REAL TIME RT-PCR WITH THE BIORAD ICYCLER AND APPLIED BIOSYSTEMS SYBR GREEN KIT

Primer Design with Beacon software:
Ahead of time: find target genes on Genbank and locate intron/exon junctions if there are any. Make sure to include primers for a positive control (such as 18S rRNA).

Tips: Use "Spidey" tool from NCBI to locate intron/exon junctions. Just type in accession numbers for mRNA and genomic sequences into the appropriate fields in the Spidey window, and the junctions will be given.

To perform BLAST search with primers, use the "search for short nearly exact matches" option. Type in the sequence for the forward primer, add a string of NNNNNNNNNN to separate the primers, and then type in the sequence of the reverse primer, with no spaces inbetween.

1. Open Beacon Designer software
2. Import sequence from Entrez: *File>Open>Sequence>From Entrez*
   Enter accession number (can enter multiple numbers separated by commas)
3. Open the first sequence
4. Select *Analyze>Primer search*
5. Set the primer parameters- set the search range approx. 75 bp before and 75 bp after intron/exon junction if desired. The product size should be between 75 and 150 bp.
6. Click "*search*"
7. Record sequence, location, and optimal annealing temperatures of desired primers.
8. Verify specificity of primers by Blast search, order primers.

RNA preparation

1. Prepare RNA by desired method (eg Trizol, Qiagen spin column, etc), resuspend in 100 µl *RNAsecure* (Ambion) and quantitate
2. If primers do not surround intron/exon boundaries, the RNA must be DNAse-treated: treat 20 µl of RNA sample with *DNAfree* (Ambion) two times

Reverse Transcription reaction with Applied BioSystems RT-PCR kit

RNase-free dH_20 with 2 mg RNA	38.5 µl

10X Taqman RT buffer	10 µl	
25 mM $MgCl_2$		22 µl
dNTP mix	20 µl	
Random hexamers	5 µl	
Rnase inhibitor		2 µl
MultiScribe		2.5 µl
		100 µl total

(divide into 2 PCR tubes if necessary)

RT program on thermocycler: 25°C/ 10 min
48°C/ 30 min
95°C/ 5 min

store cDNA at –80°C until use in PCR reactions

PCR reaction setup with Applied Biosystems SYBR green PCR kit
(note: program iCycler with desired temperature cycling before setting up reaction. Follow procedures in iCycler instruction manual.)

PCR Master Mix		12.5 µl
5 µM forward primer	1.5 µl	
5 µM reverse primer	1.5 µl	
cDNA Template		X µl
dH_20		to 20 µl total
		20 µl

Template amount varies depending on expected abundance of message
5 µl is a good starting point
1 µl is sufficient for the 18S control
aliquot into 96 well PCR plate

Notes:
reactions should all be in triplicate
include water only blank
include plasmid dilutions of cloned gene for standard curve if possible

for standard curve,
perform PCR on 1 ng, 0.1 ng, 0.01 ng, 1 pg, 0.1 pg, 0.01 pg, 1 fg of total plasmid DNA of cloned gene

BioRad iCycler operation

1. perform radiation check
2. start computer (login: user=keck user, password=keck)
3. log on to terminal
4. switch on instrument: first, turn on base unit. Next, turn on optical unit.
5. put sealed plate in iCycler
6. run PCR/melt curve program of choice

example of PCR cycling:

95°C/10 min

95°C/15 sec
annealing temp/1 min 40 cycles

link to melt curve program if desired

Data analysis:

Quantitation of product:

The cycle threshold (Ct) number is an arbitrary number of PCR cycles in which all of the PCR amplification graphs you are comparing are in the linear range. The software will pick one for you or you can pick one yourself. The Ct value is an exponent (2^{Ct}). Remember a lower Ct value means more transcript, and higher Ct value means less transcript.

To normalize to 18S, divide your gene by the 18S value

$$2^{Ct\ your\ gene} / 2^{Ct\ 18S}$$
$$= 2^{(Ct\ your\ gene\ -Ct\ 18S)}$$

For simplicity, you can just subtract the Ct values and leave it as a Ct value.

If you have done a standard curve, you can calculate how much starting mRNA you originally had in each sample.

Standard deviation calculation (adapted from online version of "ABI Prism 7700 Sequence detection system user bulletin number 2", page 15)

Follow the example below for induction of Hsp70 by heat shock:

	Hsp70	18S	□CT	□□CT	fold ↑

	Ave CT	Ave CT	Hsp70-18S[a]	□CT - □CT (no HS)[b]	rel to no HS[c]
No heat shock	30.49±0.15	23.63±0.09	6.86±0.17	0.0±0.17	1.0 (0.9 – 1.1)
Heat shock	27.03±0.06	22.66±0.08	4.37±0.10	-2.50±0.10	5.6 (5.3 – 6.0)

a. the □CT value is determined by subtracting the average 18S Ct value from the average Hsp70 Ct value. The standard deviation of the difference is calculated from the standard deviations of the Hsp70 and 18S values using the following formula:

$s = \text{square root } [(s_{1})^2 + (s_{2})^2]$

where s = standard dev

for the "no heat shock" sample:

$s_1 = 0.15$

$s_2 = 0.09$

$s = \text{square root } [\ (0.15)^2 + (0.09)^2\ ]$

$= 0.17$

b. for □□CT, simply subtract the □CT's for the test sample vs. the control. The standard deviation remains the same as for the test value.

c. The fold increase is $2^{-\square\square CT}$. The range given is determined by performing the calculation twice, once using □□CT + S value, and once using the □□CT – S value, where S = the standard deviation of the □□CT value.

Melt curve:
view melt curve graphs for each primer set to verify the formation of only one PCR product

Shut down procedure:
Switch off optical unit, then base unit
Logoff on terminal
Recover your sample
Retrieve your data from the Keck computer
Shut down computer

Reagents:

RNA reagents
Trizol or Qiagen columns for RNA preparation

<u>Ambion:</u>		
RNAsecure 10 mL	7006	$100
DNAfree	1906	$58

RT-PCR reagents

Aplied Biosystems:		
SYBR green PCR kit	4310179	$600
	(includes RT-PCR kit and SYBR green kit)	
BioRad:		
PCR plates	223-9441	$147 per pack of 25
Sealing tape	223-9444	$100 per box of 100

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