

## Real-Time PCR\*: General Considerations

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### General Information

The polymerase chain reaction (PCR) has proven to be a versatile tool in molecular biology. The use of this technique has generated unprecedented advances in gene discovery, diagnostics, and gene expression analysis. In addition, new techniques that build on PCR have further expanded its range of scientific applications.

Real-time PCR is a powerful advancement of the basic PCR technique. Through the use of appropriate fluorescent detection strategies in conjunction with proper instrumentation, the starting amount of nucleic acid in the reaction can be quantitated. Quantitation is achieved by measuring the increase in fluorescence during the exponential phase of PCR. Applications of real-time PCR include measurements of viral load, gene expression studies, clinical diagnostics, and pathogen detection.

Although the performance of PCR in more routine molecular biology applications can be relatively straightforward to optimize, several parameters must be evaluated and optimized independently to achieve the maximum potential of real-time PCR. The factors that affect real-time PCR fall into 3 categories. These are general laboratory practices, template and primer design, and reaction components and conditions. When determining which conditions to optimize, the ultimate assay goal (i.e., qualitative analysis vs. quantitation) must be considered.

To develop sensitive real-time PCR assays cost effectively, you should design and optimize the primer sets prior to developing the probe. This technical note will guide you in the development of an optimized primer set for a quantitative real-time assay.



The iCycler iQ real-time PCR detection system from Bio-Rad is an excellent instrument for quantitating your starting nucleic acid concentration.

## Considerations for General PCR Optimization

### General Laboratory Practices for Quantitative Real-Time PCR

In general, follow these practices to ensure the highest probability of success:

- Wear gloves
- Use screwcap tubes
- Use aerosol-resistant filter tips
- Use calibrated pipets dedicated to PCR
- Use PCR-grade water and use only for PCR
- Use a no-template control to verify absence of contamination
- Prepare reactions in replicate — ideally as triplicates

### Replicate Quality

To obtain good replicates, a master mix should be prepared with all reaction components — including the sample

- Use a hot-start enzyme to prevent nonspecific amplification during preparation
- Make up a master mix with sufficient volume to prepare all replicate samples
- Pipet once per well

### DNA Source

The source of the template affects the accessibility of the target sequence and must be considered during optimization. It is important to optimize the reaction for the template concentrations that will be used in your experiment.

#### Genomic DNA (Intact, High Molecular Weight DNA)

- Cut with a restriction enzyme that does not cut within the region to be amplified
- Boil DNA stock for 10 min and place immediately on ice

#### Plasmid DNA

- If there are problems with amplification, linearize the plasmid with a restriction enzyme that does not cut within the target

#### cDNA

- RNA must be free from genomic DNA contamination — treat with RNase-free DNase prior to reverse transcription. It is also helpful to design primers at splice junctions to avoid genomic DNA amplification

## Template and Primer Design

### Template Design

A successful real-time PCR reaction requires efficient amplification of the product. Both primers and target sequence affect this efficiency. Significant template secondary structure may hinder the primers from annealing and prevent complete product extension by the polymerase. Follow these guidelines:

- Amplify a template region of 75–150 bp
- Avoid secondary structure if possible
- Use an annealing temperature above the melting temperature ( $T_m$ ) for any template secondary structures
- Avoid templates with long (>4) repeats of single bases
- Maintain a GC content of 50–60%
- Analyze secondary structure with the DNA mfold server of Dr. Michael Zuker or equivalent program at:  
<http://bioinfo.math.rpi.edu/~mfold/dna/form1.cgi>

### Secondary Structure Analysis

To evaluate secondary structure of a product on Dr. Zuker's site:

1. Name the sequence — example: bactin1
2. Copy the sequence or retype it into the big text box
3. Scroll down the page to the input labeled "Folding temperature" and enter the annealing temperature of the reaction
4. Scroll down the page to the input labeled "Ionic conditions" and change the units to mM. Adjust the values to reflect the ionic conditions in the reaction (set  $[Na^+]$  to 50 mM and  $[Mg^{2+}]$  to 3 mM for most reactions)
5. Scroll down further to enter your e-mail address — you will not receive an e-mail message, but the program will not proceed without it
6. Click on the button marked "Fold DNA" next to the smiley face
7. Now you'll get a list of structures — ideally you will see only one. Pick a format such as PNG to view the structure.
8. The  $T_m$  of the structure, which appears in a separate window (Loop Free-Energy Decomposition), will tell you at what temperature this structure will form.

### Primer Design

The goal is to design primers with a  $T_m$  higher than the  $T_m$  of any of the predicted template secondary structures. This ensures that the majority of possible secondary structures have been unfolded before the primer-annealing step. Follow these parameters when designing primers:

- Design primers with a GC content of 50–60%
- Maintain a melting temperature ( $T_m$ ) between 50 and 65°C
- Eliminate secondary structure
- Avoid repeats of G's or C's longer than 3 bases
- Place G's and C's on ends of primers
- Check sequence of forward and reverse primers to ensure no 3' complementarity (avoids primer-dimer formation)
- Adjustment of primer locations outside of the target sequence secondary structure may be required
- Verify specificity using sites such as the Basic Local Alignment Search Tool (<http://www.ncbi.nlm.nih.gov/blast/>)

## Reaction Components and Conditions

### Components

Optimization conditions can vary with assay type. Therefore, these conditions should be considered when establishing a new assay:

- $MgCl_2$  concentration (3.0–6.0 mM)
- dNTP concentration (200–600  $\mu M$  each dNTP) — Increasing the [dNTP] will require an increase in  $[MgCl_2]$
- Source and concentration (1.25–4.5 U/50  $\mu l$  reaction) of *Taq* DNA polymerase
- Primer concentration (100–500 nM)
- An asymmetric primer concentration may be helpful
- Fluorescent probe or intercalation dye concentration

### Conditions

Optimization of the following amplification conditions will be required to obtain the maximum efficiency and specificity:

- Annealing temperature (50 to 65°C) and time (dependent on primer  $T_m$  and chemistry)
- Extension time (dependent on chemistry and product length)
- Denaturation temperature and time (dependent on target sequence)
- 2-step v. 3-step PCR

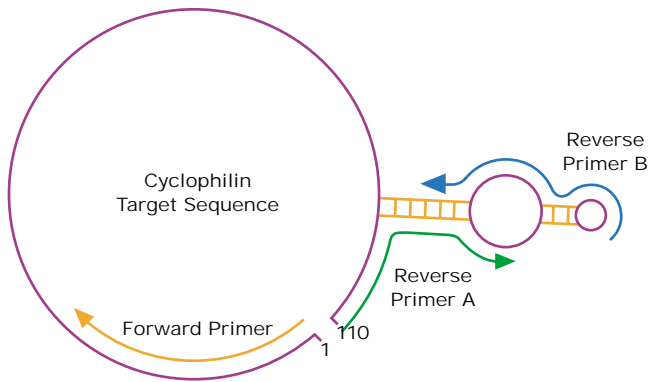


Fig. 1. Folding structure of the cyclophilin PCR product. The product sequence was folded in the Zuker site using the procedure outlined on the opposite page.

## Experimental Design and Interpretation of Results

### Primer Selection

This section demonstrates the importance of primer optimization using the human cyclophilin 40 gene. Two sets of primers, differing in location, were designed to amplify the same region of the human cyclophilin 40 gene (IMAGE Consortium clone 71154, ATCC). Figure 1 illustrates the location of the primer sets (primer sets A and B use the same forward primer).

Five replicates for a 10x dilution series ( $10^7$  to  $10^3$  copies) using identical primer concentrations (300 nM/reaction) were performed on the iCycler iQ system. The reaction mixture consisted of custom-made Life Technologies Supermix (Platinum *Taq* polymerase, 1.25 U, 20 mM Tris, pH 8.4, 3 mM  $MgCl_2$ , 0.2 mM of each dNTP, 50 mM KCl). Real-time amplification was detected using the intercalating dye SYBR<sup>®</sup> Green I (Molecular Probes; 1:75,000 dilution of the 10,000x stock solution).

Optimizing primer location to reduce template secondary structure interference increased both the sensitivity and efficiency of amplification. Using the formula  $E = (10^{-1/\text{slope}}) - 1$  to calculate efficiency (E), a reaction with 100% efficiency will generate a slope of -3.32. The amplification plot of the experiment using primer set A generated a slope of -4.53 or 66% efficiency, with a correlation coefficient of 0.995 (Figure 2, upper panel). Moving the reverse primer inwards to the location of primer B shortened the PCR product and eliminated strong secondary structure. This shift in primer location significantly improved the efficiency of the reaction to 99% (slope = -3.35) and a correlation coefficient of 0.999 (Figure 2, lower panel).

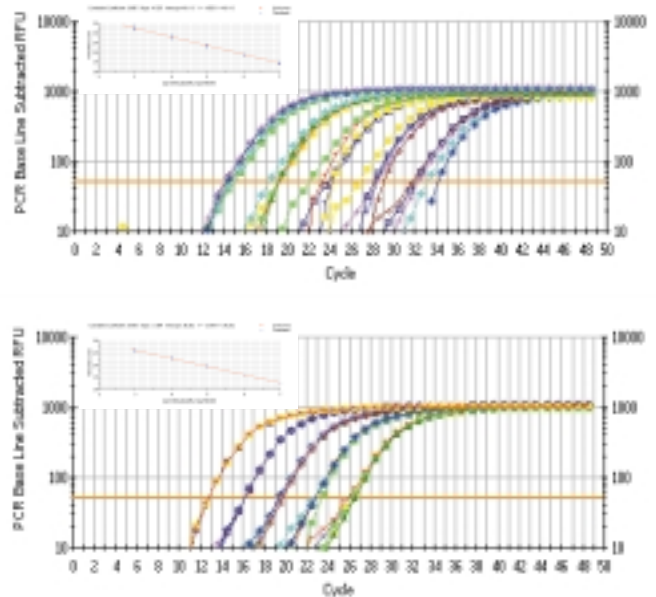


Fig. 2. Amplification plots of the 10x dilution series of the cyclophilin target with primer set A (upper panel) and primer set B (lower panel). The inset depicts the standard curve for each primer set. Data were analyzed with 95% of the data from the end of the cycle and the threshold was set at 55 RFU for both plots. Both experiments were performed on the same plate using the same plasmid dilution series.

### $MgCl_2$ Evaluation

Typically, real-time PCR requires higher concentrations of  $MgCl_2$  for optimal results. To demonstrate this, an analysis of  $MgCl_2$  concentration effects on amplification efficiency was performed. Replicate reactions at 4  $MgCl_2$  concentrations (1.5, 2.25, 3, and 4 mM) and final primer concentrations of 300 nM each (for the optimized Primer B pair from above) were prepared. Reaction conditions were 1.25 U Platinum *Taq* DNA Polymerase and 1x PCR buffer (Life Technologies), 0.2 mM each dNTP (Advantage Ultrapure dNTPs, Clontech), and SYBR Green I (Molecular Probes, 1:75,000 dilution of the 10,000x stock solution).

The magnesium concentration had a significant impact on the amplification efficiency of the PCR reactions as demonstrated in Figure 2 and Table 1. The highest PCR efficiency was achieved with a 3 mM  $MgCl_2$  concentration (Table 1). The importance of maintaining high amplification efficiencies is clearly depicted in Figure 3. The threshold cycle was shifted by 4.7 cycles at  $10^4$  copies and by 3.6 cycles at  $10^7$  copies when the  $MgCl_2$  concentration was increased from 1.5 mM to 3.0 mM. This significant shift in  $C_T$  was not observed in the 2.25 mM or 4 mM  $MgCl_2$  samples.

PCR efficiency can be affected by numerous factors. In this technical note we have evaluated 2 of these factors — secondary structure interference and free magnesium ion concentration. We have clearly demonstrated that optimization of PCR design and reaction conditions on this template has strong effects on the quality of real-time PCR assays.

Once the primer locations and the  $MgCl_2$  concentrations have been evaluated, other reaction components, such as probe concentration and protocol temperatures, may need to be evaluated. For example, a 2-step or 3-step PCR thermal protocol may yield optimal amplification detection, depending on the detection strategy. Although this initial optimization may seem cumbersome, it will result in reaction conditions that are robust and reproducible.

**Table 1. Amplification slopes, efficiencies, and correlation coefficients for 1.5, 2.25, 3.0, and 4.0 mM  $MgCl_2$  dilutions.**

Magnesium Chloride (mM)	Slope	Efficiency	Correlation Coefficient
1.50	-4.04	77%	0.993
2.25	-3.59	90%	0.996
3.00	-3.35	99%	0.999
4.00	-3.40	97%	0.999

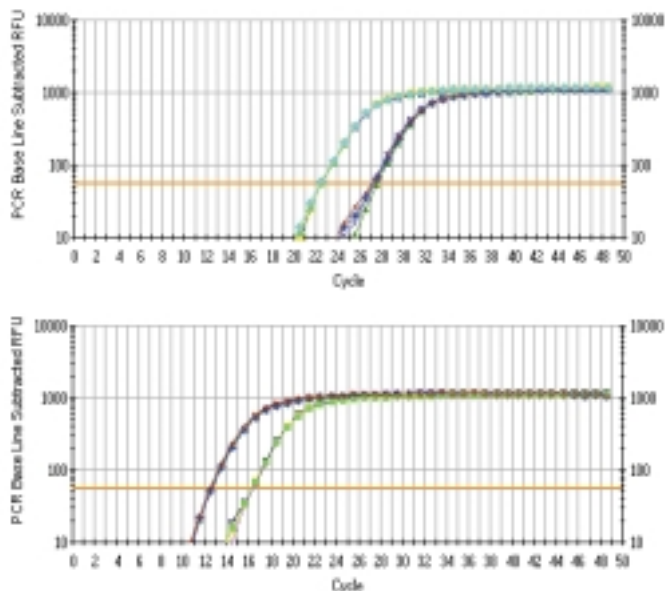


Fig. 3. Amplification plots of  $10^4$  copies (upper panel) or  $10^7$  copies (lower panel) of the cyclophilin target. In both plots the lefthand curve was generated with the 3 mM  $MgCl_2$  concentration and the righthand curve with 1.5 mM  $MgCl_2$ . Upper panel,  $C_T = 22.5 \pm 0.12$  at 3 mM  $MgCl_2$ ;  $C_T = 27.2 \pm 0.23$  at 1.5 mM. Lower panel,  $C_T = 12.6 \pm 0.06$  at 3 mM;  $C_T = 16.2 \pm 0.13$  at 1.5 mM. Data were analyzed with 95% of the data from the end of the cycle and the threshold was set at 55 RFU for both plots. Both experiments were performed on the same plate using the same plasmid dilution series. Values are averages  $\pm$  standard deviation.

## Ordering Information

Catalog #	Description	Catalog #	Description
223-9473	200 $\mu$ l Thin Wall PCR Tubes, 1,000	224-0140	2.0 ml Skirted Tubes, with installed O-ring screwcaps, sterilized, 500
223-9469	200 $\mu$ l Thin Wall PCR Tubes, 120 in strips of 8	732-6340	AquaPure Genomic DNA Isolation Kit
223-9472	8-Cap Strips, for 200 $\mu$ l tubes, 120	170-8720	iCycler Thermal Cycler, with 96 x 0.2 ml reaction module
223-9476	200 $\mu$ l Thin Wall PCR Tubes, 80 in strips of 12	170-8722	iCycler Thermal Cycler, with 2 x 48 x 0.2 ml dual block reaction module
223-9477	12-Cap Strips, for 200 $\mu$ l tubes, 80	170-8724	iCycler Thermal Cycler, with 60 x 0.5 ml reaction module
223-9466	600 $\mu$ l Thin Walled PCR Tubes, 1,000	170-8726	iCycler Thermal Cycler, with 384-well reaction module
223-9444	iCycler Optical Sealing Tape	170-8740	iCycler iQ Real-Time PCR Detection System
223-9441	96-well 200 $\mu$ l Thin Walled PCR Plates		
224-0185	0.5 ml Skirted Tubes, with installed O-ring screwcaps, sterilized, 500		

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