
   The most common method is to run a small aliquot (e.g., ~0.5 ug) on an agarose minigel. Smearing, or a relative loss of signal in the lower rRNA band (should be ideally ~1:2 ratio of 16S:28S bands) may indicate problems with the RNA prep.

   Nanodrop reading will also provide some information. For pure RNA, the $A_{260/280}$ and $A_{260/230}$ rations should be ~2. Significantly lower ratios may indicate impurities (see Nanodrop T009-Technical Bulletin)

5. **Primer pair for each gene of interest**
   These will ultimately be used as a mixture of 10uM <u>each</u>. They can be provided as a mixture if desired (in dH20), or as 100 uM individual stocks.
   We can provide some primer design assistance if needed. Some suggestions:
   - Select primers with Tms ~60ºC-65ºC (nearest-neighbor method with values of 50 mM for salt concentration and 300 nM oligonucleotide concentration).
   - Select amplicon size ~100-150

- If poly-dT will be used for RT, efficiency may be best if primers are closer to the 3' end of the message
- Be sure primers are specific for the target (e.g., according to BLAST)
- Minimize secondary structure (e.g., according to Netprimer or Primer3).
- Minimize primer dimers (e.g., according to Netprimer or Primer3)
- Note: Intron-spanning primers (can overlap intron/exon junction) may be desirable if genomic DNA contamination (below) is a concern, but note this may limit primer location options.

6. **Some additional information** to provide if you are doing relative quantitation and want the 7500 software to do the math for you:
   - Which primers are for the **endogenous control** gene (e.g., GAPDH etc.)
   - Which is the **calibrator sample** (e.g., "wild type" or "untreated" sample)

B. Services provided by the Molecular Cloning Facility

1. **cDNA production.**
   Rate is $10 per RT reaction.
   Typically we use Superscript III kit, with polyT primers. Random hexamers or specific primers can also be used if desired.

2. **SYBR Green qPCR**
   Rate is $70 for half plate, $110 for full plate
   a. **Plate setup:**
      Typically we will do duplicates PCRs of each sample, unless requested otherwise. Duplicate no-template controls will also be done for each primer set. See also "additional controls" below*****.
   b. "Run method":
      We can base a run method (i.e., annealing temps, etc.) on what has been used successfully in the past for your primers, and/or the predicted Tms of your primers, or whatever you want.
   c. Initial Analysis:
      The ABI 7500 software can do various types of analysis, and requires that the method be determined before the run is started. Relative quantization (ddCt) is frequently used. Absolute quantization (AQ plate) using a standard curve can also be done.
   d. Data Summary:
      We can provide a simple excel spreadsheet of mean Ct values for each sample, or a full Excel results "export" and/or graphical "report" pdf.

*** Extra controls**

Note that some additional controls are highly recommended, especially in the early stages and for publication-quality data:

a. No-RT control. This is an RT reaction with no reverse transcriptase added, to detect the presence of genomic DNA contamination in the sample due to inadequate DNAse treatment (also may detect amplicon or genomic contamination).

b. Primer efficiency determination. The ddCt quantitation formula typically used for relative quantitation assumes that all primers work with 100% efficiency (i.e, the DNA doubles with each cycle). If this assumption isn't correct, quantitation by this method may be misleading. Standard curves with a cDNA concentration dilution series can be used to calculate efficiency. Alternatively the LinReg program can help determine efficiency of individual reactions.