Real Time PCR (qPCR)

Introduction

Since its invention in 1983 by Kary Mullis, the PCR technique has been used in a wide variety of applications, from basic molecular cloning techniques to forensics and genetic identification. However, accurate quantitation of DNA (or RNA, by RT-PCR) proved difficult, since PCR typically reaches a plateau phase in which the same amount of product is produced regardless of the initial amount of template.

Early attempts at quantitative analysis relied on "endpoint" methods, such as gel electrophoresis, to measure amplification products during the plateau phase of PCR. These methods were not reliable, sensitive, or convenient for processing large numbers of samples.

Real-time qPCR, a variation of the original PCR process, is a quantitative method to study product amounts during the early (exponential) stages of a reaction, when the amount of product corresponds to the amount of initial template present (See Figure 1). The technique was originally developed by Russell Higuchi and coworkers in 1993, using ultraviolet detection of ethidium bromide-stained amplification products in a modified thermal cycler. Since then, qPCR technology has advanced considerably, with the use of specialized instruments designed to detect the light emitted by amplified, fluorescently labeled DNA molecules.

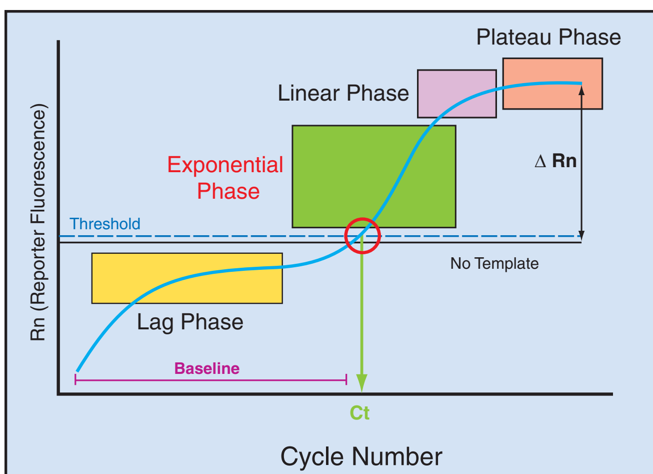


Figure 1: Profile of a qPCR Reaction.

Basic Theory

In most real-time qPCR methods, the amount of amplification product is measured at each reaction cycle. The first cycle in which the amplified product can be detected above the background signal is called the threshold cycle, and this value (denoted as Ct) is directly proportional to the amount of initial template (see figure 2).

Advantages of qPCR

Traditional methods of quantitating DNA rely on ultraviolet excitation of DNA-bound dyes, or staining of DNA, typically following gel electrophoresis. The most common method uses ethidium bromide, a dye that intercalates DNA and fluoresces upon exposure to ultraviolet light. Another fluorescent dye used is Pico Green®, which offers greater sensitivity compared to ethidium bromide. The AluQuant® System (Promega) is a specialized technique for detecting human DNA, using probes to detect repeated sequences and luciferase as the reporter system. Another probe-based detection system, QuantiBlot® (Applied Biosystems), uses biotinylated probes and subsequent colorimetric or chemiluminescent detection methods.

Real-time qPCR techniques offer several advantages over these older methods of quantitating DNA. The availability of commercial kits has made the technique easy to perform, efficient, and reliable. qPCR methods are easily adapted to high-throughput assays, allowing researchers to process large numbers of samples in a short period of time. In addition, data can be collected and analyzed using specialized software designed for the specific instrument being used, and a personal computer.

qPCR has been used for many diverse applications, including the detection of pathogenic bacteria, identification and quantitation of microorganisms from water samples, studying gene expression levels, and detection of single-nucleotide polymorphisms (SNPs) in genomic sequences, to name just a few.

Detection Methods

The most popular qPCR techniques fall into two categories: intercalating dye-based methods and probe-based methods.

Intercalating Dye (SYBR® Green I)

The first method uses SYBR® Green I, an intercalating dye that binds to the minor groove of double-stranded DNA (dsDNA) molecules, regardless of sequence. Upon binding to DNA, the intensity of SYBR® Green I fluorescent emission increases greatly (>300 fold), providing excellent sensitivity (25X the sensitivity of ethidium bromide) for the quantitation of dsDNA molecules. Because fluorescence occurs only upon binding of the dye to dsDNA, unbound dye does not contribute significantly to background noise. In its simplest form, this method is performed by adding a small amount of SYBR® Green I to a PCR reaction mixture prior to cycling. The SYBR® Green I dye becomes bound to newly synthesized dsDNA products in each cycle of the amplification process, and the products are then detected and meas-

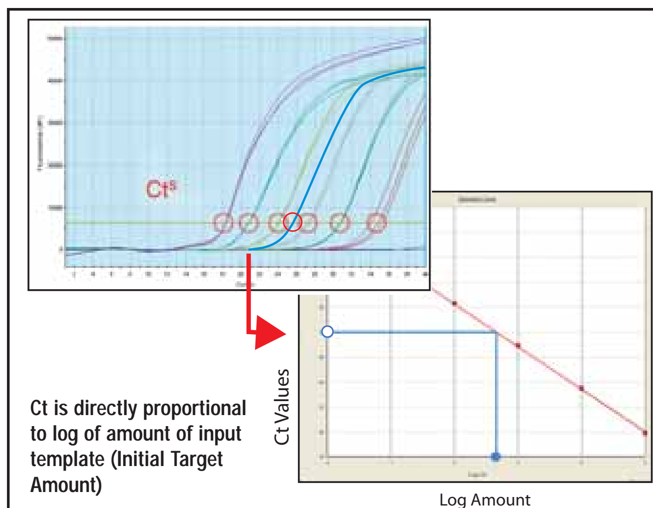
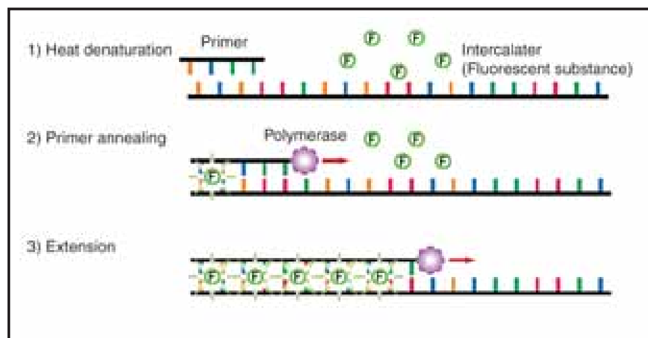


Figure 2: Demonstration of the Ct value vs Log of Amount of Input Template.

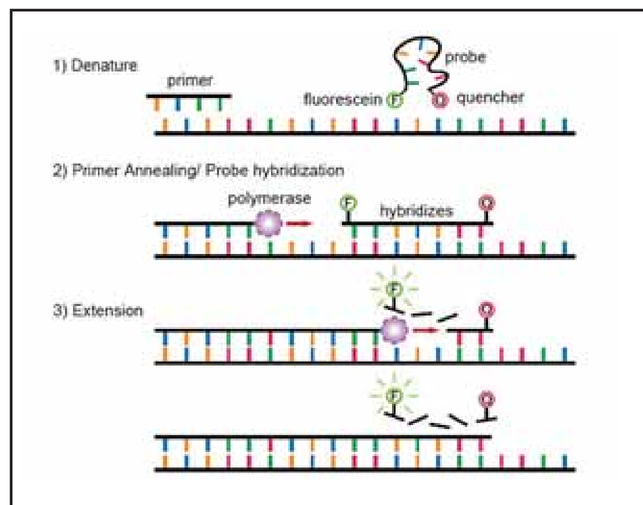
ured by the real-time PCR instrument. Takara's SYBR® Premix *Ex Taq*™ provides real-time quantitation of DNA using SYBR® Green I in a convenient, easy-to-use, premix formulation.



SYBR® Green Intercalator Detection Method

Fluorescent Probes

A second qPCR method relies on fluorescence resonant energy transfer (FRET) technology. This technology, as applied to real-time PCR and pioneered by Applied Biosystems, incorporates the use of TaqMan® oligonucleotide probes. These probes consist of a single-stranded DNA (ssDNA) molecule containing a 5' reporter dye plus a 3' quencher that inhibits fluorescence emission when located in close proximity to the reporter. The probes anneal to a specific site on the template DNA, located between the forward



Profile of Fluorogenic 5' Nuclease Assay

and reverse primer positions. During amplification, the DNA polymerase extends the PCR primer and reaches the annealed probe. The 5' exonuclease activity of the DNA polymerase cleaves the probe's terminal 5' nucleotide along with attached reporter dye, releasing it into the reaction mixture. Cleavage results in the physical separation of the reporter dye from the quencher dye and consequently, the reporter dye is able to emit strong fluorescence. TaqMan® probes are added to the PCR master mix (in addition to the normal PCR forward and reverse primers) in an excess amount, which allows for annealing of a steady supply of intact probes to newly synthesized target molecules during each amplification cycle. Thus, an exponential increase of cleaved TaqMan® probes, corresponding to the number of PCR targets amplified, is observed in each cycle. Contrary to the SYBR® Green I method, where SYBR® Green I binds to any dsDNA molecule, TaqMan® probes bind only to a specific target molecule. An advantage of probe-based methods is that multiple probes, each labeled with a different reporter dye, can be used in the same reaction. This technique is known as multiplex qPCR.

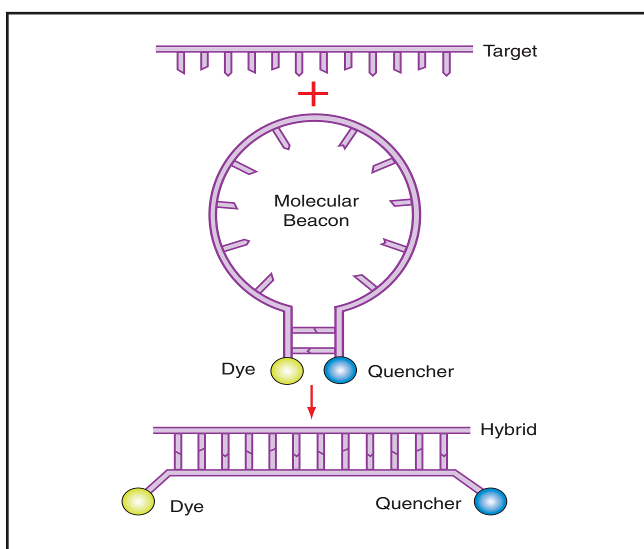
Variations of the Probe Method

Several vendors have developed qPCR technologies based on the probe detection method.

Molecular Beacons

In this method (developed by PHRI), the probe consists of a short (~30-35 base) segment of ssDNA designed to form a stem-loop structure. A fluorescent reporter dye is located at the 5' end of the beacon, with a quencher dye at the 3' end. A template-specific nucleotide sequence is located in the single-stranded loop region of the probe. When the probe is folded into a stem-loop, the quencher is in close proximity to the 5' fluor and fluorescence is quenched. However, if the probe binds to a complementary strand of DNA, the fluor and the quencher become physically separated and fluorescence is emitted. During each amplification cycle, fluorescence emissions increase as the probes hybridize to newly synthesized, complementary ssDNA targets.

Unlike the TaqMan® method, molecular beacon probes are not destroyed in each cycle but can be reused. This leads to very low background signal, making the method ideal for multiplex reactions—up to 7 probes have been used in a single reaction. However, the probes must be carefully designed so that the stem-loop structure is optimal for the specific reaction conditions used.



Profile of the Molecular Beacon

Scorpion™ Probe

This method (developed by DxS) is similar to the molecular beacons, but rather than using a separate probe, the hairpin loop is attached to the 5' end of the PCR primer sequence through a specially designed blocker. In this configuration, the quencher and fluor are in close proximity. After primer extension, the newly synthesized strand of DNA is able to adopt a new configuration in which the loop region anneals to its complementary sequence within the same DNA strand. In this structure, the fluor is no longer adjacent to the quencher, and thus an increase in fluorescence is observed.

The kinetics of the Scorpion™ probe reaction are more favorable than other probe methods, since the reaction is unimolecular (because it contains both the primer and probe). Scorpion™ probes typically give a higher fluorescence intensity compared to TaqMan® and molecular beacon probes. However, the probe design and optimization can be challenging, and the technique is not recommended for researchers who are new to qPCR.

Plexor System

The Plexor system (Promega) is a recent qPCR technology that lies between the conventional SYBR® Green chemistry and the TaqMan® method. The technology uses modified nucleotides (iso-dG and iso-dC) that are recognized by DNA polymerase and form a specific base pair with each other, but do not pair with normal nucleotides. One PCR primer is designed with a 5' fluorescent label and iso-dC residue, while the other primer is unlabeled. The PCR mixture contains iso-dG residues attached to a quencher. During amplification, incorporation of an iso-dG nucleotide paired to the iso-dC nucleotide in the primer effectively quenches the fluorescent signal. Thus, in the Plexor method, reaction progress is measured by a decrease in fluorescence, as opposed to other qPCR methods.

The Plexor system offers simplicity comparable to that of SYBR® Green, but is flexible enough to allow multiplexing.

Tips for Successful qPCR

SYBR® Green Method

SYBR® Green detection is an ideal method for researchers who are new to qPCR, or for those desiring a simple, inexpensive, and easy-to-use qPCR technique. This method also does not require the design of specialized primers for PCR. Optimization of reaction conditions is typically routine, and the method is ideally suited to initial screening of high-throughput samples (e.g. for gene expression levels).

Reaction Components

Quenchers

When designing a fluorescent probe for qPCR, it is necessary to ensure that the fluor and quencher pair is compatible with the detection chemistry. Initial quenchers included Dabcyl and TAMRA dyes; however, these quenchers contributed to background fluorescence. This problem led to the development of "dark" quenchers that emit energy absorbed from the fluor as heat, rather than light. Some popular dark quenchers include Black Hole Quenchers™ (BHQ 1-3), Eclipse, and Iowa Black. (See table on page 17).

Controls

Good controls are essential to the success of any qPCR experiment. It is important to include at least one reference gene, typically a housekeeping gene that is constitutively expressed in a wide range of cell types. The control gene should be expressed at a constant level under experimental conditions, and its expression level should be in the same range as that expected for the target gene.

In addition, controls with no template and no polymerase should be run to test for contamination or other factors that can cause an increase in background fluorescence.

Standard Curve

A standard curve should be developed, using serial dilutions of a template whose concentration is known. The template could be DNA or RNA, or a cloned PCR product; if the target region to be amplified is less than 100 bp, a synthetic oligonucleotide corresponding to the target sequence can be used. Ideally, the template used to generate the standard curve should be the same as the experimental template.

Template Quality

The purity of the PCR template is a significant factor affecting qPCR results. Degraded DNA or contaminants can affect the sensitivity of detection. In particular, for quantitative RT-PCR, it is critical that total RNA preparations be highly pure and free from degradation. (Use Takara's FastPure™ RNA Kit (TAK 9190)

High Speed qPCR

Several qPCR instruments and reagent systems have been modified to allow extremely fast (15 min or less) reactions. However, because of the small size of the products typically studied in qPCR, accelerated reaction times of 45-50 min are generally possible without extensive optimization. Takara's SYBR® Premix *Ex Taq*™ (Perfect Real Time) will work for fast PCR.

Reference Dye

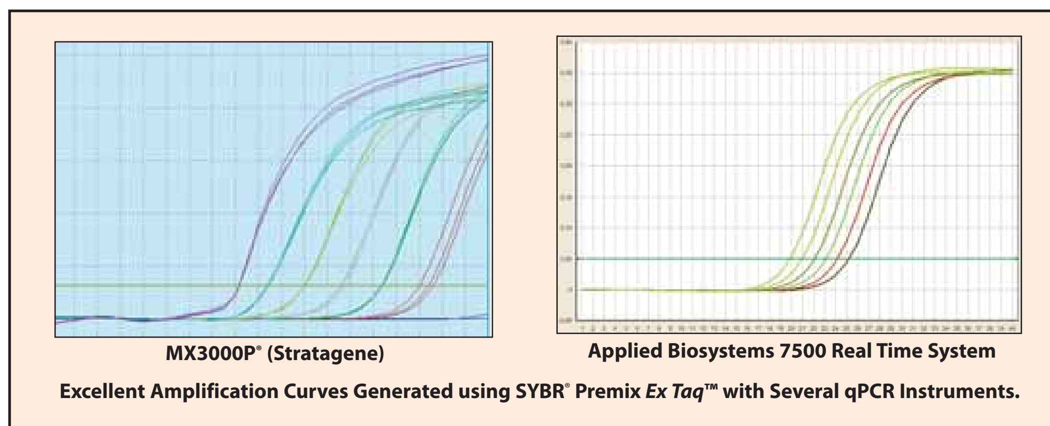
A passive fluor (e.g., ROX or fluorescein) is often used as a reference dye in fluorescence measurements. The dye is spiked into the PCR master mix at the beginning of the assay. The signal from the dye, generated by excitation at a frequency range determined by the thermal cycler, is assigned a reference value. This technique corrects for variability among samples (e.g. bubbles, sample volume, plasticware, etc.).

Sensitivity and Specificity

In general, for qPCR it is essential that the fluorescent detection system offer high sensitivity. Additionally, use of a PCR enzyme possessing high sensitivity and providing high yield will allow robust amplification of target sequences and aid in the measurement of low copy-number genes. High specificity is required, especially with SYBR® Green detection, to ensure accurate quantitation of only the product of interest. All of Takara's real-time PCR products use Hot Start *Ex Taq*™ polymerase, a high-sensitivity, high-specificity and high yield DNA polymerase, supplied with an optimized buffer system for qPCR.

Real Time PCR (qPCR)

Examples of the use of SYBR® Premix *Ex Taq*™ on Two qPCR Instruments



MX3000P® (Stratagene)

Applied Biosystems 7500 Real Time System

Excellent Amplification Curves Generated using SYBR® Premix *Ex Taq*™ with Several qPCR Instruments.

Selection Guide for Takara's Real Time PCR Enzymes

	Detection Method		qPCR Instrument								
	SYBR® Green I Detection	Probe Detection	Cepheid Smart Cycler®	Applied Biosystems 7300/7500	ABI PRISM® 7000/7700/7900 HT	Roche LightCycler®	RotorGene™	Biorad iCycler®	MJ Opticon®	StratageneMX 3000P	
SYBR® Premix *Ex Taq*™ (Perfect Real Time)	X		X	X	X	X	X	X	X	X	2X Premix* with SYBR® Green I, ROX™ reference† dyes I & II
Premix *Ex Taq*™ (Perfect Real Time)	X	X	X	X	X	X	X	X	X	X	2X Premix**, ROX™ reference† dyes I & II

* contains *Ex Taq*™ Hot Start DNA Polymerase, buffer, dNTP mix, Mg^{2+} and SYBR® Green I

** contains *Ex Taq*™ Hot Start DNA Polymerase, buffer, dNTP mix, Mg^{2+}

† ROX™ Reference DYE/DYE II is supplied to perform normalization of fluorescent signal intensities from well to well when used with Real Time instruments that have this option. Use of the ROX™ dyes is optional.

Amplification Curve (upper panel) and Melting Curve (lower panel) Comparison of SYBR® Premix *Ex Taq*™ (Perfect Real Time) with qPCR Kits from Three Competitors.

Amplification efficiency and reaction specificity were determined using Takara's SYBR® Premix *Ex Taq*™ (Perfect Real Time) and three leading competitor qPCR enzymes using three major real time instruments. The results of these experiments, performed under the manufacturer's recommended conditions respectively, can be seen in the figures below.

In **Figure 1,** Roche's real time enzyme shows poor reaction specificity when compared to Takara's SYBR® Premix *Ex Taq*™ as demonstrated by multiple peaks in the Roche melting curve, particularly when low copy number templates are amplified.

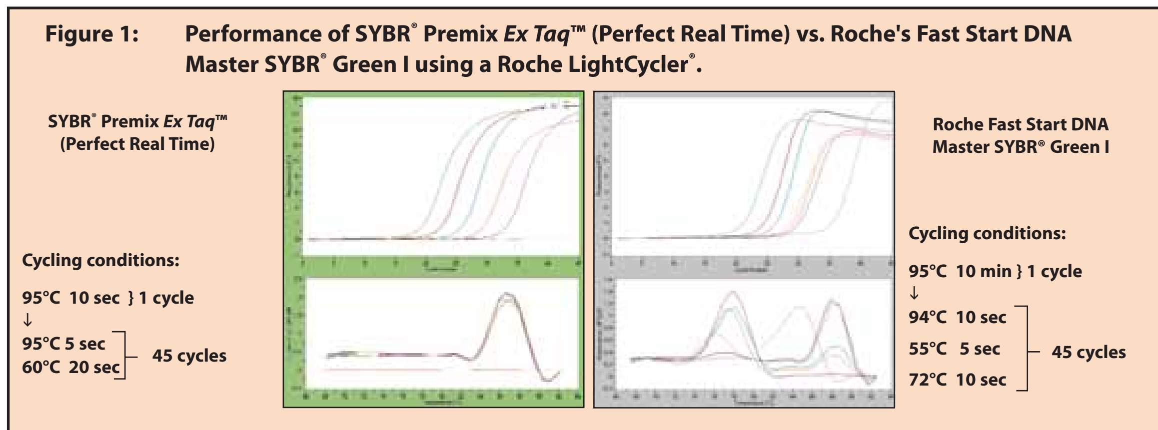


Figure 1: Performance of SYBR® Premix *Ex Taq*™ (Perfect Real Time) vs. Roche's Fast Start DNA Master SYBR® Green I using a Roche LightCycler®.

In **Figure 2**, low amplification efficiency is shown for ABI's SYBR® Green PCR Master Mix, indicated by Ct values which are shifted to the right and lower fluorescence intensity.

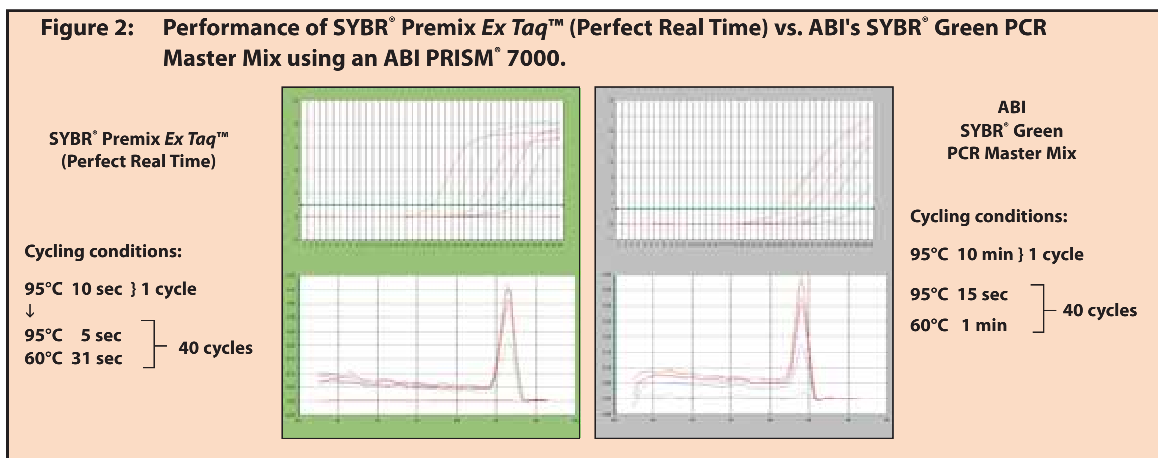


Figure 2: Performance of SYBR® Premix *Ex Taq*™ (Perfect Real Time) vs. ABI's SYBR® Green PCR Master Mix using an ABI PRISM® 7000.

In **Figure 3**, Takara's SYBR® Premix *Ex Taq*™ shows superior reaction specificity compared to Invitrogen's Real Time Supermix as demonstrated by tight peaks in Takara's melting curve.

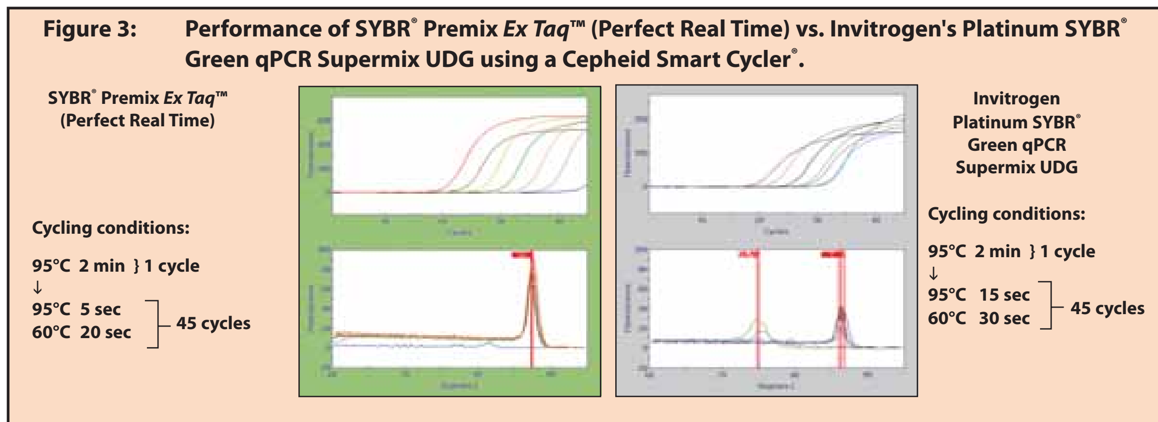


Figure 3: Performance of SYBR® Premix *Ex Taq*™ (Perfect Real Time) vs. Invitrogen's Platinum SYBR® Green qPCR Supermix UDG using a Cepheid Smart Cycler®.

These results demonstrate that Takara's SYBR® Premix *Ex Taq*™ (Perfect Real Time) exhibits superior performance in both specificity and sensitivity over three leading qPCR competitors using a variety of qPCR instruments.

Application: qPCR using Premix ExTaq™ (Perfect Real Time)

Fast qPCR Probe Detection Amplification Curve for Premix *Ex Taq*™ (Perfect Real time)

A comparison of Takara's Premix *Ex Taq*™ (Perfect Real Time) and ABI's TaqMan® Universal PCR Master Mix were performed using the Applied Biosystems 7500 Real-Time PCR System with the TaqMan® Gene Expression Assay. Two applications were performed using human ACTB and mouse GAPD as the target DNA. A dilution series of cDNA (corresponding to total RNA 1 pg–100 ng) was performed using sterile distilled water as a negative control. Cycling conditions for all reactions are included below.

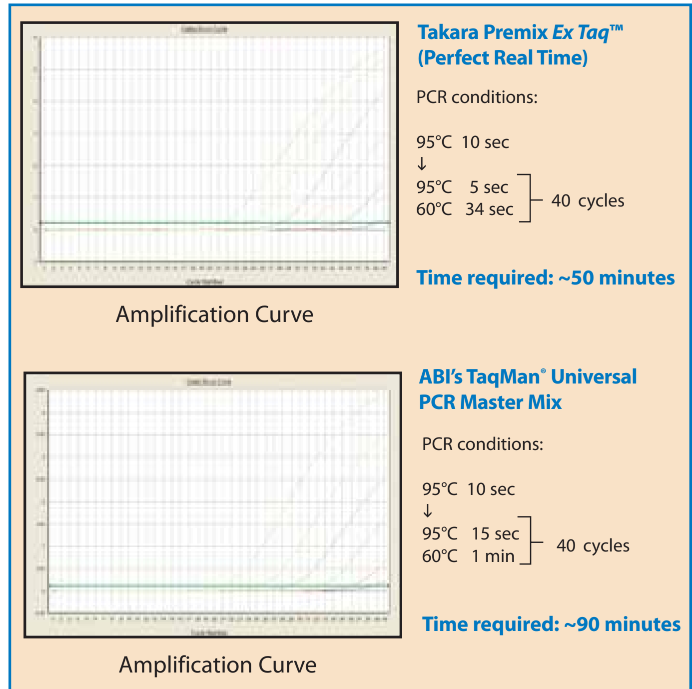


Figure 1: Performance of Premix *Ex Taq*™ (Perfect Real Time) or TaqMan® Universal PCR Master Mix with the TaqMan® Gene Expression Assays (Applied Biosystems). **Target: Human ACTB**

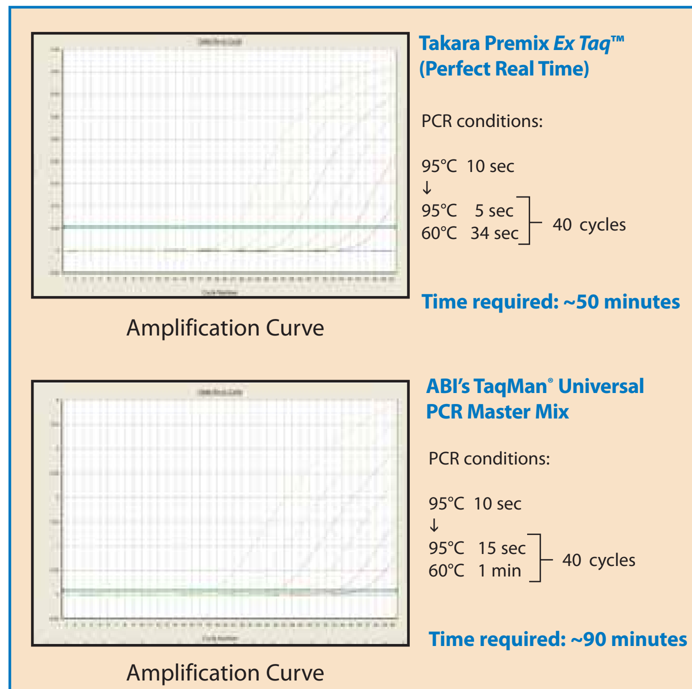


Figure 2: Performance of Premix *Ex Taq*™ (Perfect Real Time) or TaqMan® Universal PCR Master Mix with the TaqMan® Gene Expression Assays (Applied Biosystems). **Target: Mouse GAPD**

In conclusion, Takara's Premix *Ex Taq*™ (Perfect Real Time) requires half the time of the TaqMan® Universal PCR Master Mix with the TaqMan® Gene Expression Assays to achieve excellent results for this real time PCR application.

Reporter Dye/Quencher Recommended Pairing

Reporter Dyes	Quenchers				
FAM	3' TAMRA™	3' Iowa Black™ FQ	3' BHQ™-1	3' BHQ™-2	3' TAM Ester
HEX	3' Iowa Black™ FQ	3' BHQ™-1	3' BHQ™-2	3' TAM Ester	QSY7
TET	3' Iowa Black™ FQ	3' BHQ™-2	3' TAMRA™	3' TAM Ester	
Cy™3	3' Iowa Black™ RQ	3' BHQ™-2			
Cy™5	3' Iowa Black™ RQ	3' BHQ™-2			
5' CAL Fluor® Orange 560	3' Iowa Black™ RQ	3' BHQ™-2			
5' CAL Fluor® Red 610	3' Iowa Black™ RQ	3' BHQ™-2			
5' CAL Fluor® Gold 540	3' BHQ™-1	3' Iowa Black™ RQ			
5' CAL Fluor® Red 635	3' BHQ™-2 3' TAMARA	3' Iowa Black™ RQ			
Quasar 670	3' BHQ™-2				
5' CAL Fluor® Gold 590	3' BHQ™-2				
5' JOE NHS Ester	3' Iowa Black™ FQ	3' BHQ™-2	3' TAMRA™	3' TAM Ester	
5' Oregon Green® 488-X NHS Ester	3' Iowa Black™ FQ	3' BHQ™-2	3' TAMRA™	3' TAM Ester	
5' Oregon Green® 514-X NHS Ester	3' Iowa Black™ FQ	3' BHQ™-2	3' TAMRA™	3' TAM Ester	
5' ROX™ NHS Ester	3' Iowa Black™ RQ	3' BHQ™-2			
5' TAMRA™ NHS Ester	3' Iowa Black™ RQ	3' BHQ™-2			

3' Iowa Black™ quenchers are produced by Integrated DNA Technologies. The Black Hole Quenchers™ are produced by Biosearch Technologies. The reporter dyes (5' CAL Fluor®s)are produced by Biosearch Technologies. TAMRA™ is produced by Applera Corporation. Oregon Green® is produced by Invitrogen.

Real Time PCR (qPCR) Product Summary

SYBR® Premix *Ex Taq*™ (Perfect Real Time) (TAK RR041)

SYBR® Premix *Ex Taq*™ (Perfect Real Time) is a convenient (2X) premix consisting of Takara's high performance *Ex Taq*™ Hot Start DNA Polymerase, SYBR® Green I, and a newly formulated real time buffer which provides superior specificity and increased amplification efficiency in real time PCR.

Premix *Ex Taq*™ (Perfect Real Time) (TAK RR039)

Premix *Ex Taq*™ (Perfect Real Time) is a 2X concentration premix, specially designed for high speed, high sensitivity, real time PCR using various detection methods (e.g., TaqMan®, SYBR® Green I.) This premix combines high-performance *TaKaRa Ex Taq*™ Hot Start DNA Polymerase with a newly-formulated real time PCR buffer which provides increased amplification efficiency and further improved specificity for high speed real time PCR. The results are exceptional real time PCR quickly and easily.

For complete licensing information see page 56.