Quantitative Reverse-Transcription PCR (q-RT-PCR)

Adapted from Applied Biosystems protocol

Reagents

Trizol Reagent (Invitrogen #15596-026) DNase I, amplification grade (Invitrogen # 18068-015) High Capacity cDNA Reverse Transcription Kit (Applied Biosystems # 4368814) RNase Inhibitors (Applied Biosystems # N808-0119) Power SYBR Green Master Mix (Applied Biosystems # 4368577) 96-well or 384-well optically clear plate Optical plate cover DEPC-Treated H₂O Add 1 mL of 0.1% Diethylpyrocarbonate (DEPC) per 1 L H₂O Shake well and incubate overnight with shaking at 37°C Autoclave and cool to room temp before use

Primer design with MacVector software

- Try to choose a primer pair that straddles an intron to avoid amplification of genomic sequnce
- Limit amplification size to 60-175 bp
- Limit GC content to 30-70%
- The five nucleotides at the 3' end should have no more than two G and/or C bases
- Limit Tm to 57-60°C
- Check primer pair to ensure no dimers will form
- Blast primer to ensure there are no sites in the genome that have 100% identity to 18 consecutive bases

Prepare RNA for reverse transcription

- Extract total RNA using Trizol reagent according to manufacturer's protocol. Make aliquots of RNA and store at -80°C
- DNase treat 1ug of experimental and control total RNA according to manufacturer's protocol
- To demonstrate 1 ug of experimental RNA is in linear range for RT protocol and to generate a standard curve to determine relative quantities, DNAse treat 0.1 ug, 0.5 ug, 1.0 ug, 1.5 ug, and 2.0 ug of control total RNA

Generate first-strand cDNA

- Use ABI High Capacity cDNA Reverse Transcription Kit according to manufacturer's protocol using six control samples and any experimental samples
- Store reactions at -20°C until ready to prepare qPCR reactions

Perform qPCR reactions

- Optimize qPCR reaction for individual primer sets. See ABI recommendations.
 - We have found that optimal primer concentration is generally 300 nM and 1/50 of RT reaction is an appropriate amount of template to amplify most targets.

- Use plate template to indicate the reaction that will occur in each well. See ABI recommendations.
- We generally do 25 ul reactions in each well of the 96-well plates and 10 ul reactions in each well of the 384-well plates Example of a general PCR reaction in a 96-well plate:
 - o 10.5 ul primer solution
 - o 2 ul template
 - o 12.5 ul SYBR master mix
 - * Always use aerosol pipet tips and pipet in the order shown above
 - * Primer solution contains forward and reverse primers in the appropriate concentration
 - * Assemble reactions on ice
- Quickly seal plate after pipetting is complete and protect from light
- Spin down reactions in plate prior to putting plate in qPCR machine
- We use an Applied Biosystems 7900HT with SDS 2.3
 - * The user MUST be trained before logging onto this piece of equipment
 - o Open SDS 2.3 software
 - Select wells that contain reactions
 - Add SYBR detector copy to plate doc
 - Add dissociation curve to end Dissociation curves are useful to detect nonspecific amplification and primer-dimers
 - o Default parameters are as follows:
 - 1. 50° x 2min
 - 2. 95° x 10min
 - 3. 95° x 15sec
 - 4. 60° x 1min (modify this if necessary for each primer set)
 - 5. repeat steps 3 and 4 for 40 cycles
 - 6. 95° x 15sec
 - 7. 60° x 15sec
 - 8. 95° x 15sec
- Analyze PCR data using manufacturer's software and if necessary run reactions on agarose gel to confirm single correct product