Real-time PCR (SYBR green) protocol (Briana 6/2011)

Principle:
PCR technology is widely used for quantifying DNA because the amplification of the target sequence allows for greater sensitivity of detection than other methods. In an optimized reaction, the target quantity will approximately double during each amplification cycle. In quantitative PCR (qPCR), the amount of amplified product is linked to fluorescence intensity using a fluorescent reporter molecule. The point at which the fluorescent signal is measured in order to calculate the initial template quantity can either be at the end of the reaction (endpoint semi-quantitative PCR) or while the amplification is still progressing (real-time qPCR).
In real-time qPCR, a fluorescent reporter molecule (such as a double-stranded DNA binding dye or dye-labeled probe) is used to monitor the progress of the amplification reaction. With each amplification cycle, the fluorescence intensity increases proportional to the increase in amplicon concentration. The resulting plots of fluorescence vs. cycle number for all the samples are adjusted with their background fluorescence, and the threshold level of fluorescence is set above the background but still within the linear phase of amplification for all the plots. The cycle number at which an amplification plot crosses this threshold fluorescence level is called the "Ct". This Ct value can be directly correlated to the starting target concentration of the sample.
This protocol describes the real-time qPCR steps with a DNA binding dye, such as SYBR green I.

Primer design:
Add Tips for qPCR primers

Reagents:
Sybr green (Biorad, #170-8880, SAbiosciences #PA-011-12, Rouche #04913850001, Applied Biosystems #4309155)
pre-made cDNA
96 well PCR plate, Axygen #321-64-051
Optical tape (plate cover), BioRad #2239444

Setting up real-time reaction:
1. Dilute cDNA 1:9 (if you normally use 1 ul per reaction).
2. Make primer master mix in 1.5 mL eppendorf tubes:

	x1 reaction	**x # of samples per primer +1 extra**
Sybr green	10uL	calculate
Primer (10uM)	1uL	calculate

3. Make cDNA triplicate mix in PCR strip tubes:

	x1 reaction	**x3.5 (for triplicate)**
Primer master mix	11uL	38.5uL
diluted cDNA	9uL	31.5uL

4. For each sample, combine the primer master mix and cDNA as shown above, mix well and spin down.

5. Add 20uL of this real-time reaction mix to each well of the 96 well plate.
6. Spin down the plate and make sure there are no bubbles present.
7. Place on ice and bring over to the BioRad iCycler in the Kaiser lab.

Notes:

- For procedures above, reaction tubes should be placed on ice as much as possible. If you are not using the whole 96-well plate, it can be cut into pieces.

Refer to icycler manual for use: http://pmgf.osu.edu/documents/biorad_iq_manual.pdf

Real-time PCR tutorial: http://pathmicro.med.sc.edu/pcr/realtime-home.htm

Quality control steps:

- In a real time PCR assay a positive reaction is detected by accumulation of a fluorescent signal. The Ct (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceeds background level).
- Ct values < 29 are strong positive reactions indicative of abundant target nucleic acid in the sample, Cts of 30-37 are positive reactions indicative of moderate amounts of target nucleic acid, Cts of 38-40 are weak reactions indicative of minimal amounts of target nucleic acid which could represent an infection state or environmental contamination.
- Ct levels are inversely proportional to the amount of target nucleic acid in the sample (i.e. the lower the Ct level the greater the amount of target nucleic acid in the sample).
- Check the melting curve to be sure there is only one peak, if you see a smaller peak it could be primer dimers which would give you improper Ct readings.
- Ensure that the Ct values are in the linear range for both control (e.g. GAPDH) vs experimental primer sets. The control Ct values for all samples should be the same.
- When doing triplicates or duplicates for a sample, the Ct values should be no more than 0.6 Ct values apart.
- After the reaction run it on an agarose gel to make sure the size of the band you are getting is correct.

Primer design tips:

- Select the probe first and design the primers as close as possible to the probe without overlapping it. Amplicons of 50 to 150 bp are strongly recommended. If absolutely necessary product size could be increased up to 200-250bp at most.
- Keep primer/probe GC content within 30-80%.
- Avoid runs of identical nucleotides, this is especially true for guanine, where runs of four or more Gs should be avoided.
- Tm should be within 58°C to 60°C.
- The last 5 bases at the 3 prime end should have no more than two G's or C's.

- Keep the annealing tempurates of the primers as close as possible.
- Select primer pairs with minimal number of potential primer dimers and primer hairpins as possible.
- BLAST the primer sequence to be sure that they are not binding to unwanted targets.

Analysis:
http://www.biotech.illinois.edu/centers/Keck/Functional_genomics/taqman/Guide%20to%20relative%20quantitation.pdf

Comparative C_t Method (this is better since you don't have to set up extra samples to make a standard curve):
This involves comparing the C_t values of the samples of interest with a control or calibrator such as a non-treated sample or RNA from normal tissue. The C_t values of both the calibrator and the samples of interest are normalized to an appropriate endogenous housekeeping gene.

The comparative C_t method is also known as the $2^{-[delta][delta]Ct}$ method, where

$[delta][delta]C_t = [delta]C_{t,sample} - [delta]C_{t,reference}$

Here, $[delta]C_{T,sample}$ is the C_t value for any sample normalized to the endogenous housekeeping gene and $[delta]C_{t, reference}$ is the C_t value for the calibrator also normalized to the endogenous housekeeping gene.

For the $[delta][delta]C_t$ calculation to be valid, the amplification efficiencies of the target and the endogenous reference must be approximately equal. This can be established by looking at how $[delta]C_t$ varies with template dilution. If the plot of cDNA dilution versus delta C_t is close to zero, it implies that the efficiencies of the target and housekeeping genes are very similar. If a housekeeping gene cannot be found whose amplification efficiency is similar to the target, then the standard curve method is preferred.