Quantificative RT-PCR (SYBR Green)

Jan-11-2008, Hirotaka Watanabe

Background

As a quantitative PCR, we usually use SYBR Green I dye, a double-stranded DNA binding dye, to detect PCR products as they accumulate during PCR cycles. CND department utilizes '7500 Fast Real-Time PCR System' as real-time PCR machine. You have to use particular consumable supplies, so that the machine correctly works. This machine is in HIM610 (behind the Invitrogen supply shelf).

Materials

- ✓ SYBR Green PCR Master Mix, Applied Biosystems cat# 4309155, 1-Pack (5 mL), $340.00
- ✓ Fast SYBR Green Master Mix, Applied Biosystems cat# 4385612, 1-Pack (5 mL), $374.00
- ✓ MicroAmp Fast Optical 96-Well Reaction Plate, Applied Biosystems cat# 4346906, 20plates, $70.00
- ✓ MicroAmp Optical Adhesive Film, Applied Biosystems cat# 4360954, 25covers, $55.00

Reservation

Because real-time PCR machine is always busy, you need to book your convenient date via web site. Web address is as follows. Both "Sign On ID" and "Password" will be informed if you need.

http://www.localendar.com/elsie

Procedure

Primer design & preparation

Before starting an experiment, you should check whether newly designed primer pair does work in conventional PCR. Especially, be careful not to generate primer dimer band (below 100bp). This could lead to misunderstanding due to non-specific amplification. Simple tips to design primer pair for real-time PCR are as follows.

- Primer could be 18- 24 bp in length
- Primer could be 58- 62C in Tm
- Five nucleotides at 3'-end should have no more than 2 G or C
- Preferable size of amplification is 50- 200 bp

For each primer pair, make a stock solution by mixing forward and reverse primer at concentration 2.5 µM each in dH_2O. Use 2 µL per reaction tube (final concentration would be 250 nM). If primers tend to form dimers, reduce primer concentration to 1.5 µM each (final concentration: 150 nM). In the case of 18S-RNA (Ambion cat# 1717: Classic II 18S internal

standards), dilute to yield 1 µM stock (final concentration: 100 nM).

Template cDNA synthesis

You can use random hexamer as cDNA-synthesizing primer, but an oligo(dT) primer might be preferable because this could synthesize cDNAs selectively from poly(A) RNA, approximately 1-2% amount of whole RNA species.

1. Treat total RNA with DNase I at RT for 15 min

1 µg total RNA in dH_2O	8 µL
10x DNase buffer	1 µL
DNase I (Invitrogen Cat# 18068-015)	1 µL

2. Add 1 µL of 25 mM EDTA and stop the reaction at 65C for 10 min
3. Add the followings and incubate at 65C for 5 min, thereafter chill on ice

Primer (oligo(dT) or random hexamer)	1 µL
10 mM dNTP (Invitrogen Cat# 18427-013)	1 µL

4. Add the followings and incubate at 50C for 60 min (DNA oven is desirable)
   In the case of random hexamer, synthesis should be done following 10 min incubation at 25C

5x First-strand buffer	4 µL
0.1 M DTT	1 µL
RNaseOUT (Invitrogen Cat# 10777-019)	1 µL
Superscript III (Invitrogen Cat# 18080-093)	1 µL

5. Stop the reaction at 70C for 15 min
6. Add 500 µL of dH_2O to dilute cDNA (final cDNA from corresponding 2 ng RNA amount/µL)

Run real-time PCR

To decrease non-uniformity among tubes containing same template, make working mixtures of SYBR green mastermix and cDNA beforehand (e.g. 100 µL + 80 µL for 10 tubes). Aliquot 18 µL to each well and add 2 µL of primers stock. For normalization, in some of the tubes add 2 µL of 1 µM 18S-RNA primers for 'endogenous control', instead of gene-specific primers.

1. Mix the following reagents. Please be careful that maximum volume of each reaction in "Fast Plate" should be 20 µL, unlike previous protocol.

2xSYBR Green Master Mix	10 µL
cDNA template	8 µL
Primer mix stock	2 µL

2. Put each component into Fast Optical 96-Well Plate, and seal with Adhesive Film. Spin down briefly to ensure the reaction solution at the bottom of each tube. Set the plate to the equipment

correctly. *You can store the plate up to several hours in 4C refrigerator before the run.*

3. Open the program (7500Fast System SDS Software) → To create a new plate document, File > New
4. Select [Assay] "Relative Quantification (ddCt) Plate", and [Run Mode] "Fast 7500" or "Standard 7500". Accept the default settings for [Container] and [Template]. Click Next >
   You should choose the Run Mode according to the product you bought (that is to say, cat# 4309155 or 4385612).
5. Select detectors and click Add >>, and click Next >
6. Specify the detectors and tasks for each well, and click Finish.
7. Using the Well Inspector, enter the sample names per well.
8. To specify thermal cycling conditions, select the Instrumental tab, and verify the cycling condition as follows: 95°C for 10 min and 40 cycles of 95°C for 15 sec and 60°C for 1 min for "Standard 7500", and 95°C for 20 sec and 40 cycles of 95°C for 3 sec and 60°C for 30 sec for "Fast 7500".
9. Save the RQ Plate document in your desired file.
10. Click Start. You had better stay around the machine until confirm an onset of PCR run. *The reaction of Fast 7500 will finish within 1 hour, whereas that of Standard takes about 2 hours.*
11. Analyze the data.
12. Data acquisition
    Select [File- Export- Result], and check out 2 boxes to export the data.