Two-step RT-PCR analysis

1. cDNA synthesis for use in RT-PCR

Protocol taken from: Quantitect Reverse Transcription Handbook 03/2009

Principle

In the reverse transcription (RT) step, cDNA is reverse transcribed from total RNA samples using random primers from the Quantitect cDNA Reverse Transcription Kit.

Protocol

Please refer to the protocol described in the QuantiTect Reverse Transcription Handbook: (http://www.qiagen.com/knowledge-and-support/resource-center/resource-download.aspx?id=f0de5533-3dd1-4835-8820-1f5c088dd800&lang=en)

Protocol Modification:

- In "Step 2" calculate your dilution to obtain 0.8 µg RNA concentration in all samples. If some samples contain really little RNA, adjust the end concentration of all samples accordingly by diluting the more to match the low RNA concentration.

- After "Step 7" (Incubation at 95°C to inactivate Quantiscript Reverse Transcriptase), the 20 µL cDNA solution is split into two 10 µL aliquots:
  - 10 µL is stored at -20°C
  - 10 µL is diluted 8x (add 70 µL RNase-free water). This is the solution which will be used for the second step of the PCR-analysis

2. PCR-Step

Principle

In the PCR step, PCR products are synthesized from cDNA samples using the Fast SYBR® Green Master Mix.

Materials

Applied Biosystems 7500 Fast Real-Time PCR System
MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode (PN 4346906)
Multi-step and multi-channel pipettes will greatly improve the plate preparation speed.
Only use DNase/RNase free microtubes and pipette tips.

Protocol

Please refer to the protocol described in the Fast SYBR® Green Master Mix Protocol: (http://tools.lifetechnologies.com/content/sfs/manuals/cms_046776.pdf)

Protocol Modifications:

- The SYBR Green master mix prepared is based on a 10 µL reaction volume:

5 µL	SYBR® Green
0.3 µL	Forward Primer
0.3 µL	Reverse Primer
1.9 µL	RNase / DNase free water

2.5 µL	cDNA template (or water for NTC)
Total: 10 µL	Reaction volume

Make a quantity Master mix (without cDNA tamplate) that's sufficient for all samples (+NTC and dilution series) for each specific gene.

- Prepare and dilute a mixed cDNA sample:
  - Randomly select 20 samples and mix an aliquot of 4 µL of each of them to obtain 80 µL of mixed sample
  - Make a dilution series by taking 20 µL, and adding 60 µL of RNase / DNase free water. Mix well and repeat the process 4 more times.
  - This will yield a dilution series of: 1; 1/4; 1/16; 1/64; 1/256 and 1/1024
  - Run this dilution series with each tested gene.

- Only one gene per plate to enable inter - sample comparison.
- Each plate has a No Template Control (NTC) sample
- Perform a melting curve at the end of the amplification cycles.
- Include at least 4 household genes in your analysis.

Data Analysis:

Refer to the following documents for more information about analyzing your data. You can download the documents from: http://docs.appliedbiosystems.com/search.taf
If using a 7500 Fast RT PCR:
• Applied Biosystems 7300/7500/7500 Fast Real-Time PCR Systems Relative Quantification Getting Started Guide (PN 4347824)
• Applied Biosystems 7300/7500/7500 Fast Real-Time PCR Systems Absolute Quantification Getting Started Guide (PN 4347825)

- Before exporting the data, adjust the baseline and threshold according to the documents referred above. Check the NTC fluorescence levels and ensure that there is no significant amplification. Check the melt curves: if more than one peak is visible, the quality of the end products is not OK and data should not be used for gene expression calculations.
- Calculate the amplification efficiencies of the primers based on the dilution of the mixed cDNA samples according to Wong and Medrano (2005):
  Formula: $(10^{-1/slope}-1)*100$
  Accept the primer efficiencies when they are greater than 85%.
- Use multiple reference genes for data normalization. First, calculate the $2^{-\Delta\Delta Ct}$ values of the reference genes (Livak et al., 2001). Based on the $2^{-\Delta\Delta Ct}$ values, determine the expression stability of the reference genes by geNorm (Vandesompele et al., 2002). The M values should be <1, the V value should be <0.15.
- For the accepted reference genes, calculate the normalization factor (NF) for each sample:
  $(2^{-\Delta\Delta Ct}_{gene1}*2^{-\Delta\Delta Ct}_{gene2}*\ldots*2^{-\Delta\Delta Ct}_{geneX})^{1/X}$
- For the genes of interest: first calculate the primer efficiencies based on the dilution of the mixed cDNA sample.

- Calculate the $2^{-\Delta\Delta Ct}$ for each sample. Divide this value by the NF for the respective sample. Based on those values, the average expression per treatment can be calculated.

References:

Wong ML, Medrano JF. "Real-time PCR for mRNA quantification" BioTechniques 39:75-85 (July 2005).

Kenneth J. Livak and Thomas D. Schmittgen. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta Ct}$ Method. doi:10.1006/meth.2001.1262, available online at http://www.idealibrary.com

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 2002 Jun 18;3(7):RESEARCH0034. Epub 2002 Jun 18.