

QUANTITATIVE RT-PCR PROTOCOL (SYBR Green I)

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REVERSE TRANSCRIPTION

1. Add 1 ng in vitro transcribed RNA (human gene H2, GeneBank Accession #: [AA418251](#)) to the RNA sample (500 ng – 1000 ng)
2. Add DEPC H₂O to the RNA sample to 29.5 μ l
3. Add 0.5 μ l 1 μ g/ μ l random hexamer and 1 μ g/ μ l poly dT respectively
4. Incubate the mixture at 65°C for 10 min and then put on ice immediately for 5 min. Let it stand at room temperature for 10 min.
5. Add 18.5 μ l pre-mixture, which contains 2.5 μ l 10mM dNTP mix, 10 μ l 5 x 1st strand buffer, 5 μ l 0.1M DTT and 1 μ l RNase OUT.
6. Mix and spin, stand at room temperature for 2 min.
7. Add 1 μ l Superscript II RT and mix gently.
8. Spin down and let it stand at room temperature for 10 min.
9. Incubate at 42°C for 50 min, followed by heat inactivating at 70°C for 15 min.
10. Add 1 μ l RNase H, mix gently and spin down.
11. Block at 37°C for 30 min.

Take partial of the reaction products and dilute 100 times, for example, take 5 μ l and dilute to 500 μ l, to use as a template for the next steps. Store the rest 1st strand cDNA at -20°C.

PRIMER DESIGN CRITERIA

- (1) T_m: 58°C to 61°C, not bigger difference than 2°C in the primer pair
- (2) Primer lengths of 19-24 bp
- (3) Guanine cytosine (GC) contents: 45-55%
- (4) PCR amplicon lengths: 100-200 bp
- (5) Flank intron if DNA is eliminated (or cover an exon-exon junction if DNA can not be eliminated totally)
- (6) All designed primers were blasted to MAGI/NCBI database to make sure they are specific to the target gene
- (7) Order primers with standard desalting purification.

PRELIMINARY PRIMER TEST

1. Dilute primers to 5 μ M
2. Do conventional PCR by using genomic DNA and cDNA as templates to test primers

PCR setup:

Template (genomic DNA/cDNA)	5 μ l
10 x PCR buffer	2.5 μ l
50 mM MgCl ₂	0.5 μ l
10 mM dNTP	0.5 μ l
5 μ M primer forward	2 μ l
5 μ M primer reverse	2 μ l
Taq polymerase	1 μ l
H ₂ O	11.5 μ l
TOTAL	25 μ l

PCR condition (change the condition if you have special requirement):

80°C	forever	
(To pre-warm the block for hot start, manually go to the next step)		
94°C	3 min	
94°C	30 sec	} 32 cycles
60°C	30 sec	
72°C	1 min, 30sec	
72°C	10 min	
4°C	forever	

3. Running 1% agarose gel and take gel picture
4. Select those primers, which generate single band with correct length for cDNA for further experiment.

| **QUANTITATIVE REAL-TIME PCR (qRT-PCR)**

| 1. Do qRT-PCR and test the selected primers

| (1) qRT-PCR set up: Do two reactions for each pair of primers by using cDNA and H₂O as templates separately. Use primer final concentration of 200nM. All procedures should be done on ice.

Template: cDNA	6.8µl	Template: H ₂ O	6.8µl
2 x SYBR Green mix	10µl	2 x SYBR Green mix	10µl
5µM primer Forward	0.8µl	5µM primer Forward	0.8µl
5µM primer Reverse	0.8µl	5µM primer Reverse	0.8µl
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H ₂ O	1.6µl	H ₂ O	1.6µl
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Total	20µl	Total	20µl

| (2) qRT-PCR conditions and instrument setup

- a. Open the Mx4000 program and set up. Define the reactions of cDNA templates as “Unknown” and H₂O templates as “NTC” (No Template Control).
- b. The default PCR condition is as below. Don’t change it for this experiment but need to select the button for dissociation curve.

50°C	2 min	1 cycle
95°C	10 min	1 cycle
95°C	15 sec	40 cycles
60°C	1 min	40 cycles

60°C: dissociation curve starting temperature

- c. Select dissociation curve panel. Save your setup before running (The program will save all fluorescence data during PCR automatically).
- d. Run qRT-PCR as soon as possible after set up.

(3) Running 3% agarose gel after [qRT-PCR](#)

(4) Analyze [qRT-PCR](#) data: Select those primers with one band in the gel picture and one peak in the dissociation curve for further experiments. At the same time, have a look at the Ct value to have a rough idea about how much RNA/cDNA is enough for the further experiments. If the Ct value is high (>30), it's better to increase the amount of template for further experiments.

2. Do [qRT-PCR](#) to obtain dilution curve and calculate PCR efficiency for each pair of primer

(1) Make 7 serial five-fold dilutions of cDNA (could be any cDNA sample, but it's better to use the mixture of the target samples to reduce the difference caused by templates). The cDNA amount units of the templates could be the dilution values 1, 0.2, 0.004, 0.0008, and so on.

(2) Set up PCR reactions. Do 3 duplicated reactions for each diluted templates and NTC.

(3) Each reaction set up is the same as above.

(4) [qRT-PCR](#) conditions and instrument setup are also the same as above.

(5) Data analysis by using Mx4000 software

a. Check dissociation curve to make sure that every reaction just has single peak

b. Check amplification curve to make sure there is no product formed for NTC (no contamination)

c. Set up proper threshold to get the Ct values in the range of exponential amplification (usually 0.3 works fine)

d. Export Ct values to Excel file (The default format is “.CSV”)

(6) Data analysis by using Excel

a. Input the dilution values and calculate log values for them in the exported Excel file of Ct values

b. Select the log dilution values and Ct data

- c. Draw an XY (scatter) plot on the work sheet with the log input amount as the X values and Ct as the Y values
- d. Open the insert menu and select trendline to plot a line through the data point and select linear regression
- e. Go to the Options page and select the boxes for display Equation on Chart and display R² value on chart. If the R² value is lower than 0.98, you need to redo the experiments or do not use the primers for further experiments or analysis
- f. Calculate E values according the following equation (E = PCR efficiency + 1)

$$E = 10^{-(1/slope)}$$

- g. Save the file as “.xls” file
3. Do qRT-PCR for the above primers by using the samples you want to compare as templates separately
 - (1) Make a certain diluted templates for cDNA samples according to previous results (Don't use original cDNA samples directly for qRT-PCR because the buffers left after RT reaction would affect the qRT-PCR results).
 - (2) Set up PCR reactions. Do at least 3 duplicated reactions for each sample of each pair of primers. Each reaction is set the same as above.
 - (3) “qRT-PCR conditions and instrument setup” are the same as above
 - (4) Data analysis by using Mx4000 software
 - a. Check dissociation curve to make sure that every reaction just has single peak
 - b. Set up proper threshold to get the Ct values in the range of exponential amplification (usually 0.3 works fine)
 - c. Export Ct values to Excel file (The default format is “.CSV”)

- d. Calculate the fold-change of each gene for the two samples and normalize them by using the spiking human gene as reference

$$Ratio = \frac{E_{TARGET}^{\Delta C_T(TARGET)(Control-sample)}}{E_{REF}^{\Delta C_T(REF)(control-sample)}}$$

Note: “control-sample” could be “sample 1-sample 2”.

- (5) Data analysis to determine the difference: If you have enough biological replicates for the real time PCR, consult Dr. Dan Nettleton for statistical analysis. If you don't, the following is a way to calculate the lower and upper limits of foldchanges. One way to determine the difference is (lower limit, upper limit) does not span 1.

$$\log ll(LOWER LIMIT) = \log Fc - 2.78 \times \sqrt{(\log E_{TARGET})^2 \times \left(\frac{SD_1^2}{3} + \frac{SD_2^2}{3}\right) + (\log E_{REF})^2 \times \left(\frac{SD_{REF1}^2}{3} + \frac{SD_{REF2}^2}{3}\right)}$$

$$\log ul(UPPER LIMIT) = \log Fc + 2.78 \times \sqrt{(\log E_{TARGET})^2 \times \left(\frac{SD_1^2}{3} + \frac{SD_2^2}{3}\right) + (\log E_{REF})^2 \times \left(\frac{SD_{REF1}^2}{3} + \frac{SD_{REF2}^2}{3}\right)}$$

MAIN REAGENTS AND EQUIPMENTS

- Oligonucleotide Primers. Gene specific primers are retrieved from Primer3. These primers are ordered from IDT. Primer purification: standard desalting.
- SuperScript First-Strand Synthesis System for RT (Invitrogen).
- SYBR Green PCR master mix (Applied Biosystems, cat log: 4309155).
- 96-well plates (Stratagene, cat.: 410021)
- 8-tube strip tubes (Stratagene, cat.: 410022)
- Optical strip caps (Stratagene, cat.: 410024)
- 3% Agarose Gel.
- Agarose gel electrophoresis apparatus.

TIPS AND NOTES

1. If using different instrument or fluorescent dye, the protocol needs to be modified.
2. Find an adjusted pipette and keep it for the qRT-PCR set up.
3. Eliminate bubble before running PCR because it will affect the final Ct results.
4. If the initial set of 200nM primer doesn't work, you can try to optimize the primer concentration.
5. Make mixtures for PCR setup to reduce the differences among reactions.
6. Before determining the reference gene, select several housekeeping genes and 18S rRNA to do some preliminary experiments.
7. Because the RNA I obtained is amplified RNA, I did the RT with random hexamer. If starting RNA is total RNA, it's better to use polydT primer and modify the procedures.
8. If the dissociation curve gives multiple peaks (even though some are very short or weak), don't use the primers for further experiments. Because it either means there is primer dimers or non-specific products. If it is only the primer dimer, then it may be fixed by optimizing primer concentration. If the curve contains very ambiguous peaks, it is also possible that there are non-specific products. To use these kinds of primers, it's better to do sequencing before further experiments. For example, there is more than one product by sequencing for the qRT-PCR amplicons, which has the dissociation curve as showed in the following figure.

