SOP: SYBR Green-based real-time RT-PCR

By

Richard Yu

Research fellow

Centre for Marine Environmental Research and Innovative Technology (MERIT)
Department of Biology and Chemistry
City University of Hong Kong
(Email: bhrichyu@cityu.edu.hk)

A. Development and assessment of assays

1. **Design of gene-specific primers**
    - **Primer3**
      http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi
      This is used for picking up primers from an input gene sequence. Below are the recommended settings for selecting real-time PCR primers:
        - "*Product size range*" = 80–150 bp
        - "*Primer size*" = Min: 18 Opt: 20 Max: 23
        - "Primer Tm" = Min: 58 Opt: 60 Max: 63
        - From the output primer list, select the primers which end with G/C at their 3′-ends or avoid the primers ended with several consecutive A/T at their 3′-ends.
    - **Oligoanalyzer**
      http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/Default.aspx
      This is used for more detailed analyses for your selected primers for undesirable structures and specificity (e.g. hairpin, self-dimer, hetero-dimer and BLAST check).

2. **Validation of primer efficiency and specificity by dilution curve construction**

- Prepare 4 concentrations of tissue cDNA template by serially diluting a standard RT reaction product (see Appendix 1) or plasmid DNA (10^4–10^7 copies/reaction) as follows:

Dilution	Recipe
A (1/5):	4.5 µl RT rnx product + 18 µl water
B (1/20):	6 µl Dilution A + 18 µl water
C (1/80):	6 µl Dilution B + 18 µl water
D (1/320):	6 µl Dilution C + 18 µl water

Add 5 µl of each concentration in triplicate to appropriate wells on a 96-well PCR plate.

	Triplicate		
Conc.	1	2	3
A	○	○	○
B	○	○	○
C	○	○	○
D	○	○	○

- Prepare a PCR master mix as follows:

	1 rnx.(µl)	Master mix 13 rnx. (µl)
2× iQ SYBR Green Supermix*	12.5	162.5
10 µM Forward primer	0.5	6.5
10 µM Reverse primer	0.5	6.5
Water	6.5	84.5
Diluted cDNA (A, B, C, or D)	5	(pre-add to well)
	Total = 25	

* For other PCR machines which require ROX for normalization (e.g. ABI 7500), use other ROX-containing supermixes (e.g. ABI SYBR Green Master Mix).

3. Mix 20 µl PCR master mix with each cDNA aliquot by gently pipetting up and down several times (Note: Try your best to minimize bubble formation).

4. Seal the 96-well plate with an optical tape (BioRad, Cat# 2239444 or equivalent). Spin down all droplets by centrifugation at 4,000 rpm for 2 min.

5. Warm up the iCycler system for 15–20 min before use. Use the following protocol for a general SYBR Green PCR assay:

Cycle 1:	**(1X)**	
Step 1:	95.0ºC	for 00:30
Cycle 2:	**(45X)**	
Step 1:	95.0ºC	for 00:20
Step 2:	60.0ºC	for 00:30
Step 3:	72.0ºC	for 00:30

Data collection and real-time analysis enabled.

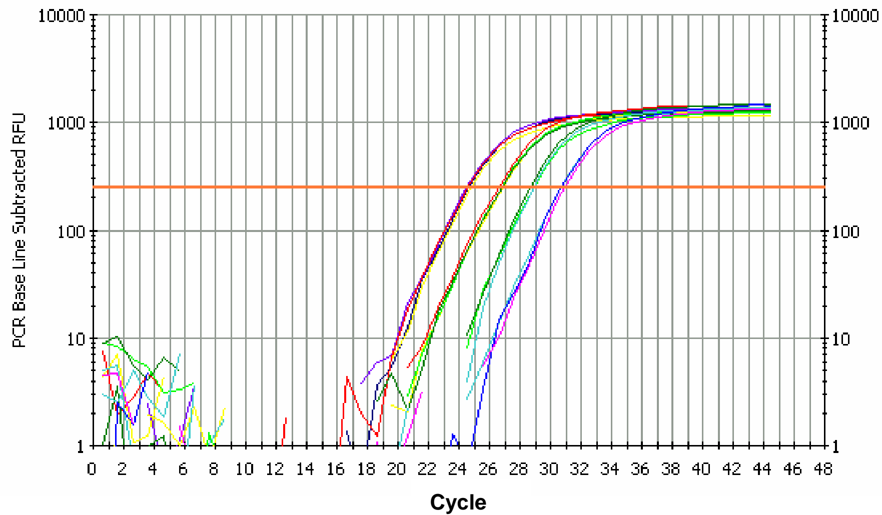
Melt curve analysis:

Cycle 3:	**(1X)**	
Step 1:	95.0ºC	for 01:00
Cycle 4:	**(1X)**	
Step 1:	55.0ºC	for 01:00
Cycle 5:	**(80X)**	
Step 1:	55.0ºC	for 00:10

Increase setpoint temperature after cycle 2 by 0.5ºC
Melt curve data collection and analysis enabled.

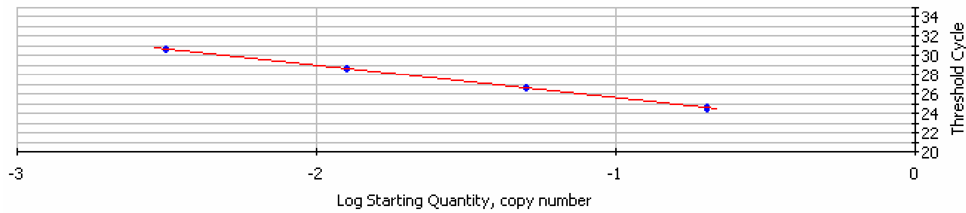
6. **Typical results of a successful assay:**

a. PCR amplification plot (PCR Base Line Subtracted)



Note: The threshold line should be placed within the **exponential increase phase** (which looks linear in the log transformation) of the amplification curve. Check from the plot that the curves DO NOT overlap with the **baseline range** (e.g. preset to be **2–10 cycles** in iCycler).

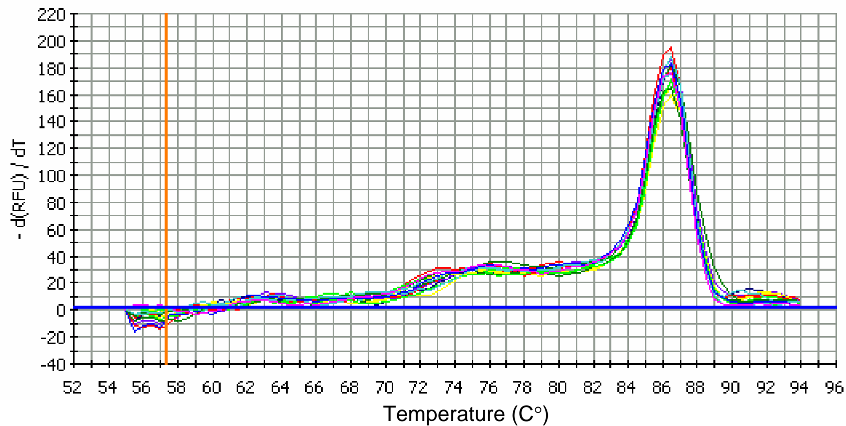
b. Standard curve (PCR Base Line Subtracted)



PCR efficiency and $\mathbf{R^2}$ are calculated automatically by the iCycler software from the dilution curve. Otherwise, use the following formula for the calculation of PCR efficiency in other real-time PCR systems:

$$\text{PCR efficiency} = (10^{-1/\text{slope}} - 1) \times 100\%$$

c. Melt-curve analysis



Specific amplification should be represented by a **single peak** emerged at about 80–90°C. Minor peaks at lower and higher temperatures implies the presence of primer dimers and larger non-specific PCR products, respectively.

7. **Optimization of PCR reaction**

A. *If PCR efficiency is less than 90% (i.e. low sensitivity of the assay):*
- ➢ Increase primer concentration from 200 nM to 400–600 nM; or
- ➢ Re-design primers

B. If more than 1 peak in the melt curve:
- ➢ Decrease primer concentration from 200 nM to 100 nM if primer dimer formation occurs; or
- ➢ Increase annealing temperature from 60°C to 62°C if other non-specific product form; or
- ➢ Re-design primers

C. *If target gene expression in the selected tissue is too low for dilution curve construction:*

- ➢ Use plasmid DNA containing the target gene insert as a template (concentration range: 10^6, 10^5, 10^4 and 10^3 copies/rxn) for dilution curve construction.
- ➢ Use the following formula for copy number calculation:
  No. of molecules/µl
  = (plasmid conc. in ng × 1.515 ÷ N_{bp}) × 6.023× 10^{11}
  where N_{bp} = size of vector + size of insert in bp

8. **Verification of amplification specificity**

- Agarose gel electrophoresis (there should be one band seen on the gel)
- Cloning of the PCR product for automated DNA sequencing
  Note: Don't clone PCR product generated from UDG-containing PCR supermixes because bacteria fail to recognize using uracil-containing template for DNA replication. If you use UDG-containing PCR supermixes, direct sequencing of your PCR product is the only choice.

B. Gene expression measurements

1. Dilute RT product (i.e. cDNA) by 10 times as follows:
   1.6 µl RT product + 14.4 µl H_2O

   If the expression of your target gene is low, dilute RT-product by 5 times. In general, 1/5 dilution is the highest concentration which you can input for PCR. Concentrations higher than 1/5 will not be linearly related to C_T as predicted from the dilution curve.

2. Add 5 µl of the diluted RT product in triplicate to the wells of a 96-well PCR plate.

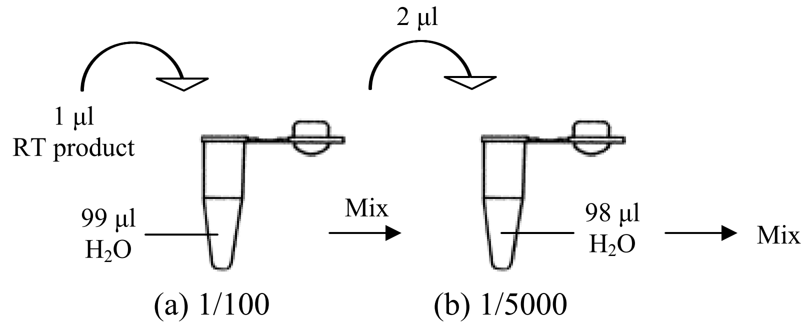
3. See above for other procedures and record the C_T values for samples (Use the same threshold for all samples).

C. 18S rRNA Normalization

1. Dilute RT products by 5000 times as follows:

   (a) Prepare a 1/100 dilution by adding 1 µl RT product (dispense using a P20 autopipette) to 99 µl water. Mix thoroughly by vortexing.

   (b) Transfer 2 µl of the diluted RT product to 98 µl water aliquoted in a new tube. Mix thoroughly by vortexing. This tube contains RT product at 1/5000 dilution.



2. Add 5 µl of the diluted products to the wells of a 96-well PCR plate.

3. Prepare a master mix as follows:

	1 rnx.(µl)	Master mix x+1 rnx. (µl)
iQ SYBR Green Supermix	12.5	
10 µM om18S-F	1	
10 µM om18S-R	1	
Water	5.5	
1/5000 diluted cDNA	5	(pre-add to well)
	Total = 25	

Oryzias melastigma-specific 18S rRNA primer:
om18S-F (5′-CCTGCGGCTTAATTTGACCC-3′)
om18S-R (5′-GACAAATCGCTCCACCAACT-3′)

4. Add 20 µl of master mix to each well. Mix by pipetting up and down for several times.

5. See above for other procedures and record the C_T values for samples (Use the same threshold for all samples).

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D. Calculation of gene expression

For each experimental sample, the expression level of a target gene was normalized to 18S rRNA, resulting in a mean normalized expression (MNE) value as derived from Equation (1):

$$\mathrm{MNE} = \frac{(\mathrm{E_{reference}})^{\mathrm{C_T}_{\mathrm{reference,mean}}}}{(\mathrm{E_{target}})^{\mathrm{C_T}_{\mathrm{target,mean}}}} \quad (1)$$

where E_{target} and $E_{reference}$ ($=10^{-1/slope}$) (Simon, 2003). Final data were calculated as mean normalized target gene expression relative to the control (Equation 2):

$$\text{N-fold change} = MNE_{exp}/MNE_{con} \qquad (2)$$

Reference

Simon, P., 2003. Q-Gene: processing quantitative real-time RT-PCR data. Bioinformatics 19, 1439–1440.

Appendix 1 First-strand cDNA synthesis by reverse transcription

A. DNase Digestion

1. Set up the DNase digestion as follows:

Total RNA	1 µg (in 8 µl H_2O)
10x Reaction buffer*	1 µl
DNase (1 U/µl)*	1 µl
Total:	10 µl

*provided in the RQ1 RNase-Free DNase kit (Promega, Cat.# M6101)

2. Incubate at 37°C for 30 min.

3. Add 1 µl Stop Solution to terminate the reaction.

4. Incubate at 65°C for 10 min to inactivate the DNase.

B. Reverse transcription

1. Add 3 µl random primer (40 ng/µl) to each tube containing the DNase digestion product.

2. Heat to 70°C for 5 min. Cool quickly on ice for 5 min.

3. Prepare a **"RT master mix"** as follows:

	1 reaction	Master mix *n*+1 reaction
5x Reaction buffer*	5	
10 mM dNTP	1.25	
RNaseOut (Invitrogen)	1	
Water	2.75	
M-MLV RT (H-)*	1	

*M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant (Promega, Cat.# M3683)

4. Add 11 µl RT master mix to each tube in (3). Pipette up and down to mix the contents (total vol. = 25 µl).

5. Incubate at room temperature for 10 min.

6. Incubate at 42°C for 50 min.

7. Inactivate the reaction at 70°C for 15 min.

8. Store at –20°C until use.

For more information, please go to my website, Molbio-Spot (http://www.geocities.com/richyuhk/) and see under PCR technology: Real-time PCR.