Bauer Core Standard Protocol		
Title: Guidelines for Designing Real Time PCR Experiments		
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1. Purpose

This document is designed to provide an introduction to real-time PCR. New users may use these guidelines for the many steps in designing their experiments (platform selection, primer design, template preparation, etc.). Instructions for operating the Bauer Core's Real time PCR instruments (the MJ Opticon 2 and Stratagene MX3000P (MJ Research) can be found in separate protocols.

2. Materials

Template (DNA or RNA depending on the type of reaction)
Primers
Probe (for TaqMan or Molecular Beacons assays)
QPCR mix (homemade or commercial e.g. Qiagen # 204143)
For homemade use SYBR Green Dye (Molecular Probes # S-7563)
Low Profile Opaque PCR Plates (e.g. MJ Research # MLL-9651 or # HSP-9655)
Optical Caps (MJ Research # TCS-0803)

3. Instrumentation

DNA Engine Opticon (MJ Research)
Centrifuge with plate/slide adapter (e.g. Sorvall Legend RT model)

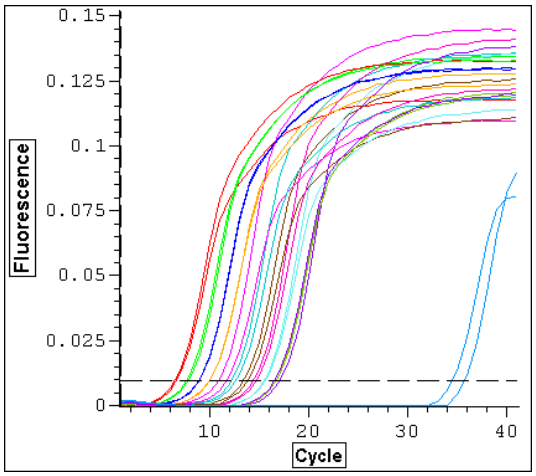
4. Reagent preparation

None.

5. Procedure

5.1 The Basic Theory Behind Real Time PCR.

All real time PCR chemistries operate using the same basic principle: a fluorescent dye is included in the PCR reaction mix that allows for the detection of the PCR product at each cycle of the reaction. As the reaction proceeds through each cycle, more and more product is formed and is detected by the fluorescent signal.
A plot can be made of fluorescent intensity over the cycle number, which will look something like this:



The dotted line in the graph represents the "threshold" above which the product of a reaction is deemed to be significant. The Cycle-Threshold (C_T) is the numeric value for the cycle at which the product crossed the threshold. Reactions with lower C_T values contain more of the gene of interest since they took less time to amplify. In the same way, samples with a higher C_T contain less of the gene of interest.

5.2. Real-Time PCR Chemistries.

5.2.1. SYBR Green is the simplest and cheapest chemistry. This dye binds to the minor groove of double stranded DNA and fluoresces 1000 times brighter when bound than when unbound. As the PCR generates more double stranded product, the SYBR Green signal increases. Many vendors sell SYBR Green master mixes which contain the dye, buffer, dNTPs and enzyme need for the real time reaction. You can make a home made mix yourself using SYBR Green Dye from Molecular Probes. The disadvantage to the SYBR Green system is that it is non-specific. Primer dimers and non-specific PCR products will give a signal identical to that of the real product. A melting curve can be preformed to determine the composition of the reaction product, but will not correct the results. Good primer design with PCR optimization is essential. Finally, genes that are expressed in very small numbers can be hard to detect with SYBR Green experiment since it can be hard to overcome the primer dimer problem. Many people start with SYBR green, but move on to one of the other chemistries for their "difficult" genes.

5.2.2. TaqMan (5'-3' exonuclease assays) is one of these other chemistries. In this system, a probe specific for each gene is added to the PCR mix. Attached to the probe are a reporter dye and a quencher dye. The reporter will not fluoresce as long as it is on the probe with the quencher dye. As the polymerase extends the primer and approaches the probe, it uses its 5'-3' exonuclease activity to cleaves off the reporter. This allows the reporter to fluoresce. The probe gives the TaqMan assay another degree of specificity. The disadvantage to this system is that the cost of the probes makes it more expensive than the SYBR green. Also, it requires a bit more effort since one must design a probe for each gene as well as the primers.

5.2.3. Molecular Beacons provides yet another chemistry. In this case, a reporter and quencher are kept in close proximity by a hairpin loop, allowing FRET to occur. When the probe binds to the specific sequence in the gene-of-interest, the hairpin loop comes apart, increasing the distance between the reporter and quencher and allowing the reporter to fluoresce without FRET.

5.3. Template

Real Time PCR uses a Taq DNA polymerase to amplify a DNA template. Often, however, people want to use real time PCR to measure the amount of RNA in a sample (as for gene expression studies). In this case, the RNA template is transcribed into DNA before the real time PCR begins (this is real time RT-PCR). Both DNA and RNA templates should be pure, and this can be checked by measuring the $OD_{260}/OD2_{80}$ ratio of the sample using a spectrophotometer. Pure DNA and RNA have ratios of 1.8 to 2. RNA samples should also be free of contaminating genomic DNA which can be removed using a DNAse enzyme (for instructions on how to do this, see the Bauer Center's DNAse Protocol). Note that DNAse reactions do not have to be performed if the primers have been designed to span an intron or cross and intron/exon boundary (see section 5.5 on primer design). RNA samples should also be undegraded. One way to check for RNA integrity is to run the samples on a gel or on an Agilent Bioanalyzer chip. The ratio of 28S to 18S is 2.0 in an undegraded sample.

5.4. One-Step vs. Two-Step Reactions.

5.4.1. One Step reactions include an RT enzyme in the same tube as the RNA template, the polymerase enzyme and the detection dyes. A reverse transcription step is performed before the PCR cycling begins. This option is useful for people with limited amounts of RNA samples. If a person only has enough RNA to do one reaction per sample, this is a good way of simplifying the process.

5.4.2. In a two-step reaction, an RT is performed prior to the amplification, and a portion of the resulting cDNA is used as a template for the real time PCR. For most applications, a two-step reaction is preferred since it generally produces higher yields and is more reliable than a one-step. Often, people will make a fairly large batch of cDNA which they can then use as the template of several different real-time reactions. In this way, the behavior of several genes can be assessed using the same batch of cDNA.

5.5. Primer Design

Designing primers is one of the most important steps of a real-time PCR experiment. Several software packages are available to help with the design including Primer3, a free system from MIT that can be found at this link: http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi. There are several considerations when designing real-time PCR primers. The product should be short (for TaqMan, about 100bp, for SYBR Green 200 to 300 bp is ideal), the two primers should have similar T_ms (ideally within 0.5^oC of each other but no more than $1\ ^oC$ apart), and the primers should have low or no self complementarity (to avoid primer dimers). In addition, many people try to design primers that span introns or cross intron/exon boundaries. In this way, only cDNA from mRNA gene transcripts will be amplified, not genomic copies of the gene.

5.6. Controls

5.6.1. No Template Controls (NTCs) should be included on every plate for every primer set to check for contamination. These wells, which include primers and mastermix but no template, should show little or no amplification. If the NTC well shows amplification with a C_T lower than 38, steps should be taken to reduce contamination. These measures can include using dedicated pipettors and bench space, or setting up reactions in a hood that is sterilized by UV.

5.6.2. –RT reactions should be performed to check for genomic DNA contamination. Every sample that was reverse transcribed should also be run in a mock reverse transcription reaction that contains no RT enzyme. In this way, the only DNA template in that sample would be from contaminating gDNA. If subsequent real-time PCR detects a signal from these –RT samples (a C_T above 35), the samples should be treated more thoroughly with DNAse prior to the RT. The one exception to this rule is reactions for which the primers were designed to span an intron or fall over an intron/exon boundary. Since these primers will not amplify gDNA, it is not necessary to perform a –RT control

5.6.3. Housekeeping Genes are often used to control for variability in the efficiency of reverse transcription reactions. The housekeeping genes (HKGs) are genes whose expression levels do not change in the conditions being studied. Any variation in expression of the HKGs is attributed to experimental variation and is compensated for during normalization. It is important to verify that the levels of the HKG gene truly are static in the conditions studied, since fluctuating HKGs will throw off subsequent analysis.

5.6.4. Some users performing relative quantification choose a calibrator sample against which they make all their relative comparisons. Examples of a calibrator include untreated samples in a drug treatment study, time 0 in a time course, or a pool of many tissues in tissue specific expression studies.

5.6.5. Finally, it is important to run every reaction (controls as well as unknowns) in at least duplicate to control for pipetting error.

5.7. Quantification Methods:

5.7.1. Absolute quantification uses a carefully measured standard curve to assign quantities to each unknown. The standard must be made from a pure DNA or RNA sample of known quantity. Accurate pipetting is important, as is the stability of the DNA or RNA sample. Results are often reported as “molecules per mg of tissue” or “copies per 1000 cells”.

5.7.2. Relative quantification compares the expression level of two samples. ABI's "user bulletin #2" gives an explanation of how to do this using a relative standard curve or by using the “ΔΔCt method”. Follow this link for instructions: http://groups.yahoo.com/group/qpcrlistserver/files/ABI%20User%20Bulletin%202.pdf.

5.7.3. Standard Curves should be run at least once for every primer pair (regardless of the type of quantification being performed) in order to check the efficiency of the PCR reaction. A graph is made of the standard curve by plotting the Ct values on the y axis and the log of the input amounts on the x axis. The slope of the line of this plot will give the efficiency of the reaction according to the equation E=10^-(1/slope) – 1. A reaction of 100% efficiency will have a slope of –3.33. Usually people will accept values

of –3.33 +/- 0.3 (or those with greater than 90% efficiency), but what is most important is that the slopes of all the genes being studied show similar efficiencies.

5.7.4. The Bauer Center's MJ Opticon plots the standard curve inversely to the convention by default, with the Ct values on the x axis and the log of the input quantity on the y axis. For this reason, a perfect slope given by the Opticon graph is the inverse conventional -3.33 which is -0.3). The equation for finding the efficiency of the reaction on the MJ Opticon is E=10^-(slope) – 1. The software does include a toggle button to plot the curve in the conventional manner.

5.7.5. A relative standard curve can be run using several different template sources. Often, users will choose to make a dilution series of an abundant sample that they can aliquot store so that they always use the same material for standard curves. If samples are hard to generate, another option is to perform a PCR using primers outside the primers that will be used for the real time reaction. This PCR product can be diluted and used as a standard curve template. One should not use the real-time primers to generate a standard curve template because some bases may be lost off either end of the product and this could impact the reaction efficiency. Finally for short TaqMan products, it is possible to order an oligo of the desired sequence to use as a standard curve template.

Protocol for Real-Time RT-PCR

This protocol describes the detailed experimental procedure for real-time RT-PCR using SYBR Green I as mentioned in Xiaowei Wang and Brian Seed (2003) A PCR primer bank for quantitative gene expression analysis. Nucleic Acids Research 31(24): e154; pp.1-8. Please refer to this paper and the PrimerBank Help page for more background information. The procedure begins with reverse transcription of total RNA. The cDNA is then used as template for real-time PCR with gene specific primers. You may need to modify this protocol if you use different reagents or instruments for real-time PCR.

Time required

cDNA synthesis: 2 hours.
real-time PCR: 2 hours.
Dissociation curve analysis: 0.5 hour.

Reagents and Equipments

- Oligonucleotide Primers. Gene specific primers are retrieved from PrimerBank (http://pga.mgh.harvard.edu/primerbank/). These primers are ordered from the MGH DNA Core facility (https://dnacore.mgh.harvard.edu/synthesis/index.shtml). All the primers are desalted and both UV absorbance and capillary electrophoresis are used to assess the quality of primer synthesis.
- Mouse total liver RNA (Stratagene).
- Mouse total RNA master panel (BD Biosciences / Clontech).
- SYBR Green PCR master mix, 200 reactions (Applied Biosystems).
- Optical tube and cap strips (Applied Biosystems).
- SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen).
- 25 bp DNA ladder (Invitrogen).
- ABI Prism 7000 Sequence Detection System (Applied Biosystems).
- ABI Prism 7000 SDS software (Applied Biosystems).
- 3% ReadyAgarose Precast Gel (Bio-Rad).
- Agarose gel electrophoresis apparatus (Bio-Rad).

Detailed procedure

Reverse Transcription

Reverse Transcription is carried out with the SuperScript First-Strand Synthesis System for RT-PCR. The following procedure is based on Invitrogen's protocol.

1. Prepare the following RNA/primer mixture in each tube:

Total RNA	5 μg
random hexamers (50 ng/μl)	3 μl
10 mM dNTP mix	1 μl
DEPC H_2O	to 10 μl

2. Incubate the samples at 65°C for 5 min and then on ice for at least 1 min.
3. Prepare reaction master mixture. For each reaction:

10x RT buffer	2 μl
25 mM $MgCl_2$	4 μl
0.1 M DTT	2 μl
RNAaseOUT	1 μl

4. Add the reaction mixture to the RNA/primer mixture, mix briefly, and then place at room temperature for 2 min.
5. Add 1 μl (50 units) of SuperScript II RT to each tube, mix and incubate at 25°C for 10 min.
6. Incubate the tubes at 42°C for 50 min, heat inactivate at 70°C for 15 min, and then chill on ice.
7. Add 1 μl RNase H and incubate at 37°C for 20 min.
8. Store the 1st strand cDNA at -20°C until use for real-time PCR.

Real-time PCR

1. Normalize the primer concentrations and mix gene-specific forward and reverse primer pair. Each primer (forward or reverse) concentration in the mixture is 5 pmol/μl.
2. Set up the experiment and the following PCR program on ABI Prism SDS 7000. Do not click on the dissociation protocol if you want to check the PCR result by agarose gel. Save a copy of the setup file and delete all PCR cycles (used for later dissociation curve analysis). Please note the extension steps are slightly different from described in our paper.
    1. 50°C 2 min, 1 cycle
    2. 95°C 10 min, 1 cycle
    3. 95 °C 15 s -> 60 °C 30 s -> 72 °C 30 s, 40 cycles
    4. 72°C 10 min, 1 cycle

3. A real-time PCR reaction mixture can be either 50 μl or 25 μl. Prepare the following mixture in each optical tube.

25 μl SYBR Green Mix (2x)		12.5 μl SYBR Green Mix (2x)
0.5 μl liver cDNA		0.2 μl liver cDNA
2 μl primer pair mix (5 pmol/μl each primer)	OR	1 μl primer pair mix (5 pmol/μl each primer)
22.5 μl H_2O		11.3 μl H_2O

4. After PCR is finished, remove the tubes from the machine. The PCR specificity is examined by 3% agarose gel using 5 μl from each reaction.
5. Put the tubes back in SDS 7000 and perform dissociation curve analysis with the saved copy of the setup file.
6. Analyze the real-time PCR result with the SDS 7000 software. Check to see if there is any bimodal dissociation curve or abnormal amplification plot.

Troubleshooting

Here I listed a few major causes for real-time PCR failures. Please read the PrimerBank Help page for more details.

Little or no PCR product. Poor quality of PCR templates, primers, or reagents may lead to PCR failures. First, please include appropriate PCR controls to eliminate these possibilities. Some genes are expressed transiently or only in certain tissues. In our experience, this is the most likely cause for negative PCR results. Please read literature for the gene expression patterns. One caveat is that microarrays are not always reliable at measuring gene expressions. After switching to the appropriate templates, we obtained positive PCR results in contrast to the otherwise negative PCRs (see our paper for more details).

Poor PCR amplification efficiency. The accuracy of real-time PCR is highly dependent on PCR efficiency. A reasonable efficiency should be at least 80%. Poor primer quality is the leading cause for poor PCR efficiency. In this case, the PCR amplification curve usually reaches plateau early and the final fluorescence intensity is significantly lower than that of most other PCRs. This problem may be solved with re-synthesized primers.

Primer dimer. Primer dimer may be occasionally observed if the gene expression level is very low. If this is the case, increasing the template amount may help eliminate the primer dimer formation.

Multiple bands on gel or multiple peaks in the melting curve. Agarose gel electrophoresis or melting curve analysis may not always reliably measure PCR specificity. From our experience, bimodal melting curves are sometimes observed for long amplicons (> 200 bp) even when the PCRs are specific. The observed heterogeneity in melting temperature is due to internal sequence inhomogeneity (e.g. independently melting blocks of high and low GC content) rather than non-

specific amplicon. On the other hand, for short amplicons (< 150 bp) very weak (and fussy) bands migrating ahead of the major specific bands are sometimes observed on agarose gel. These weak bands are super-structured or single-stranded version of the specific amplicons in equilibrium state and therefore should be considered specific. Although gel electrophoresis or melting curve analysis alone may not be 100% reliable, the combination of both can always reveal PCR specificity in our experience.

Non-specific amplicons. Non-specific amplicons, identified by both gel electrophoresis and melting curve analysis, give misleading real-time PCR result. To avoid this problem, please make sure to perform hot-start PCR and use at least 60°C annealing temperature. We noticed not all hot-start Taq polymerases are equally efficient at suppressing polymerase activity during sample setup. The SYBR Green PCR master mix described here always gives us satisfactory results. If the non-specific amplicon is persistent, you have to choose a different primer pair for the gene of interest. You are also encouraged to report bad primers to Xiaowei Wang (xwang@molbio.mgh.harvard.edu).