

# Quantitative Real-Time PCR (qPCR) Using DNA Engine Opticon II

(Originated by Xiaoji Luo/Yang Bi/Ni Tang; updated by Fang Deng @ 12/11/2013; commented by TCH)

## Materials and Supplies

- 1) **DyNAmo SYBR Green qPCR Mix** (2x, Cat.#: Thermo **F-400**, from Thermo Fisher; 100 $\mu$ l aliquots kept at -20°C freezer)
- 2) **DyNAmo Buffer** (2x stock, homemade, from 10x DyNAzyme Optimized buffer, Cat# **F-511S**, from Thermo Fisher, 300 $\mu$ l aliquots kept at -20°C freezer)
- 3) **qPCR low-profile tubes** (white tubes with clear flat lids; **Cat #: 249970**, from Research Products International Corp; or DOT Scientific, **Cat# 400LP-8PCR-W**)
- 4) **pUC19 DNA Standards**: #1=5.0pg/reaction, #2=1.0pg/ reaction, #3=0.2pg/reaction, #4=0.04pg/reaction, #5=0.008pg/reaction (aliquots kept at -20°C)
- 5) **pUC19 Standard qPCR primers**: **qPCR1857** and **qPCR1858**

## Preparation of RT cDNA Templates

For average abundant transcript: original RT cDNA reaction =20 $\mu$ l, add ddH<sub>2</sub>O→**100 $\mu$ l stock** (kept at -20°C or -80°C) →**1:5 to 1:50 dilutions**, use **2.5 $\mu$ l per reaction**.

## Setting up Real-Time PCR Reactions

DyNAmo SYBR Green qPCR mix	<b>2.0 <math>\mu</math>l</b>	} premix to make cocktail: <b>5.0<math>\mu</math>l/reaction</b>
DyNAmo Buffer (Homemade)	<b>3.0 <math>\mu</math>l</b>	
Primer #1 (20ng/ $\mu$ l)	1.25 $\mu$ l	} premix to make cocktail: <b>2.5<math>\mu</math>l/reaction</b>
Primer #2 (20ng/ $\mu$ l)	1.25 $\mu$ l	
<u>cDNA template (see above)</u>	<u>2.5 <math>\mu</math>l</u>	
<b>[Total volume</b>	<b>10.0 <math>\mu</math>l]</b>	

Add 10-20 $\mu$ l mineral oil (using the individually wrapped fine-tip plastic Pasteur pipettes)

Briefly spin down

## Typical Cycling Program

1. Incubate at 95.0°C for 00:05:00
2. Incubate at 94.0°C for 00:00:05
3. Incubate at 55.0°C for 00:00:10
4. Incubate at 72.0°C for 00:00:15
5. Incubate at **76.0°C** for 00:00:01\*\* (see the **NOTE**)
6. **Plate Read**
7. Goto Line 2 for 34 more Times

8. Perform **Melting Curve** from 50.0°C to 99.0°C; read every 1.0°C; hold for 00:00:01 between reads.

**END**

### Quick Tips for Data Analysis

- 1) Check **melting curve** first for product specificity and reaction quality when the reactions are done.
- 2) For **Subtract Baseline**, choose “**average over cycle range**”, and change “**cycle range**” (e.g., usually cycle #1 to cycle #7).
- 3) Move **cycle threshold line** to fit the standard curve with the highest possible **R<sup>2</sup> value** (1 being the best, while >0.96 acceptable).
- 4) From “Quantitation” of the menu, choose all wells, click calculation and copy the quantitation data to Excel spreadsheets.
- 5) The “**Opticon Monitor 3**” software program can be freely downloaded from Bio-Rad website (we have a copy in MOLab) and installed on your personal computers for data analysis.

### NOTE:

- 1) DyNAmo mix should be **the last reagent** to be added to the PCR tube. The essence to ensure best results is to add reagents accurately and quickly.
- 2) Fang found that adding mineral oil to the PCR tubes is very helpful (especially for small volume reactions) although most protocols do not recommend (but who cares!). Please make sure add small volume (10-20µl) and be consistent for all tubes.
- 3) Although the actual reaction volume is **10.0µl**, the reaction volume should be set as **20µl** volume in the plate program.
- 4) The temperature of **Plate Read** should be set according to the location of product peak in the **melting curve**. We are using 20mer primers and the expected products sized 150 ~ 250bp, so the **plate read temperature** can be increased up to 80°C [although **76.0°C** should be fine in the most cases].
- 5) Yang and Ni tested the primer concentrations, and found that the use of **20ng/per reaction/per primer (i.e., half of the primers used in previous protocol)** yields more reproducible results (lower noise levels). Thus, it is highly recommended that **20ng primer/reaction be used as a part of the routine protocol**.
- 6) It's necessary to remove buRIP backup files periodically from the hard drive: **C:\Programs Files\Opticon Monitor 3\instrument\Opticon2\buRIP folders**