

A close-up photograph of a flower, likely a lily, with vibrant purple petals and bright yellow stamens. The image is positioned on the left side of the cover, partially overlapping the dark blue background.

Successful PCR Guide

3rd Edition

Routine PCR

Real Time PCR (qPCR)

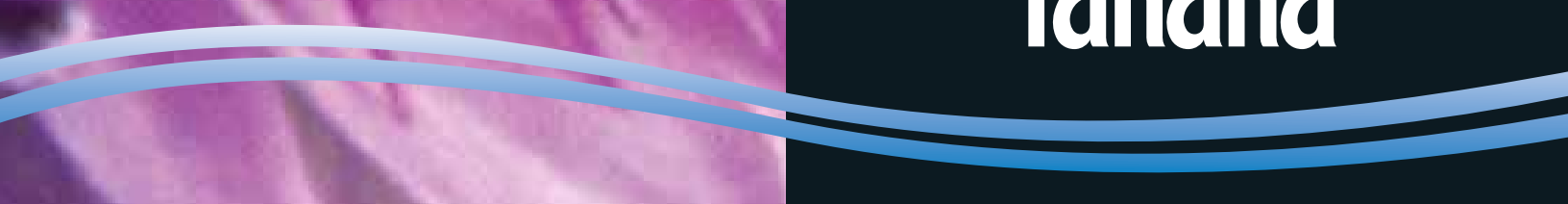
High Fidelity PCR

High Performance PCR

Hot Start PCR

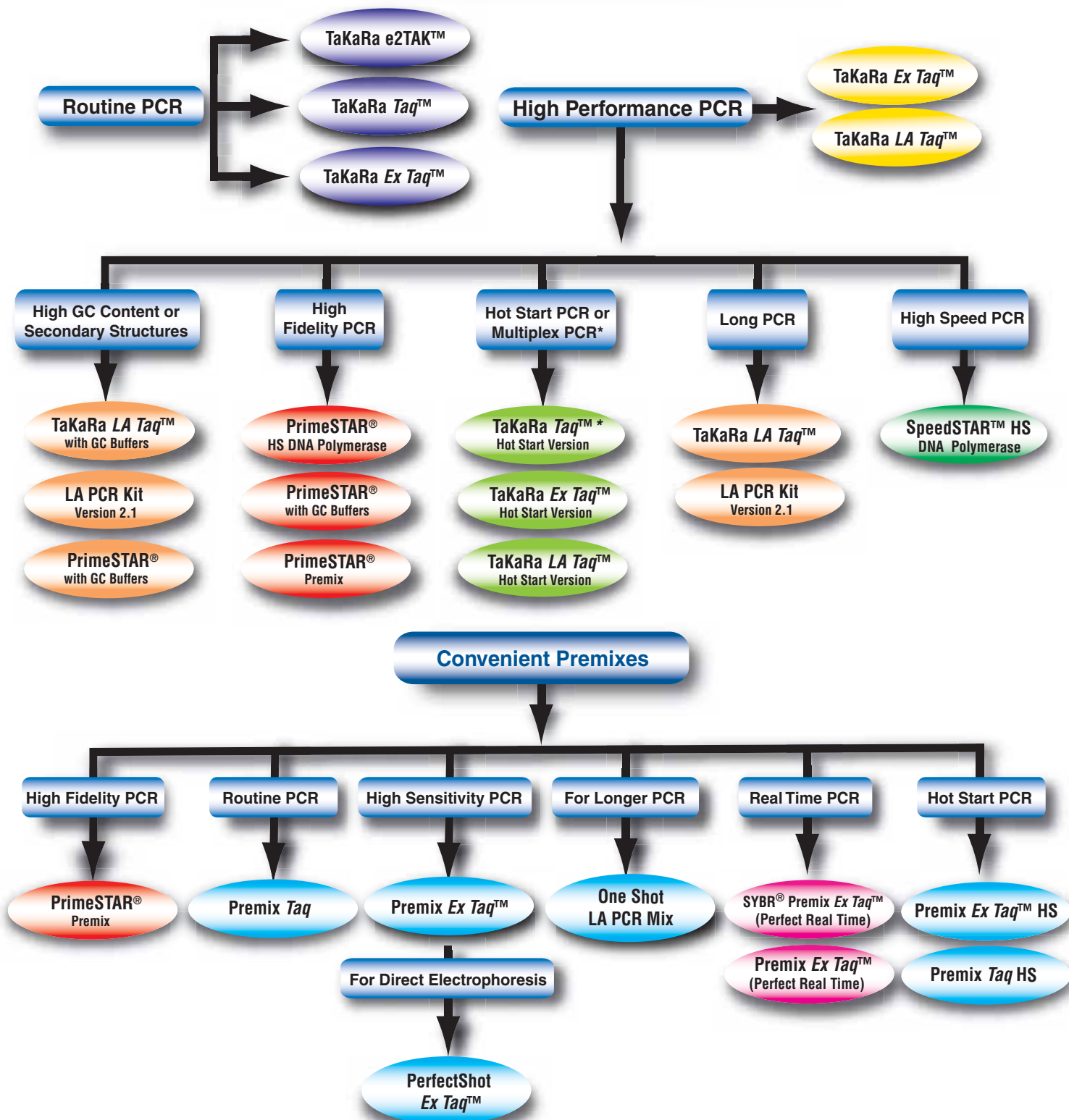
RT-PCR

PCR Cloning

A decorative blue wavy line that spans the width of the cover, located at the bottom.

TaKaRa

How to Select The Best PCR Enzyme for Your Application



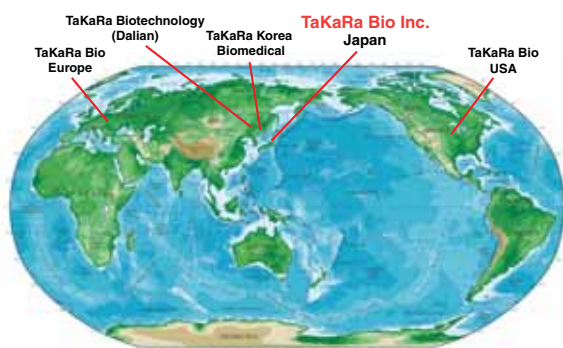
*Hot start enzymes contain an anti-Taq antibody to minimize non-specific amplification

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ABOUT TAKARA BIO USA

Takara Bio Inc. is a world class supplier of life science research products headquartered in Otsu, Shiga, Japan. Takara Bio was the first domestic manufacturer to introduce restriction enzymes to the Japanese market in 1979, and has consistently developed novel, cutting edge life science technologies and products. This talent for innovation, combined with Takara Bio's unwavering commitment to quality, has resulted in an outstanding line of unique, dependable products for life science research.

Takara Bio holds worldwide patents on Long and Accurate (LA) PCR, and has built a portfolio of PCR licensed high-performance PCR reagents and kits, including *Ex Taq*[™], *LA Taq*[™], PrimeSTAR[®], SpeedSTAR[™], e2TAK[™], SYBR Premix *Ex Taq*[™] (Perfect Real Time) and Premix *Ex Taq*[™] (Perfect Real Time).



Takara Bio Inc., Otsu Shiga, Japan

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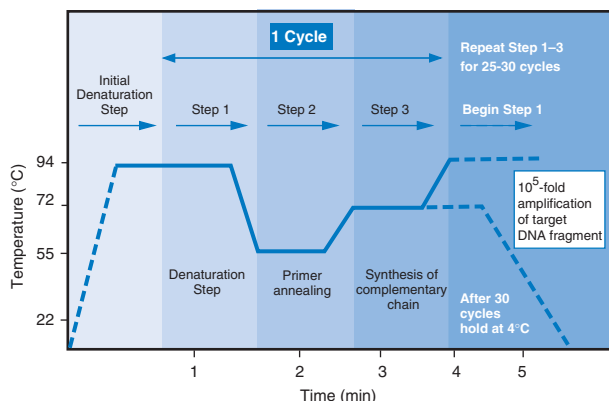
For a complete description of Takara Bio USA's product offering, please visit our website at www.takarabiousa.com.

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Profile of PCR Reactions

Profile of Routine PCR Reaction



Step 1: Denaturation. Double-stranded DNA fragment is denatured in a reaction mixture containing primers, dNTP and polymerase.

Step 2: Annealing. Primers are annealed to denatured single-stranded DNA.

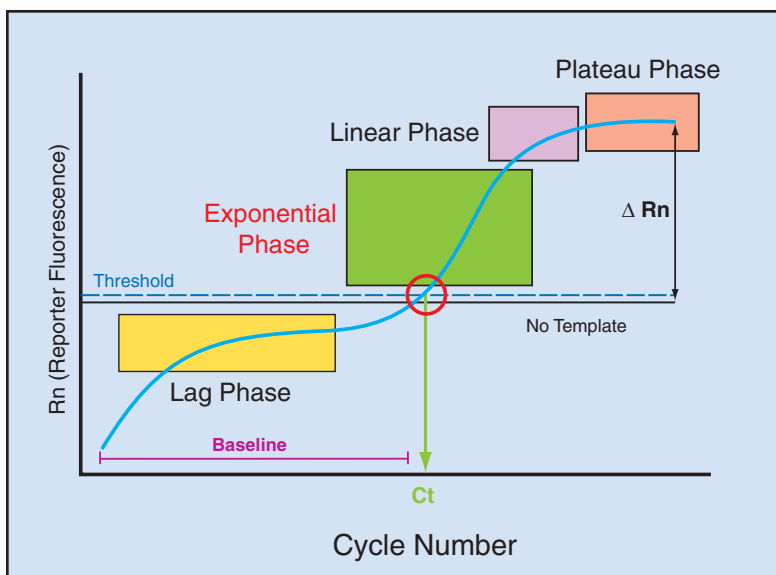
Step 3: Extension. Annealed primers are extended with DNA polymerase.

Cycling parameters must be empirically determined as optimum conditions for PCR vary depending on the DNA template and primers used.

PCR Reaction Efficiency

Cycle #	100% efficiency	90% efficiency	80% efficiency
0	1	1	1
1	2	2	2
2	4	4	3
3	8	7	6
4	16	13	10
5	32	25	19
6	64	47	34
7	128	99	61
8	256	170	110
9	512	323	198
10	1,024	613	357
11	2,048	1,166	643
12	4,096	2,213	1,157
13	8,192	4,205	2,082
14	16,384	7,990	3,748
15	32,768	15,181	6,747
16	65,536	28,844	12,144
17	131,072	54,804	21,859
18	262,144	104,127	39,346
19	524,288	197,842	70,824
20	1,048,576	376,900	127,482
21	2,097,152	714,209	229,468
22	4,194,304	1,355,998	413,043
23	8,388,608	2,578,296	743,477
24	16,777,216	4,898,763	1,338,259
25	33,554,432	9,307,650	2,408,866
26	67,108,864	17,684,534	4,335,969
27	134,217,728	33,600,615	7,804,726
28	268,435,456	638,941,168	14,048,505
29	536,870,912	121,298,220	25,287,311
30	1,073,741,824	230,466,618	45,517,160

Profile of qPCR Reaction



ΔRN = change in reporter fluorescence

Ct = Cycle Threshold

Baseline = a linear function subtracted from the data to eliminate background signal.

Points to Consider

Although PCR has become routine in many laboratories, careful experimental design is still critical for a successful outcome. Preliminary experiments to optimize reaction conditions are essential (including determination of reaction buffer pH, cycling parameters, concentrations of key components such as Mg^{2+} , dNTP, primers and DNA polymerase). PCR success also depends upon individual template-primer combinations for Endpoint PCR and template, primer, probe and detection method for qPCR.

The following chapter discusses the most common issues which should be addressed when designing a PCR experiment.

ENDPOINT PCR USING REGULAR Taq

PCR (Polymerase Chain Reaction) is a simple and powerful tool for amplification of DNA *in vitro*. The PCR method is performed in a thermocycler which repeats three incubation steps at different temperatures. The three steps include:

- 1. Denaturation Step:** The double-stranded target DNA is heat denatured. 94°C for 30 sec.
- 2. Annealing Step:** The two primers complementary to the target segment are annealed to the template DNA at low temperature. 55°C for 30 sec.
- 3. Extension Step:** The annealed primers are then extended at an intermediate temperature by a DNA polymerase. The target copy number doubles upon each cycle, resulting in exponential amplification and potentially billions of copies of the original DNA fragment (see PCR reaction efficiency table). 72°C for 1 min/kb.

Getting Started

It is ideal to have a room dedicated for PCR use only. However, this is not possible in many research labs. Use of barrier filter tips and dedicated pipettes are imperative. Contamination from dirty pipettes is one of the most common causes of experimental failure. The PCR bench area used should be decontaminated frequently using a product which removes DNA, such as DNA-OFF™ (TAK 9036), as well as cleaned with ethanol (70%) before and after the assembly of the reaction. Care should be taken to avoid careless contamination from the outside environment.

Template DNA

Successful PCR of a target DNA depends on the purity and/or quality of the DNA template and the quantity of template DNA used. Many common DNA purification protocols utilize reagents (such as organic solvents, detergents, salts, etc.) which are inhibitors of DNA polymerases. These reagents must be removed (generally by ethanol precipitation) before inclusion of the template in a PCR experiment.

After removal of these reagents, the DNA should be ready for use in PCR. However, special care must be taken when working with longer targets (>10 kb) during the DNA preparation to avoid shearing of the intact molecules.

After DNA purification, it is important to use the appropriate amount of template. The use of either excessive plasmid DNA or insufficient genomic DNA template are two of the most common PCR mistakes. A minimum of 10^4 copies of target sequence must be used to obtain a signal in 25–30 cycles for a final concentration of DNA at 10 ng/μL.

Primer Preparation

The melting temperature (T_m is defined as the temperature at which half of the primer binding sites are occupied) of a DNA hybrid depends somewhat upon its length, and the primer sequence should be designed with the recommended primer length in mind (i.e., primers that are too long and, therefore, too stable, are problematic).

Recommended PCR primer lengths range from 18–25 bases for fragments smaller than 5 kb, and 20–30 bases for fragments greater than 5 kb. These parameters allow the T_m differences between the template and the unstable primer to be minimized, allowing for more efficient PCR.

The following list provides additional guidelines for primer sequence:

1. Primers should end (3') in a G or C, or CG or GC. This design increases the efficiency of priming by forming a tight G/C bond.
2. Design primers with balanced melting temperatures (within 2–3°C of each other). Temperatures between 65–70°C are preferred, as higher annealing temperatures increase reaction specificity.
3. Avoid complementarity in the 3'-ends of primers, as primer dimers will be preferentially synthesized because of short lengths in a reaction.
4. Avoid primer self-complementarity (ability to form secondary structures, such as hairpins) which effectively reduce primer concentration.
5. Avoid runs of three or more C's or G's at the 3' ends of primers, which may promote mispriming at G or C-rich sequences (because of the stability of annealing).

Primer Annealing Temperature

Many formulas are available to determine the theoretical T_m of nucleic acids. The following commonly-used formula can be used to estimate the melting temperature for any oligonucleotide:

$$T_m = 2^\circ\text{C} \times (\text{number of A+T}) + 4^\circ\text{C} \times (\text{number of G+C})$$

A more technical formula is

$$(T_m = 81.5 + 16.6 \cdot (\log_{10}[\text{Na}^+]) + 0.41 \cdot (\%G+C) - 675/n)$$

where $[\text{Na}^+]$ is the molar concentration of monovalent cations ($[\text{Na}^+] = [\text{K}^+]$) and n = number of bases in the oligonucleotide.⁽¹⁾

For example, to calculate the melting temperature of a 22mer oligonucleotide with 60% G+C in 50 mM KCl:

$$\begin{aligned} T_m &= 81.5 + 16.6 \cdot (\log_{10}[0.05]) + 0.41 \cdot (60) - 675/22 \\ &= 81.5 + 16.6 \cdot (-1.30) + 24.60 - 30.68 \\ &= 53.84^\circ\text{C} \end{aligned}$$

Polymerase Amount

The optimal amount of polymerase for use in a given reaction is dependent upon the template size and the type of template. For genomic or plasmid templates <5 kb in length, use the following enzyme concentrations:

Units	Rxn Size	Enzyme
1.25 U	50 μL	Ex Taq™
2.5 U	50 μL	LA Taq™
1.25 U	50 μL	PrimeSTAR®
1.25 U	50 μL	e2TAK™

Excess enzyme may facilitate non-specific amplification which can result in a diffuse smear of bands. In contrast, insufficient enzyme lowers the efficiency of amplification which may result in low or no product yields.

Points to Consider

Cycle Numbers

For most PCR reactions, the optimum cycle number is 25–30 cycles. The exact number should be determined by considering the quantity or complexity of template DNA and the length of the target DNA fragment. Insufficient cycles may result in low product yield, whereas excess cycles may encourage amplification of secondary products or contaminants, resulting in spurious bands or a diffuse smear upon electrophoresis.

Denaturation Conditions

When *Takara Ex Taq™* or *Takara LA Taq™* are used, denaturation for 10 seconds at 98°C is generally recommended. There may be applications that require a lower temperature for a longer time. When using thin-walled tubes, a shorter denaturation time (i.e. 20 seconds at 94°C) is recommended. *Takara's e2TAK™* DNA Polymerase and *PrimeSTAR®* HS DNA Polymerase both have 98°C denaturation times for 10 sec. These enzymes are made from a different thermostable enzyme compared to *Taq*.

It is critical that complete strand separation occur during the denaturation step to assure successful PCR. A denaturation time that is too short or a denaturation temperature that is too low may cause either diffuse smearing (due to the inability to generate full-length product) or poor amplification efficiency.

A denaturation time that is too long or a denaturation temperature that is too high may inactivate the polymerase, resulting in reduced levels of product.

Magnesium Concentration

Magnesium concentration is a critical factor in a PCR reaction. Optimal magnesium concentration may be affected by dNTP and template concentration, template-primer combinations, and chelating agents (i.e. EDTA) carried over with template DNA.

Magnesium affects the annealing of the oligo primer to the template DNA by stabilizing the oligo-template interaction. It also stabilizes the replication complex, which consists of polymerase with template-primer.

Excess Mg^{2+} tends to cause non-specific priming of template DNA and primer/primer interaction (smears on a gel), while insufficient Mg^{2+} may generate fewer or no amplified products.

The Mg^{2+} concentration along with the dNTP concentration can affect the fidelity of the polymerase and should be considered when problems with fidelity occur.

Primer Concentration

Optimal primer concentration ranges from 0.1 to 1.0 μM . At lower than optimum concentrations, amplification yield may be poor. At a higher than optimal concentration, non-specific reactions may outperform primer-specific amplifications.

dNTPs Concentration

dNTP's are the building blocks for DNA. It is important that they are pure and stable. An optimal dNTP premix that has been pre-dispensed works best as it can be added directly to the amplification reaction with minimal pipetting steps and errors. Optimal dNTP concentration in most PCR reactions is 200 μM or less. At lower than optimum concentrations, amplification yield may be poor. At a higher than optimal concentration, the degree of nucleotide misincorporation will increase.

Conditions for Annealing and Extension using *Taq*, *Ex Taq™* or *LA Taq™*

Determine the optimum annealing temperature experimentally by varying temperatures in 2°C increments over a range of 45°–68°C.

To perform a combined anneal-extension step at 68°C (i.e. Two Step or Shuttle PCR and omitting the denaturation step) the recommended time setting is 30–60 seconds per one kilobase of target sequence. When the temperature is set below 68°C, longer steps will be required as enzyme activity is reduced.

Annealing temperatures that are too high generate no amplification products, while temperatures that are too low may generate non-specific products. An extension time that is too short may generate no amplification products or predominantly non-specific, short products; while excessive extension times increase amplification of non-specific products, resulting in diffuse, smeared electrophoresis bands.

As both *Takara Ex Taq™* and *Takara LA Taq™* show good activity from 60°–68°C, Shuttle PCR can be performed within this range.

When long PCR amplification is performed (>5 kb), a significant increase in amplification efficiency may be obtained by using the Autosegment Extension Method (See Appendix II: FAQ).

Conditions for Annealing and Extension using *e2TAK™* and *PrimeSTAR®*

The annealing temperature for these two enzymes are different than the *Taq* based enzyme. *Takara* recommends using 55°C as the initial annealing temperature. The time for initial annealing is between 5-15 sec. and depends on the calculation of the primer T_m . When the $T_m > 55^\circ C$, set the time at 5 sec. When the $T_m < 55^\circ C$ set the annealing time at 15 sec.

Enhancing Reagents

Several additives are commonly used to enhance PCR performance. They include dimethyl sulfoxide, ACS grade (DMSO), bovine serum albumin (BSA), betaine, and glycerol. DMSO, betaine and glycerol act similarly by "melting" secondary structures and decreasing non-specific products, thus improving amplification efficiency as well as specificity.

Recommended final concentrations are:

- up to 5% for DMSO,
- 1% for glycerol, and
- 1M for betaine.
- BSA in concentrations of up to 0.8 $\mu g/\mu l$ have been shown to increase efficiency of the PCR reaction (even more than DMSO) by binding PCR inhibitors and acting as a nonspecific enzyme stabilizer⁽⁴⁾.

The usefulness of these adjuvants must be tested in each experiment to determine their utility.

Points to Consider

PCR Inhibitors and Their Concentrations (5)

Substance	Inhibitory concentration
SDS	>0.005% (w/v)
Phenol	>0.2% (v/v)
Ethanol	>1% (v/v)
Isopropanol	>1% (v/v)
Sodium acetate	> or = to 5 mM
Sodium chloride	> or = to 25 mM
EDTA	> or = to 0.5 mM
Hemoglobin	> or = to 1 mg/ml
Heparin	> or = to 0.15 i.U./ml
Urea	>20 mM
RT reaction mixture	> or = to 15%

REAL TIME PCR (qPCR)

In quantitative PCR (qPCR), PCR products are labeled using a fluorescent reporter molecule, and the quantity of product determined by measuring the fluorescence intensity of the reaction. Fluorescent signal can be measured at the end of the reaction (as in Endpoint PCR) or during the amplification process (real time qPCR).

Real Time qPCR analysis is performed during the exponential stage of an amplification reaction, where a direct relationship between amount of product, signal intensity, and quantity of initial template present exists. At this stage, the amount of product generated by the reaction is not limited by depletion of required reagents, accumulation of inhibitors, or inactivation of the polymerase. (All of these factors influence the amount of product generated in Endpoint PCR analysis, making it variable and unreliable). Real Time PCR has become widely used because it provides sensitive, reproducible results, even at very low amounts of input template.

A real time qPCR reaction includes four stages (see "Profile of a qPCR Reaction" on page 2). The initial phase is called the "Lag Phase". In this phase, the amount of product is doubling at each cycle, but the total amount of fluorescent signal incorporated into products is still too low to be detected by the instrument.

The second phase of the reaction is called the "Exponential Phase". In this phase, the reaction is very specific and the amount of template continues to double at each cycle. Quantitative measurements are taken in this phase.

The third phase is called the "Linear Phase", and here reaction components (dNTPs, primer, polymerase) are becoming depleted. The final phase is the "Plateau Phase". At this point, the reaction components are exhausted and the relationship between the amount of product and initial amount of template is most variable. The plateau phase is where End Point PCR is analyzed, and generally fails to provide accurate quantification.

Reporter Molecules or Detection Method

Generally, there are two general detection methods used for qPCR. The Intercalator Method uses a non-specific DNA binding dye such as SYBR® Green I, which fluoresces upon intercalation into dsDNA. As the amount of dsDNA in a PCR reaction increases (by specific amplification), the amount of SYBR® Green I fluorescence observed will increase as well.

The second detection method, Probe Detection, uses a double-

labeled sequence-specific probe composed of an oligonucleotide labeled with a fluorescent dye plus a quencher (see page 17). This probe fluoresces only when the probe hybridizes to a specific target. As the amount of target DNA in a reaction increases (via PCR amplification), the amount of fluorescence observed from probe hybridization will also increase.

Target DNA

The ideal qPCR target length is from 80 to 150 bp. It is possible to amplify longer targets if reaction times are adjusted, but this will give higher changes in reporter signal, (ΔR_n) due to an increase in SYBR® Green I incorporation (Intercalator Method). The GC content of the product should be between 40–60%, and obvious regions of secondary structure should be avoided.

Primer Design for Intercalator Assays

Care should be used when designing the primers for assays using a non-specific DNA binding dye (SYBR® Green II) for detection. This is because amplification of primer dimers and non-specific amplification products will be detected and could make the results inaccurate. The following parameters should be considered when designing primers for these assays:

- Primer length should be between 18–30 bases, with 40–60% GC content
- Primer annealing temperatures should be between 58–62°C
- The T_m difference between the primers should be less than 4°C
- The primers should exactly match the target sequence (no mismatches)
- Avoid runs of identical bases (i.e. AAAAA)
- Avoid T bases at the 3' end of the primer (this allows mismatching)
- Avoid complementarity within and between the primers so secondary structures and primer-dimers are avoided

Probe Design for Probe Detection Assays (TaqMan Assay)

For these assays, it is generally best to design the amplification primers first and then the probe. Also, although the presence of primer-dimers and non-specific amplification products will not be detected in these assays, they may influence the PCR dynamics and efficiency and should be avoided.

The following parameters apply for probe design in Probe Detection assays:

- Probe length should be between 18–30 bases with an optimal length of 20 bases
- Probe GC content should be between 30 and 80%
- The probe should contain more C than G bases
- G bases should be avoided on the probe 5' end (because of potential fluorophore quenching)
- The annealing temperature of the probe should be 8–10°C higher than the T_m of the primers
- The probe should be placed as close as possible to a primer without overlapping
- Avoid any complementarity with the primers
- Avoid continuous runs of a single base (especially G bases)

Points to Consider

Primer Design for Probe Detection Assays

General recommendations for primer design for Probe Detection Assays include:

- Primer length should be between 18–30 bases
- Primer GC content should be between 40–60%
- Primer annealing temperature should be between 58–62°C
- The T_m difference between the primers should be less than 4°C
- The primers should exactly match the target sequence (no mismatches)
- Avoid runs of identical bases (i.e. AAAAA)
- Avoid T bases at the 3' end of the primer (this allows mismatching)
- Avoid complementarity within and between the primers so secondary structures and primer-dimers are avoided
- Run a BLAST search on all primer and probe sequences to make sure they do not anneal to other targets

Choosing Reporter Dye and Quencher for Probes

For new users, SYBR® Green I is probably the best detection method, as the experimental design is more similar to that used in standard PCR assays, and the assay requires less optimization and expense as compared with Probe Detection.

However, if higher specificity is required, several manufacturers (i.e. Applied Biosystems) supply pre-optimized kits for popular targets using their Probe Detection technologies. Although expensive, if one of these systems is available for your target and is compatible with your instrument, its use may save a lot of time in initial experimental design and optimization.

If these kits do not neatly fit your application, the first step in experimental design is careful selection of a probe technology. The best choice will depend strongly on your target sequence, desired specificity and sensitivity, throughput, and instrumentation. (See Chapter 3 for more information on various Probe Detection Technologies and their advantages and disadvantages.)

Also important is identification of the available fluorescent reporter dyes and quenchers that are compatible with each other and with your instrument. Common reporter dyes include: FAM (fluorescein), HEX (hexachlorofluorescein), TET (tetrachlorofluorescein), Texas Red, Cy3 and Cy5.

TAMRA is a widely-used quencher, and a combination of FAM and TAM is used in the widely used fluorogenic 5' nuclease assay (TaqMan Assay, Applied Biosystems). However, use of “dark quenchers”, which are non-fluorescent quenchers which overlap with the reporter dyes' emission spectrum, have been recently gaining popularity. These include Dabcyl (azobenzene dye), the Black Hole Quencher™ (Biosearch Technologies) with three spectrum ranges, the Eclipse™ Dark Quencher (Nanogen) and Iowa Black Quenchers (IDT). See page 17 for a table of Compatible Reporter and Quencher Dyes.

Calculating the Quantity of the Target Gene

There are two techniques used to calculate the initial quantity of the target gene: Absolute Quantification and Relative Quantification.

Absolute Quantification uses a Standard Curve of Ct values derived from serial dilutions of a known standard to calculate the absolute quantity (i.e. number of copies present) of an experimental sample. Relative Quantification (comparative Ct method)

compares the difference in Ct values between two samples (most often an experimental gene and a “housekeeping gene”) to calculate relative amounts of template present.

Analysis of Data

I. Absolute Quantitation

- Standard sample must be accurately quantitated
- Multiplex analysis required; amplification of the internal control and of the gene(s) of interest is performed in a single tube
- The final result is usually reported relative to a defined unit (copies per ng of total RNA, per genome, per cell or mg of tissue)
- Uses: for viral load determination and inter-lab comparisons

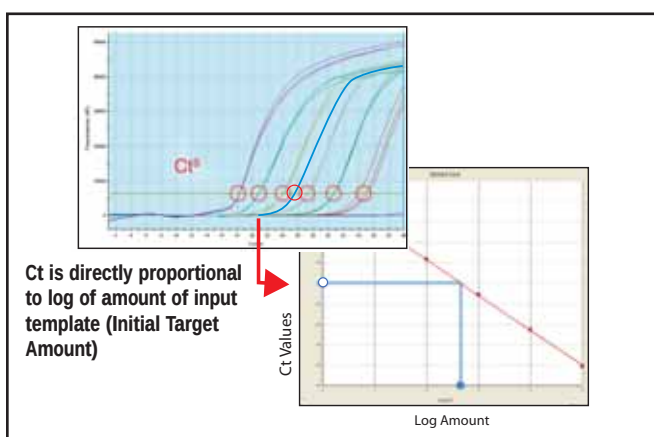
II. Relative Quantitation

- Results usually reported as a ratio of Gene of Interest/Endogenous Reference (Housekeeping Gene)
- Best used for gene expression studies

Number of Cycles vs Initial Target Concentration

When analyzing qPCR data, the basic principle is that an accurate estimate of initial target concentration can be determined by measuring the number of cycles required to reach a fixed concentration of reaction product.

Therefore, the number of cycles required to reach a given fluorescence intensity should correlate well with initial target concentration, as the fluorescence intensity values correlate with the concentration of the PCR product (see Demonstration of the Ct Value vs Log of Amount of Input Template below). The value at which the amount of product reaches a detectable level is called the “threshold fluorescence”, and the number of cycles required for any one reaction to reach it is the Ct or “threshold cycle”. These values are the key ones used in analysis of qPCR data.



Demonstration of the Ct Value vs Log of Amount of Input Template

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2. White, B., (1993) *PCR Protocols: Current Methods and Applications Methods in Microbiology*, Vol. 15.
3. Dieffenbach, C.W., and Dveksler, G. (1995) *PCR Primer: A Laboratory Manual*, Cold Spring Harbor Laboratory Press.
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5. “Critical Factors for Successful PCR” pg.29, Qiagen Inc.

Routine PCR

PCR Introduced

First introduced by Kary Mullis at a scientific conference in 1985, the Polymerase Chain Reaction (PCR) is a procedure in which a single DNA molecule can be replicated to over a billion copies. By combining double-stranded DNA with a thermostable polymerase and DNA primers (short complementary single-stranded DNA molecules that bind to the target DNA template), repeated cycles of temperature-controlled steps (i.e. denaturation, annealing and extension) result in mass production of the original DNA molecule. PCR has had far reaching consequences since 1985, and is used today in such diverse studies as taxonomy, evolution, medicine, ecology, archeology and forensics. Dr. Mullis won the 1993 Nobel Prize in Chemistry for his invention.

Cycling Reactions

The general premise of PCR is simple and founded upon the thermostability of a DNA double helix within a 55°C–94°C temperature range. An outline of the general steps taken during PCR amplification of DNA fragments ≤5kb is shown to the left.

Components of the PCR reaction mixture include:

1. A template or "target" DNA to be amplified;
2. A set of primers;
3. A buffer solution containing one or more salts (including Mg^{2+} , which influences the binding affinity of the primers to the DNA template);
4. dATP, dCTP, dGTP and dTTP (nucleotides to be added to the growing double-stranded duplex); and,
5. A thermostable DNA polymerase (e.g. *Taq* DNA Polymerase) to catalyze the reaction.

Routine *Taq* Based PCR steps:

Initial Denaturation Step:

94°C 1 minute

30 Cycles:

Denaturation Step:

94°C 30 sec

Annealing Step:

55°C 30 sec

Extension Step:

72°C 1 minute/kb

An Initial Denaturation step followed by a 3-step cycle (consisting of Denaturation, Annealing and Extension) comprise the four basic steps of the PCR reaction. During the Initial Denaturation Step, the double helix of the DNA template is destabilized at 94°C (i.e., melted) resulting in the production of two single-stranded DNA molecules. Failure to perform this Denaturation Step thoroughly can result in partially denatured substrates which contain regions unavailable for amplification and can lead to up to 50% loss in yields. It should be noted that temperatures above 95°C are not recommended for *Taq* based PCR denaturation due to the thermal stability properties of *Taq* DNA polymerase (*Taq* half-life at 95°C = 35 min vs. 97.5°C = 7 min). Takara's two non *Taq* based PCR enzymes (e2TAK™ and PrimeSTAR®) required 98°C denaturation temperature. Additionally, some templates (>5 kb and/or GC-rich templates) may require addition of an enhancing reagent (see page 5) to facilitate complete denaturation.

After initial denaturation of the template, a 3-step cycle consisting of Denaturation, Annealing and Extension (see outline above) is performed and repeated 30 times. The Denaturation Step functions to denature newly synthesized PCR products (i.e., products from the previous amplification cycle). Following denaturation, temperatures are lowered from 94°C to 55°C (or the T_m determined for primers used) for 30 seconds during the Annealing Step. This temperature drop allows binding of the primers to complementary sequences of the target region of each single-

stranded DNA template. During the Extension Step of a 3 step protocol, the temperature is then raised to 72°C, the optimal temperature for nucleotide (dATP, dCTP, dGTP, or dTTP) addition to the 3' end of the annealed primer by thermostable *Taq* polymerase. Nucleotides continue to be added to the 3' end of the strand until the temperature is raised again to 94°C (Denaturation Step), beginning the next round of cycling. Because *Taq* polymerase synthesizes the complementary strand of a growing DNA duplex at a rate of 1 kb/min, the Extension Step is generally performed at 68–72°C for 1 min/kb of target DNA to be amplified. This 3-step cycle is generally repeated ~30 times, and ultimately results in the production of ~1 billion copies of the target molecule (see diagram on page 3).

Routine PCR Steps for e2TAK™:

3-Step PCR

98°C	10 sec	} 30 cycles
55°C	5 sec or 15 sec	
72°C	1 min/kb	

2-Step PCR

98°C	10 sec	} 30 cycles
68°C	1 min/kb	

Routine PCR

Routine PCR can be defined as any PCR application that does not present special demands of length, fidelity, sensitivity, yield, template quality or sequence complexity. Enzyme fidelity refers to the ability of DNA polymerase to faithfully replicate the original template DNA sequence without error. The major advantages of performing Routine PCR are a minimal need for optimization and the ability to use a low cost enzyme like *Taq* or e2TAK™ DNA polymerase for amplification reactions, thus saving money, particularly if many reactions are being performed.

Many PCR Polymerases are cloned in *E. coli*, the quality of the enzyme needs to be confirmed especially for reactions using bacterial DNA templates. Testing for contamination from *E. coli* genomic DNA may need to be performed. Takara's PCR enzymes are tested and confirmed to be LD (low DNA) enzymes (≤10 fg *E. coli* DNA) as confirmed by nested PCR of the Ori region of the *E. coli* genome (see application on page 10).

However, even with a good quality routine polymerase, unforeseen problems can arise which will compromise the amplification process. For example, DNA fragments that possess secondary structure or have high GC content may prove difficult to amplify. In these cases, the addition of the organic solvent DMSO (dimethyl sulfoxide, ACS grade) to a final concentration of 5% or betaine at a 1M concentration in the PCR reaction, often helps to relieve the tension on the DNA molecule and allows the polymerase to proceed with synthesis.

Non-Specific Primer Design

Additional amplification problems can also arise due to non-specific primer design. Particularly when genomic DNA is used as the template DNA, it is possible that primers may share partial sequence similarity to regions of the genome other than the target fragment. Assembly of the reaction mixture at room temperature can facilitate annealing of primers at these undesirable regions, and these duplexes can be extended by *Taq* polymerase (partial activity exists at room temperature). This annealing results in the amplification of unwanted "secondary" PCR prod-

Routine PCR

ucts. To avoid such false starts, use of Hot Start Technology blocks *Taq* polymerase activity prior to the initial PCR denaturation step. Takara offers an antibody-mediated Hot Start Technology in which *Taq* DNA polymerase is supplied bound to a *Taq* antibody. The antibody is released from the enzyme during the Initial Denaturation Step of the PCR reaction. Thus, the enzyme remains sequestered during reaction assembly and is only released when the reaction mixture is heated during the Initial Denaturation Step, which allows primers to bind to the correct target sequences before synthesis begins.

Low Yield of PCR Product

Another common problem sometimes experienced with Routine PCR is low yields of PCR product. Because *Taq* lacks proofreading ability (i.e. the ability to replace incorrect nucleotides that have been inserted at the 3' end of the molecule with correct nucleotides), DNA synthesis can become temporarily stalled when nucleotide misincorporations occur. Such stalling trans-

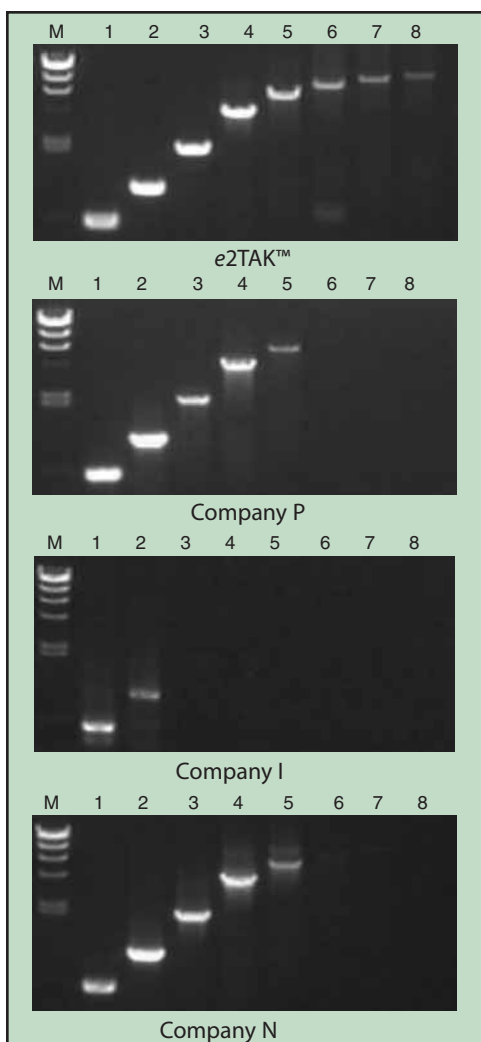
lates into a fewer number of mature PCR products being produced and, thus, lower PCR yields. Therefore, use of a PCR enzyme that crosses over into the High Performance category may prove useful for problematic low yield reactions. Such cross-over enzymes are often enzyme "cocktails." That is, they are composed of *Taq* DNA polymerase plus one or more proofreading polymerases. Cross-over enzymes provide some of the qualities of High Performance enzymes while still offering an attractive reduced cost.

Takara Taq™ DNA Polymerase and *e2TAK™* DNA Polymerase are high quality, versatile, thermostable DNA polymerases suitable for a variety of Routine PCR applications. *Takara Taq™* is also available in standard, premix and hot start versions.

For Routine PCR reactions that require higher yields with minimal optimization, *Takara Ex Taq™* DNA polymerase is an excellent Routine PCR enzyme that crosses over into the High Performance PCR category, but at a very affordable price.

Application: Routine PCR using *e2TAK™*

Amplification of Various Size λ Fragments using *e2TAK™* and Three Competitors



e2TAK™ provides high yield and excellent sensitivity in amplification of fragments up to 8 kb in size. The results are shown below.

PCR Conditions:

e2TAK™

98°C 10 sec
68°C 5 sec
72°C 1 min/kb

30 cycles

Company P

95°C 2 min
↓
95°C 30 sec
60°C 30 sec
72°C 1 min/kb
↓
72°C 5 min

30 cycles

Company I

94°C 2 min
↓
94°C 30 sec
60°C 30 sec
68°C 1 min/kb
↓
68°C 10 min

30 cycles

Company N

94°C 2 min
↓
94°C 30 sec
60°C 30 sec
72°C 6 min
↓
72°C 5 min

30 cycles

Fragment Sizes:

λ DNA: 1 ng

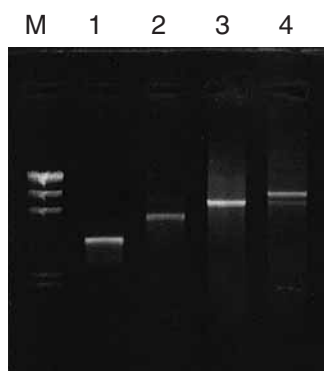
1: 0.5 kb
2: 1 kb
3: 2 kb
4: 4 kb
5: 6 kb
6: 8 kb
7: 10 kb
8: 12 kb
M: λ -Hind III digest

Amplification λ DNA Fragments of Various Size (0.5 kb-12 kb) using *e2TAK™* and Three Competitors. *e2TAK™* successfully amplified all 8 fragments with substantial yield compared to all three competitors.

Application: Routine PCR

Amplification of λ DNA using *TaKaRa Taq*TM DNA Polymerase.

The figure below demonstrates the versatility of *TaKaRa Taq*TM DNA Polymerase in generating PCR products up to 10 kb in length. Lanes 1-4 contain PCR products obtained using 1 ng of λ DNA template amplified using *TaKaRa Taq*TM DNA Polymerase with various primer sets. PCR products were analyzed by agarose gel electrophoresis.



Versatility of *TaKaRa Taq*TM DNA Polymerase in Amplification of λ DNA fragments up to 10 kb.

PCR Conditions:

4–6 kb	94°C	30 sec.	} 30 cycles
	60°C	30 sec.	
	72°C	3.5 min.	
8–10 kb	94°C	30 sec.	} 30 cycles
	60°C	30 sec.	
	72°C	6 min.	

Fragment Sizes:

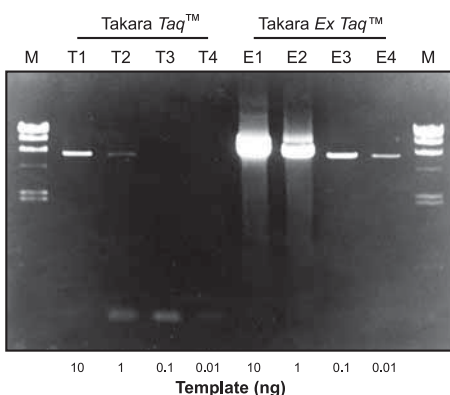
lane M:	λ -Hind III DNA Markers.
lane 1:	4 kb
lane 2:	6 kb
lane 3:	8 kb
lane 4:	10 kb

Reaction Mix:

<i>TaKaRa Taq</i> TM (5 units/ μ L)	0.25 μ L
10X PCR Buffer (Mg ²⁺ Plus)	5 μ L
dNTP Mixture (2.5 mM each)	4 μ L
λ DNA	1 ng
Primer 1	0.2 μ M
Primer 2	0.2 μ M
Sterilized dH ₂ O	up to 50 μ L

Amplification of a 6 kb Target from *E. coli* Genomic DNA with *TaKaRa Taq*TM and *TaKaRa Ex Taq*TM DNA Polymerases.

Significant increases in the amount of PCR product obtained can be observed when a high performance thermostable polymerase (i.e. *Ex Taq*TM) is used for routine amplifications. In the figure below, amplification of a 6 kb target from *E. coli* genomic DNA was performed using *TaKaRa Taq*TM vs. *Ex Taq*TM DNA Polymerases. Each 50 μ L reaction contained 1.25 units of enzyme and varying amounts of template DNA. Takara's robust *Ex Taq*TM enzyme-buffer system resulted in high product yields from even very small amounts of starting DNA (0.025 ng). This system also allows amplification of DNA from problem organisms and sources, including high polysaccharide plants, algae, and human biopsy and fecal specimens (see Appendix V: References).



Amplification of a 6 kb Target from *E. coli* Genomic DNA with *TaKaRa Taq*TM and *Ex Taq*TM DNA Polymerases.

PCR Conditions:

94°C 1 min	} 30 cycles
↓	
98°C 10 sec.	
68°C 10 min.	
↓	
72°C 10 min	

Template Concentration:

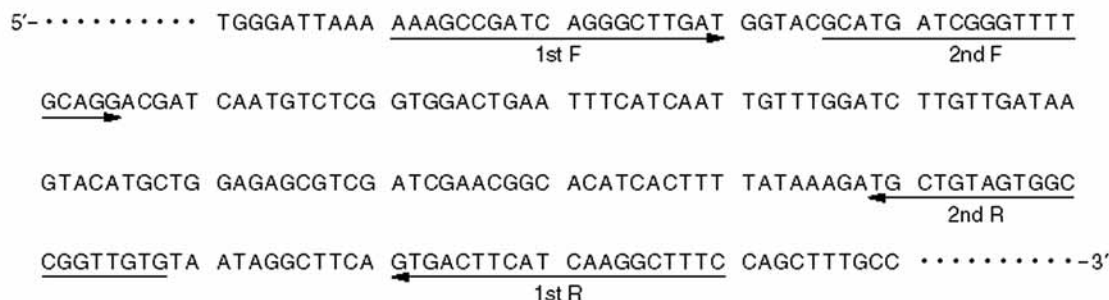
lane M:	λ -Hind III DNA Markers
lane T1:	10 ng
lane T2:	1 ng
lane T3:	0.1 ng
lane T4:	0.01 ng
lane E1:	10 ng
lane E2:	1 ng
lane E3:	0.1 ng
lane E4:	0.01 ng

Reaction Mix:

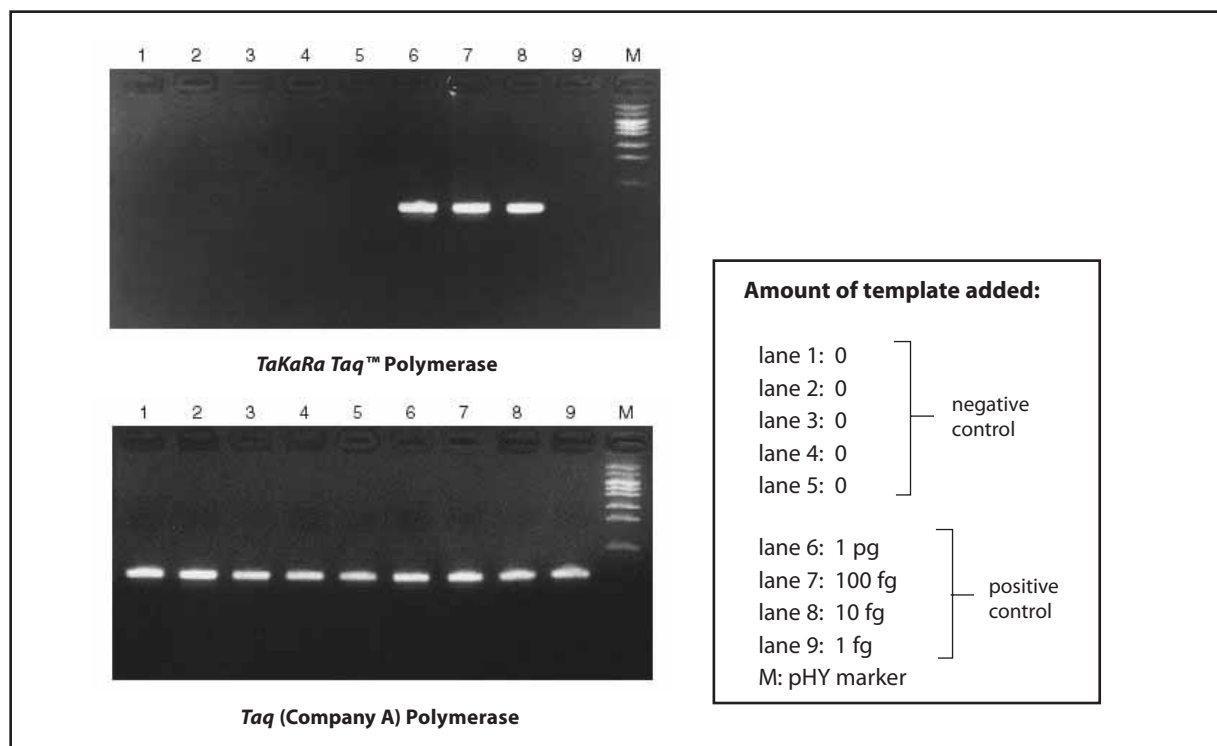
<i>TaKaRa Taq</i> TM or <i>Takara Ex Taq</i> TM (5 units/ μ L)	0.25 μ L
10X PCR or 10X <i>Ex Taq</i> TM Buffer (Mg ²⁺ Plus)	5 μ L
dNTP Mixture (2.5 mM each)	4 μ L
Template	0.01–10 ng
Primer 1	0.2 μ M
Primer 2	0.2 μ M
Sterilized dH ₂ O	up to 50 μ L

Determination of Polymerase Bacterial DNA Contamination

TaKaRa Taq™ is confirmed to be a low DNA contamination grade enzyme. Because typically *Taq* DNA polymerase is cloned in *E. coli*, it is especially important for bacterial amplifications to test *Taq* polymerase for the presence of contaminating *E. coli* genomic DNA. Quality control testing of *TaKaRa Taq™* for *E. coli* DNA contamination is performed by nested PCR of the *E. coli* genomic DNA Ori region.



Nested PCR of *E. coli* Ori region: 143 bp (2nd PCR)



Routine PCR Product Summary

e2TAK™ DNA Polymerase (TAK RF001)

Takara's e2TAK™ DNA Polymerase is a novel, economical and efficient PCR enzyme which provides excellent product yield, sensitivity, and product length (up to 8 kb human genomic DNA) for routine PCR applications. e2TAK™ also possesses superior priming efficiency, which allows shorter annealing time and high specificity.

TaKaRa Ex Taq™ DNA Polymerase (TAK RR001)

TaKaRa Ex Taq™ DNA Polymerase combines the proven performance of TaKaRa Taq™ DNA Polymerase with an efficient

3' → 5' exonuclease activity for excellent PCR performance. Ex Taq™ DNA Polymerase is a high yield-high sensitivity enzyme for increased fidelity and reproducible results in your PCR application.

TaKaRa Taq™ DNA Polymerase (TAK R001)

Takara Taq™ Polymerase is a recombinant, thermostable, 94 kDa DNA polymerase encoded by the DNA polymerase gene of the *Thermus aquaticus* YT-1 strain which has been cloned into *Escherichia coli*. It has been shown to have essentially the same characteristics as native *Taq* DNA polymerase.

For complete licensing information see page 56.

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

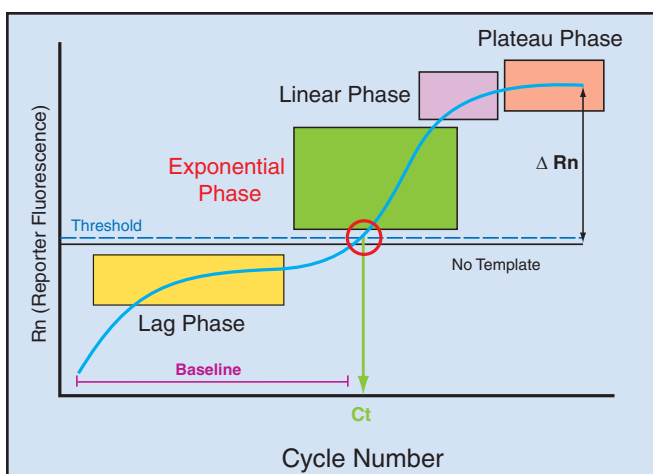


Figure 1: Profile of a qPCR Reaction.

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.

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 C_t) 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-

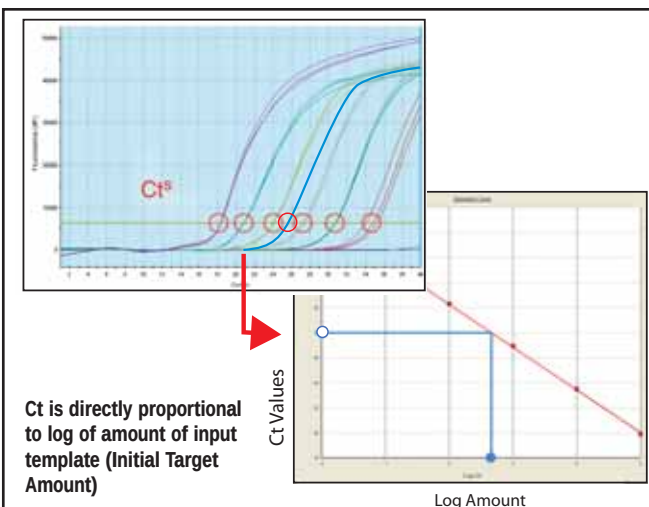
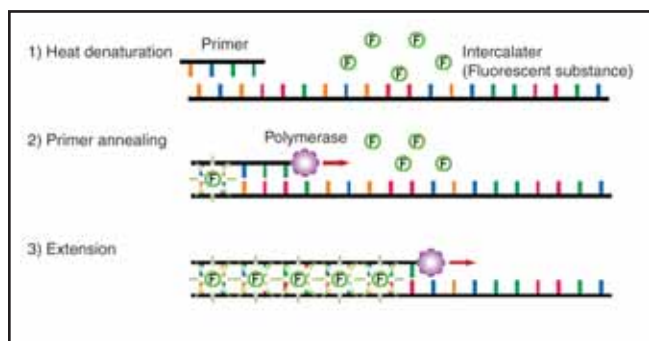


Figure 2: Demonstration of the C_t value vs Log of Amount of Input Template.

Real Time PCR (qPCR)

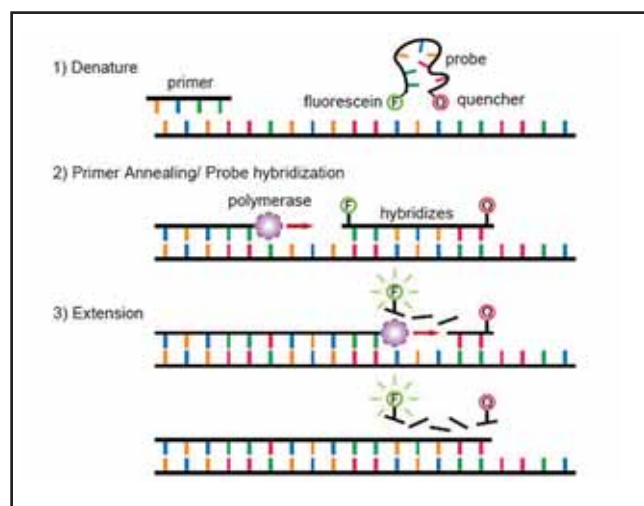
ured by the real-time PCR instrument. Takara's SYBR® Premix Ex Taq™ provides real-time quantitation of DNA using SYBR® Green I in a convenient, easy-to-use, premix formulation.



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

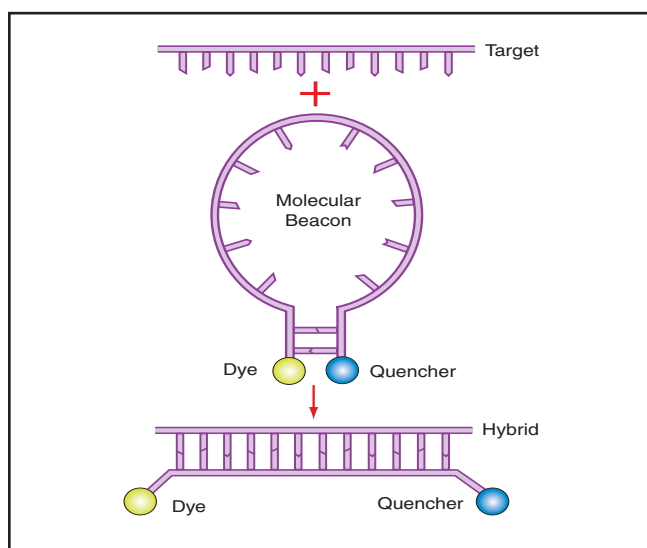
TaqMan® probes, corresponding to the number of PCR targets amplified, is observed in each cycle. Contrary to the SYBR® Green I method, where SYBR® Green I binds to any dsDNA molecule, TaqMan® probes bind only to a specific target molecule. An advantage of probe-based methods is that multiple probes, 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

Real Time PCR (qPCR)

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

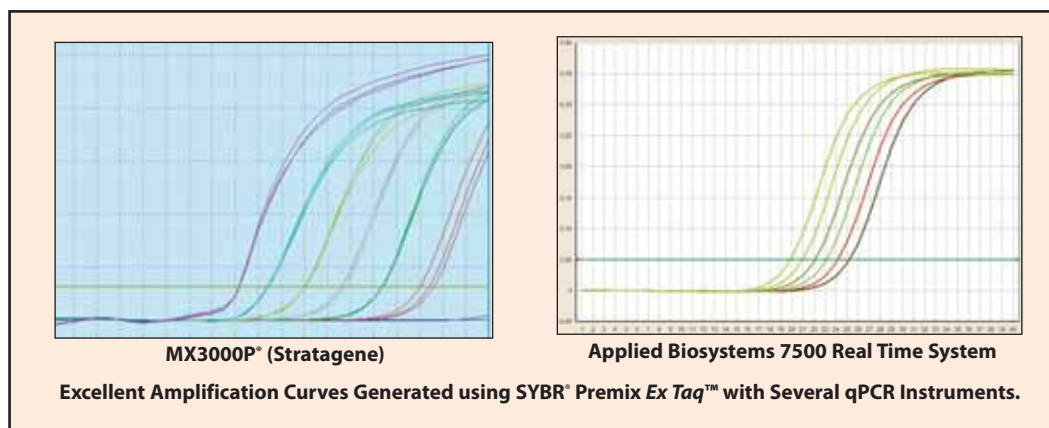
A passive fluor (e.g., ROX or fluorescein) is often used as a reference dye in fluorescence measurements. The dye is spiked into the PCR master mix at the beginning of the assay. The signal from the dye, generated by excitation at a frequency range determined by the thermal cycler, is assigned a reference 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



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 <i>Ex Taq</i> ™ (Perfect Real Time)	X		X	X	X	X	X	X	X	X	2X Premix* with SYBR® Green I, ROX™ reference† dyes I & II
Premix <i>Ex Taq</i> ™ (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, Mg²⁺ and SYBR® Green I

** contains *Ex Taq*™ Hot Start DNA Polymerase, buffer, dNTP mix, Mg²⁺

† 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.

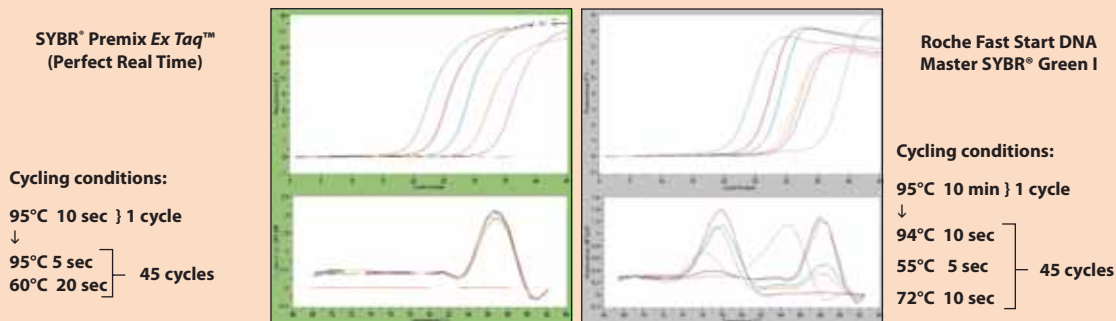
Application: qPCR using SYBR® Premix Ex Taq™ (Perfect Real Time)

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.

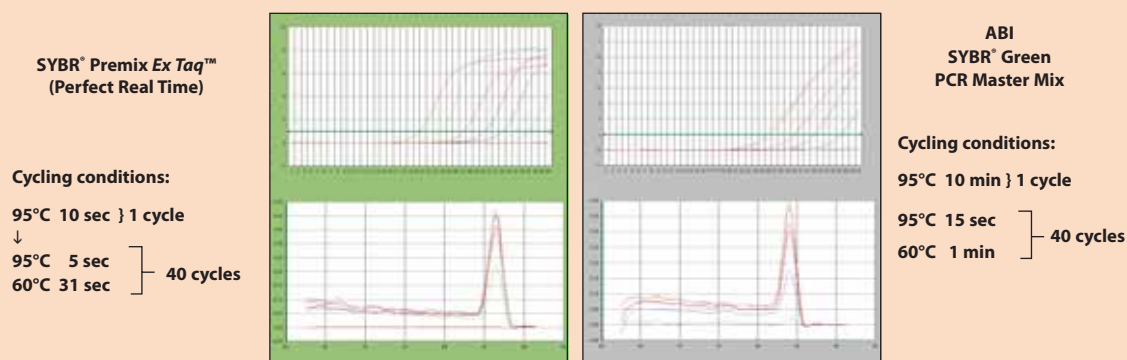
In **Figure 1**, Roche's real time enzyme shows poor reaction specificity when compared to Takara's SYBR® Premix Ex Taq™ as demonstrated by multiple peaks in the Roche melting curve, particularly when low copy number templates are amplified.

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®.



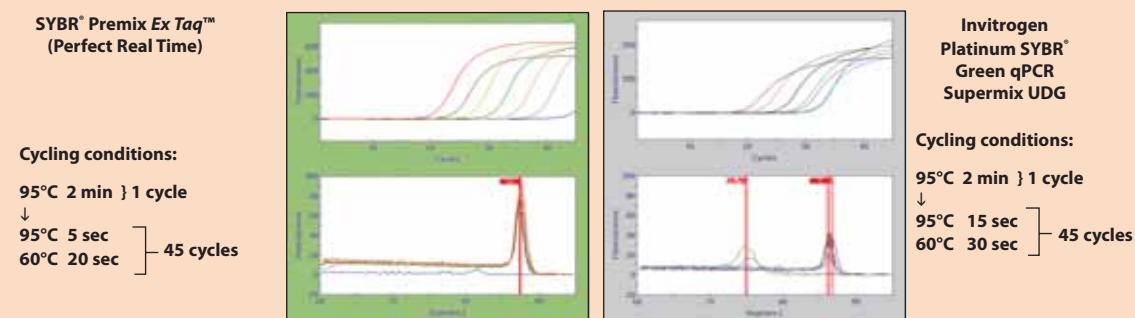
In **Figure 2**, low amplification efficiency is shown for ABI's SYBR® Green PCR Master Mix, indicated by Ct values which are shifted to the right and lower fluorescence intensity.

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 Ex Taq™ (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**

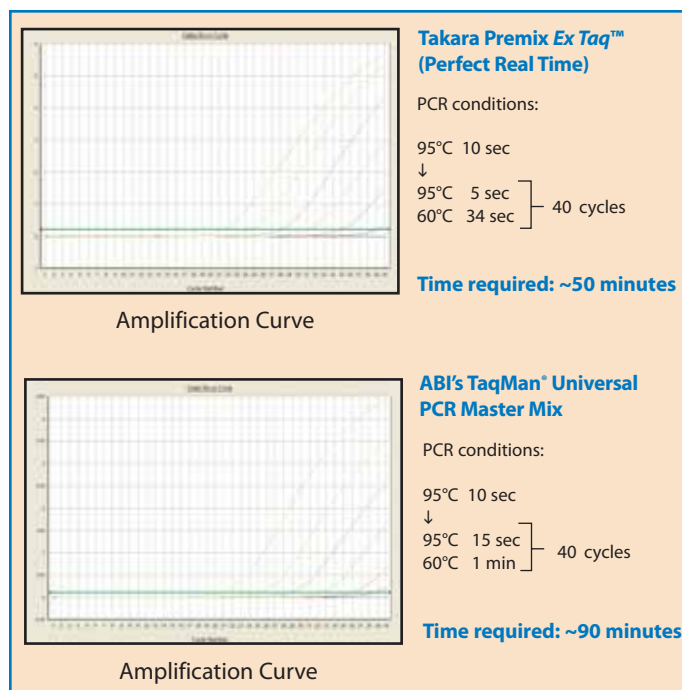
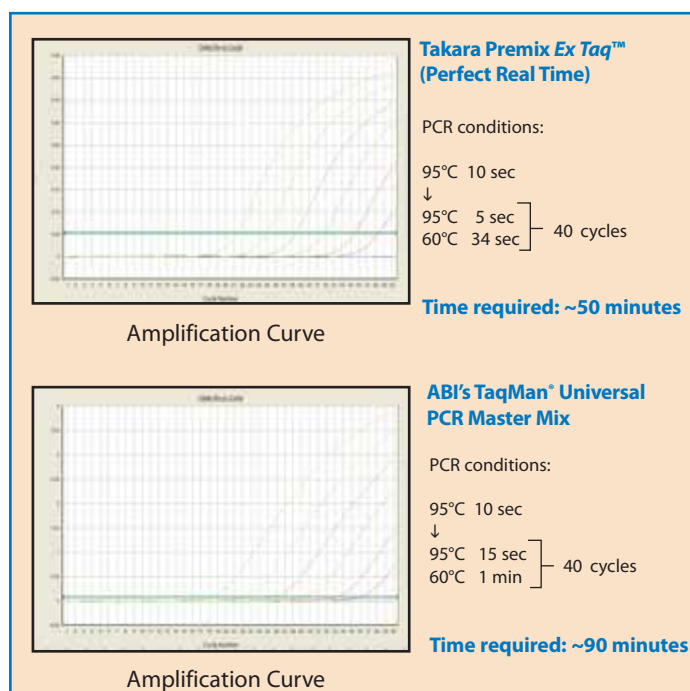


Figure 2: Performance of Premix Ex Taq™ (Perfect Real Time) or TaqMan® Universal PCR Master Mix with the TaqMan® Gene Expression Assays (Applied Biosystems). **Target: Mouse GAPD**



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.

Real Time PCR (qPCR)

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.

High Fidelity PCR

PCR has become a basic laboratory procedure, being performed thousands of times each day in laboratories worldwide. *Taq* Polymerase was the first thermostable polymerase to be made available to researchers, and is still the most widely-used PCR enzyme. It is a highly processive enzyme, suitable for many routine PCR applications. However, *Taq*'s performance is not adequate for other more demanding PCR applications, such as high-fidelity PCR, high-sensitivity PCR, or the synthesis of long or complex DNA targets.

Importance of High Fidelity

High polymerase fidelity (i.e. a low rate of base misincorporations, or errors) is most important in PCR applications where downstream sequencing or gene expression of the amplified product is desired. It is also significant in applications requiring amplification of low-copy-number templates (requiring many rounds of amplification), longer target sequences, or amplification and rare transcripts or allelic mutants. cDNA library construction, site directed mutagenesis, and mutation detection are also particularly sensitive to error rate.

Enzyme fidelity can be influenced by a variety of factors, including template sequence (i.e. GC-rich templates generally have increased error rates), cycling parameters, and reaction conditions (i.e. pH, Mg^{2+} , dNTP concentration). However, in controlled studies, polymerases exhibit characteristic rates of base misincorporations, rates of extension from those misincorporations, and 3'→5' exonuclease or proofreading activity. These factors together result in an intrinsic "error rate" for each polymerase.

Polymerase Fidelity

Taq polymerase and related *Thermus* family polymerases generally possess a high rate of base misincorporations, a low rate of extension from these misincorporations, and lack a 3'→5' exonuclease or "proofreading" function. Their error rates are the highest among the most widely-studied viral and bacterial polymerases. Additionally, the low extension rate actually acts somewhat as a de facto proofreading function, as incorrect templates fall out of the amplifiable pool. However, this results in lower yield and sensitivity, particularly on longer products.

Using conventional mutant-based fidelity assays, the recorded error rates of about 10^{-4} are common for *Taq*. This number may seem low, but this means that after one fairly typical 10^6 fold PCR amplification of a 200 bp target, up to 33% of the resulting products may contain errors.

The expected fraction of PCR-induced mutants can be calculated according to the following formula:

$$F(>1) = 1 - e^{-bdf}$$

b= length of target sequence

f= error rate

d=number of doublings

Pyrococcus sp. polymerases (also called "proofreading" polymerases) have an even higher initial misincorporation rate than *Taq*, but because they contain a 3'→5' exonuclease activity, they generally possess much lower error rates than *Taq* Polymerase or other *Thermus*-family polymerases. However, these enzymes often display low processivity, resulting in low product yield, reduced product length, and difficulties in optimization.

Mixing a proofreading polymerase with *Taq* polymerase has been shown to increase amplification performance, and is the basis for several widely-used enzymes, including *TaKaRa Ex Taq™* and *LA Taq™*. These blends provide superior amplification efficiency and product length as compared to *Taq* or the proofreader alone. Fidelity is also much improved over *Taq* polymerase alone, but may still be problematic in some applications.

Calculated Error Rate

Error rate and fidelity are calculated via the following formulas:

$$\text{Error Rate} = \frac{\# \text{ misincorporated bases}}{\# \text{ bases synthesized}}$$

$$\text{Fidelity} = 1/\text{error rate}$$

Most quoted error rates are experimentally based on indirect phenotypic measurements of mutant frequency, and vary widely. For example, one common method calculates the frequency of observed mutants by identifying the number of phenotypically altered colonies following bacterial transformation with a PCR-amplified DNA fragment. However, lethal amino acid substitutions derived from misincorporation of one or more incorrect bases during the PCR reaction will go unnoticed and uncounted, as they result in cell death. Fidelity rates calculated via this method are also subject to an additional level of inaccuracy because some nucleotide changes will not result in clear phenotypic changes of the expressed protein (usually beta-galactosidase). Therefore, these conventional methods of calculating error rates can provide useful comparisons within a single set of reaction conditions, but actual results may vary widely from predicted numbers.

Takara Bio recently introduced PrimeSTAR® HS DNA Polymerase, a novel DNA polymerase which offers very high fidelity as well as excellent amplification efficiency and extended product length (8.5 kb for human genomic DNA; 22 kb for λ DNA). PrimeSTAR® is the only currently available DNA polymerase whose error rate (only 15 errors per 480,000 bases on a GC-Rich template) is determined by DNA sequencing.

PrimeSTAR® HS DNA Polymerase

PrimeSTAR® HS is a recombinant enzyme expressed in *E. coli*. It was derived from a proprietary thermostable bacterial strain, and was chosen by Takara after studying a panel of bacterial strains which had been identified as potential producers of high fidelity polymerases. It has a very strong 3'→5' exonuclease activity, high replication accuracy, and extremely high priming efficiency. It also contains an antibody which inactivates both the polymerase and exonuclease functions during reaction assembly. This prevents false initiation events due to mispriming or primer digestion, resulting in lowered background and increased reproducibility.

High Fidelity PCR

Takara's fidelity assay for PrimeSTAR® is as follows:

Eight arbitrarily selected GC-rich regions were amplified with PrimeSTAR® HS and other enzymes, using *Thermus thermophilus* HB8 genomic DNA as a template. Each product (approx. 500 bp each) was then cloned into a suitable plasmid. Multiple clones were selected and subjected to sequence analysis.

Sequence analysis of DNA fragments amplified using PrimeSTAR® HS demonstrated only 15 mismatched bases per 480,000 total bases. This is higher fidelity than *Thermococcus kodakaraensis* DNA Polymerase (KOD), *Pfu*, and 10X higher fidelity than *Taq* DNA polymerase.

References:

- (1) Cha, R.; Thilly, W. in *PCR Primer, A Laboratory Manual*, 1995, 34-51.
- (2) Keohavong, P.; and Thilly, W. *Proc. Natl. Acad. Sci. USA*, 1989, 86:9253-9257.
- (3) Pavlov, R.; et. al. *TRENDS in Biotechnology*, 2004, 22:254-261.
- (4) Barnes, W. *Proc. Natl. Acad. USA*, 1994, 91:2216-220.

Application: High Fidelity PCR

Comparison of PrimeSTAR® HS Amplification Efficiency with Three Competing Enzymes on a 2 kb Human Genomic DNA Fragment.

Comparisons of the amplification efficiency of PrimeSTAR® HS DNA polymerase versus several competing high fidelity DNA polymerases were performed using the human DNA cross-link repair 1A gene (DCLRE1A), a 2 kb human genomic DNA fragment, and a high GC-content *Thermus* genomic template. The results are shown below. PrimeSTAR® demonstrated excellent specificity and high efficiency when amplifying the DCLRE1A 2 kb fragment.



PCR Conditions:

98 °C, 10 sec
60 °C, 5 sec
72 °C, 1 min/kb

30 cycles

Reaction Mix:

PrimeSTAR® (2.5U/μL)
5X PrimeSTAR Buffer (Mg⁺plus)
dNTP mixture
Primer 1
Primer 2
Template
dH₂O

Vol.

0.5 μL
10 μL
4 μL
10-15 pmol
10-15 pmol
~500 ng
up to 50 μL

Final

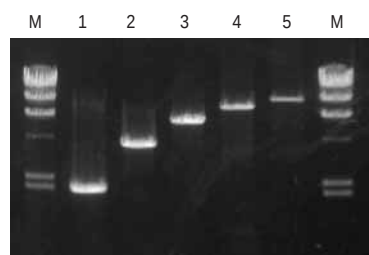
1.25U/50 μL
1 X
200 μM each
0.2-0.3 μM
0.2-0.3 μM

Template Concentration:

Lane 1: 0 ng (dH₂O)
Lane 2: 100 pg
Lane 3: 1 ng
Lane 4: 10 ng
Lane 5: 100 ng

Amplification of Varying Sizes of *E. coli* Genomic DNA Targets using PrimeSTAR® HS DNA Polymerase.

PrimeSTAR® was used to amplify varying sizes of *E. coli* Genomic DNA ranging from 2 kb to 10 kb. Excellent sensitivity, yield and specificity are demonstrated in the results below.



Amplification of *E. coli* DNA using PrimeSTAR®.

PCR Conditions:

98°C 10 sec
60°C 5 sec
1 min./kb } 30 cycles

Template DNA:

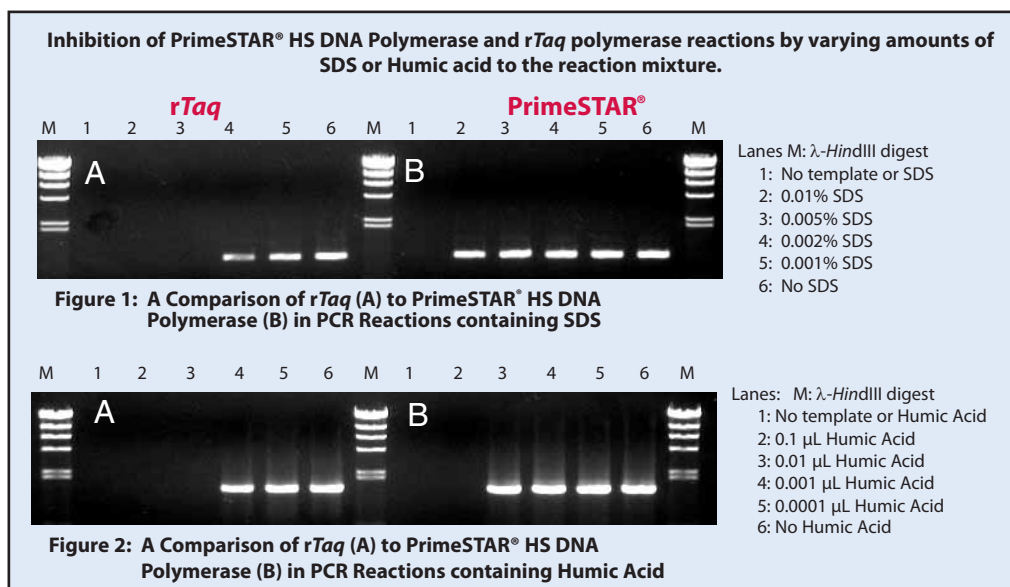
100 pg *E. coli* genomic DNA

Amplified Sizes:

M: λ -Hind III digest
1: 2 kb
2: 4 kb
3: 6 kb
4: 8 kb
5: 10 kb

Amplification of a 1.5 kb *E. coli* Genomic Fragment in the Presence of Varying Quantities of SDS and Humic acid using PrimeSTAR® HS

PrimeSTAR® has been used in many applications including protocols that require using samples with contaminating SDS or Humic (known PCR inhibitors). Humic acid can be found in environmental samples such as soil or marine sediments. It is an alkali-soluble and acid-insoluble fraction of humus soil and a reddish brown or blackish brown organic fraction in marine sediments. Even minute quantities of humic acid strongly inhibit PCR reactions. Special care should be taken when performing PCR from a DNA sample that could possibly be contaminated with humic acid.



Experiment:

A 1.5 kb *E. coli* genomic DNA target was used in PrimeSTAR® and *rTaq* amplifications in the presence of varying quantities of SDS and Humic Acid. (Figure 1 & 2) Figure 1 shows complete inhibition of *rTaq* in the presence of SDS at concentrations of 0.005% or higher (Figure 1A). In contrast, PrimeSTAR® HS DNA Polymerase amplification was not affected even when the SDS concentration was 0.01% (Figure 1B).

A "quick and dirty" crude extract containing humic acid from soil was diluted and added to a standard PCR reaction mixture, and inhibition of PrimeSTAR® HS DNA Polymerase and *rTaq* in PCR reactions was assessed. A known standard test control 1.5 kb *E. coli* genomic DNA fragment was used. (Figure 2) The *rTaq* reaction was inhibited when a solution equivalent to 0.001 μ L of humic acid was included in the reaction mix (Figure 2A). In contrast, PrimeSTAR® HS DNA Polymerase reaction was successful at levels up to a solution equivalent to 0.01 μ L of humic acid was added (Figure 2B).

Application: High Fidelity PCR

Amplification of a 3005 bp High-GC (73.2%) TthHB8 Genomic DNA Template using PrimeSTAR® with GC Buffer.

PrimeSTAR® HS DNA Polymerase with GC Buffer was developed for high-fidelity amplification of high-GC ($\geq 75\%$) templates. Fidelity is often reduced in high-GC amplifications. The new GC buffer formulation facilitates robust extension through even very high GC regions efficiently and with high accuracy. PrimeSTAR® HS DNA Polymerase with GC buffer provides reliable amplification, high accuracy, and high specificity in applications where amplification of high-GC DNA templates for cloning or library construction is required.



Amplification of a 3005 bp High-GC (73.2%) TthHB8 Genomic DNA Template. TthHB8 DNA; 3005 bp product, 73.2% GC. The performance of high fidelity, high-GC enzymes from Companies A, B, and C were compared with PrimeSTAR® HS DNA Polymerase with GC Buffer on a 3005 bp high-GC TthHB8 genomic DNA template. Lanes 1, 2, and 3: 100 pg, 1 ng, 10 ng human genomic DNA template.

PCR Conditions:

98°C 10 sec
60°C 5 sec
72°C 1 min/kb

30 cycles

Template Concentration:

M: λ -Hind III digest
1: 100 pg
2: 1 ng
3: 10 ng

Template DNA: Human genomic DNA

Reaction mix:

2 × PrimeSTAR® GC Buffer (Mg2+ plus)
dNTP Mixture (2.5 mM each) 4 μ L
Primer 1
Primer 2
Template
PrimeSTAR® HS DNA Polymerase (2.5 U/ μ L)
Sterilized dH₂O

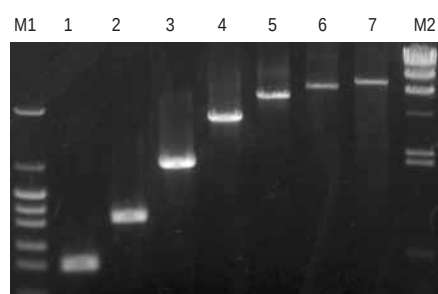
Volume

25 μ L
200 μ M
10~15 pmol
10~15 pmol
< 200 ng
0.5 μ L
up to 50 μ L

Final Conc.

1 X
0.2-0.03 μ M
0.2-0.03 μ M

Amplification of Various Sized Human Genomic DNA Fragments of Varying Sizes (0.5 to 8.5 kb) using PrimeSTAR® HS DNA Polymerase.



Amplification of Genomic DNA using PrimeSTAR®.

Template DNA:

100 ng human genomic DNA

PCR Conditions:

Template size
DNA: 0.5–6 kb
98°C 10 sec
60°C 5 sec
72°C 1 min/kb
DNA: 7.5–8.5 kb
98°C 10 sec
68°C 8 min

30 cycles

30 cycles

Fragment Sizes:

Lane M1: pHY Marker
Lane 1: 0.5 kb
Lane 2: 1 kb
Lane 3: 2 kb
Lane 4: 4 kb
Lane 5: 6 kb
Lane 6: 7.5 kb
Lane 7: 8.5 kb
Lane M2: λ -Hind III digest

High Fidelity PCR Product Summary

PrimeSTAR® HS DNA Polymerase (TAK R010)

TaKaRa PrimeSTAR® HS DNA Polymerase is a novel new high fidelity PCR enzyme which provides maximum fidelity as well as extended product length (8.5 kb for human genomic DNA; 22 kb for λ DNA). Targeted for demanding cloning (i.e. amplification of cDNA libraries) and sequencing applications, it offers extremely high accuracy, and fidelity calculated by direct sequence analysis.

PrimeSTAR® HS with GC Buffer (TAK R044)

PrimeSTAR® HS with GC Buffer was developed for high-fidelity amplification of high-GC ($\geq 75\%$) templates. The new GC buffer formulation facilitates robust extension through even very high-GC regions efficiently and accurately. Inclusion of a monoclonal antibody suppresses both the DNA polymerase and 3'→5' exonuclease activities prior to the first denaturing

step, preventing false initiation events during reaction assembly and primer digestion. PrimeSTAR® HS with GC buffer provides reliable amplification, high accuracy and high specificity in applications where amplification of high-GC DNA templates for cloning or library construction is required.

PrimeSTAR® HS DNA Premix (TAK R040)

PrimeSTAR® HS DNA Premix is a convenient 2X formulation containing PrimeSTAR® HS, PCR Buffer, MgCl₂, and dNTPs. The 2X premix solution of enzyme and reaction components provides the same high performance as the standard formulation and simplifies reaction assembly, minimizes the risk of contamination and increases reaction reproducibility.

For complete licensing information see page 56.

High Performance PCR

High Performance PCR Defined

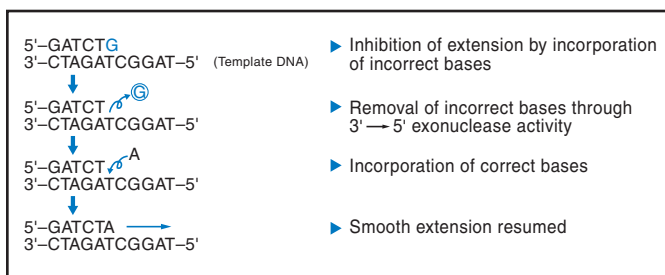
High performance PCR can be defined as any amplification that presents special demands of PCR product length, sensitivity, yield, template quality or sequence complexity. Takara Bio owns the patent rights to LA PCR technology which is the basis for most major high performance enzymes. Long and Accurate PCR technology uses a mixture of *Taq* polymerase with a proofreading polymerase to generate products with improved sensitivity, speed, fidelity, yield and length.

LA PCR Technology

Taq polymerase is the 94 kDa DNA polymerase derived from the extreme thermophile *Thermus aquaticus* YT-1, which has special applications in PCR amplification due to its ability to remain stable at temperatures close to 95°C. *Taq*, however, lacks significant 3'→5' exonuclease activity and, thus, displays a moderately high error rate during DNA synthesis. *Taq*'s error rate corresponds to approximately 1 mismatch/1000 bases. Since the initial discovery of *Taq*, other thermostable DNA polymerases have been identified (i.e., *Pfu*) which possess a much improved fidelity (i.e. 8 fold) as compared to *Taq*. However, most lack the processivity of *Taq* polymerase, are difficult to optimize and have poor reaction reproducibility. Mixing *Taq* with one or more of these proofreading enzymes yields a hybrid with performance characteristics superior to either enzyme alone.

Principle of LA PCR Technology

The key to LA PCR technology lies in the design of the PCR enzyme. Both *TaKaRa Ex Taq™* and *LA Taq™* are thermostable DNA polymerases which possess 3'→5' exonuclease activity, or proofreading activity. Polymerase efficiency declines drastically when incorrect bases are incorporated. Addition of a 3'→5' exonuclease activity removes these misincorporated bases and allows the reaction to proceed smoothly with increased yield, sensitivity, product length and fidelity.



PCR Fidelity

Fidelity, which is a measurement of the extent to which successful replication of a DNA strand occurs without introduction of sequence errors, is determined and affected by several factors, including: 1) the proofreading ability of the PCR polymerase; 2) the DNA template sequence itself; and, 3) the reaction mixture properties and components (e.g. pH and salt composition/concentration). During DNA proofreading, a 3'→5' exonuclease activity of the DNA polymerase excises and replaces a mismatched nucleotide that has been incorrectly added to the 3' end of a growing double-stranded chain. This process helps to ensure that the original template DNA sequence is perpetuated without error in all duplicated molecules.

Takara's High Performance Enzymes

Takara has three lines of high performance PCR enzymes that offer increased robustness, sensitivity, product length and speed over traditional *Taq*. *TaKaRa Ex Taq™* and *LA Taq™* DNA polymerases are enzyme "cocktails" composed of *Taq* plus one or more high fidelity enzymes, which allows increased performance and yield compared to traditional *Taq*. *TaKaRa Ex Taq™* DNA polymerase provides 4.5X** the fidelity* of regular *Taq* polymerase with very robust yields in both routine and high performance PCR. Additionally, amplifications of 1–30 kb fragments can be obtained with minimal optimization. *TaKaRa LA Taq™* is a high performance enzyme which offers 6.5X** the fidelity* of regular *Taq*, and is particularly suited for amplification of long DNA fragments (10–40 kb), although <1–10 kb size fragments can also be amplified well. *TaKaRa LA Taq™* is also available with 2 GC buffers designed specifically for use with templates that are GC-rich or contain secondary structure.

High Speed PCR

A typical PCR reaction consists of three steps: denaturation, annealing and extension, which are typically repeated 25–35 times. The reaction could take anywhere from 2 to 8 hours depending on template size. High speed PCR polymerases have been created but may be limited by equipment, template size and sensitivity (See Page 52 for a technical article on High Speed PCR). Takara's SpeedSTAR™ HS DNA Polymerase is a polymerase blend which allows extension times as short as 10 sec/kb and can amplify a 2 kb fragment in as little as 30 minutes. It can reduce reaction times by two-thirds without specialized instruments required by other high speed enzymes. The hot start formulation provides increased specificity and reduced background. SpeedSTAR™ is able to amplify fragments from <1.0 to 20 kb size fragments and is robust, fast, sensitive and reliable, which makes it ideal for high speed PCR.

*Fidelity is dependent upon many factors including template sequence, magnesium and dNTP concentrations, and may need to be empirically determined for your template.

**Fidelity was determined using the Cline and Kunkel methods ^(1,2).

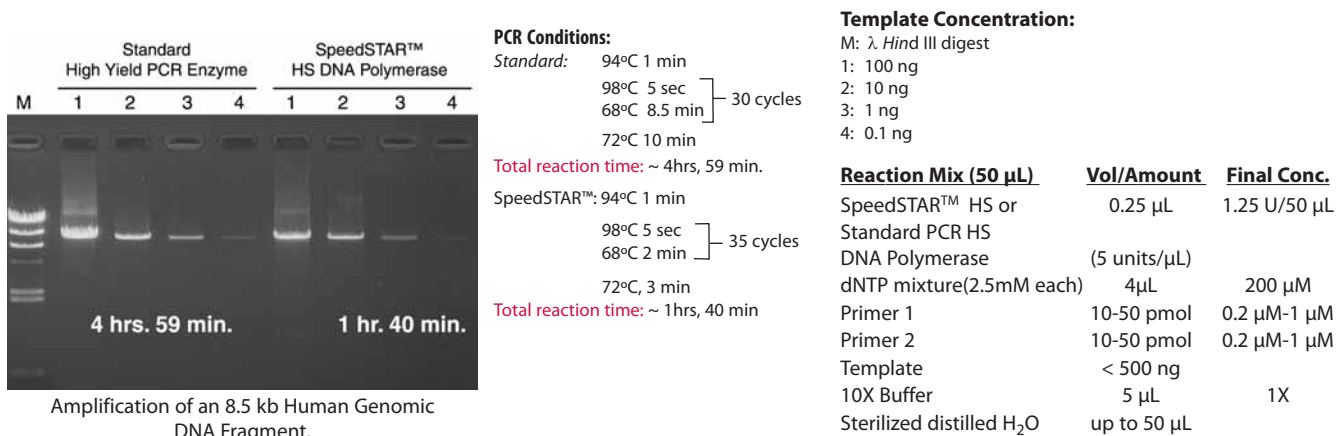
References:

1. Cline, J. et al. 1996. *Nucleic Acids Res.* **24**:3546-3551.
2. Kunkel, T.A. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 488.

Application: High Speed PCR

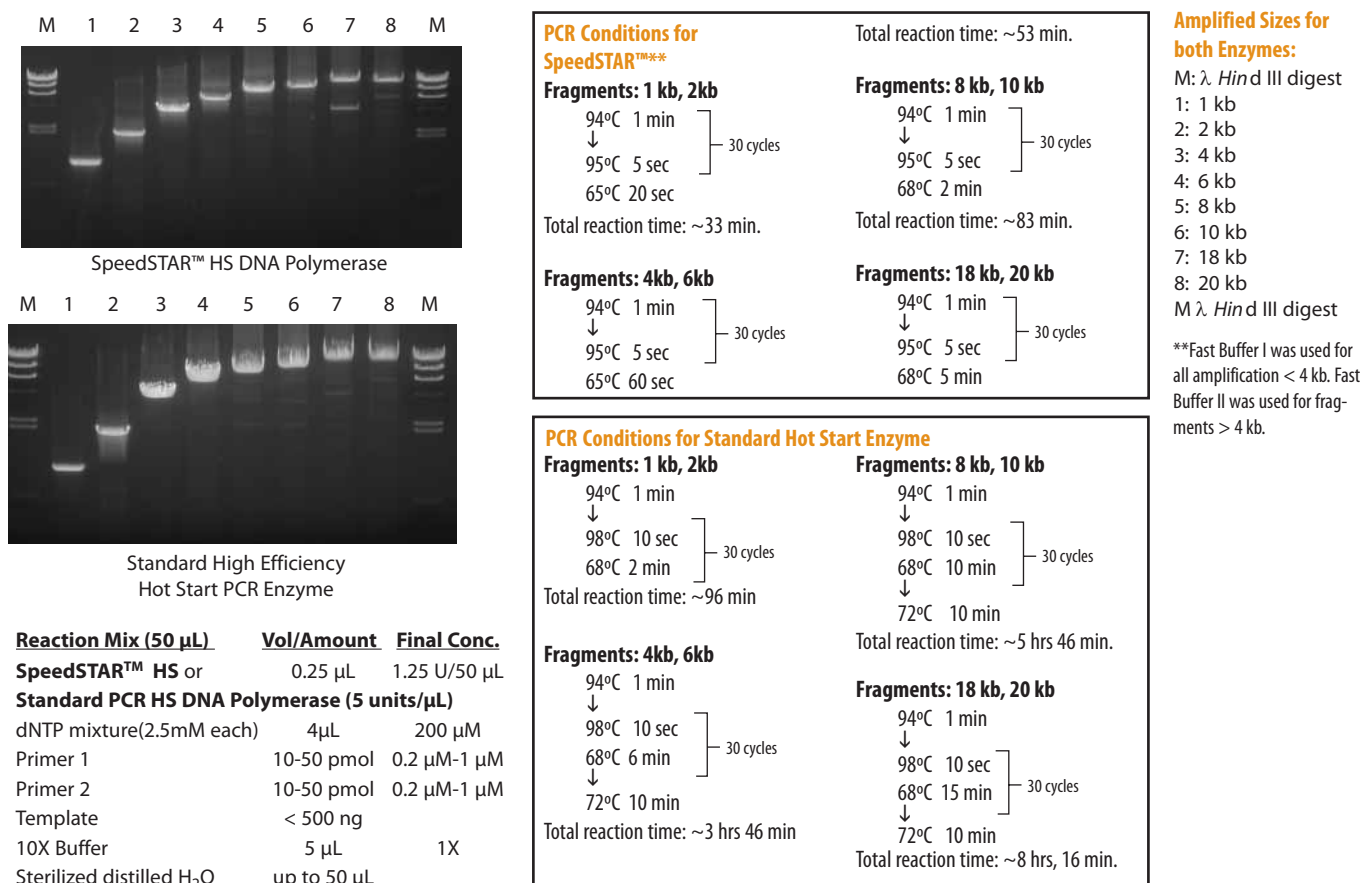
Amplification of an 8.5 kb Human Genomic DNA Fragment using a Standard High Yield Polymerase and SpeedSTAR™.

Comparison of SpeedSTAR™ and a Standard High Yield PCR enzyme were used to amplify a 8.5 kb human genomic DNA fragment. SpeedSTAR™ amplified the 8.5 kb fragment ~3 times faster than the Standard PCR enzyme with the same accuracy and yield.



Comparison of SpeedSTAR™ and a Standard High Efficiency PCR Enzyme was in Amplification of Fragments of Varying Sizes.

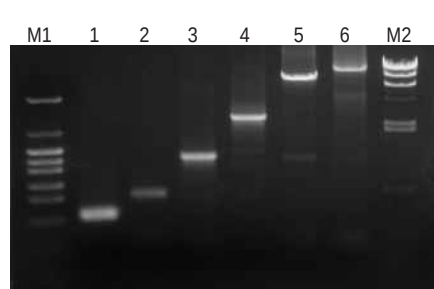
A comparison of detection sensitivity and reaction speed between Takara's SpeedSTAR™ HS DNA Polymerase and a standard high efficiency hot start DNA Polymerase was performed using *E. coli* genomic DNA targets of varying sizes. SpeedSTAR™ amplified these product fragments at the same sensitivity level as the high efficiency hot start enzyme, but required reaction times that were only one-third of those required for the other enzyme. All experiments were performed on the Takara DICE thermal cycler.



Application: High Speed PCR

Amplification of Human Genomic DNA Targets of Varying Sizes using SpeedSTAR™.

High speed amplification is especially valuable when amplifying large targets. The data below illustrates SpeedSTAR™ amplification of human genomic DNA targets from 0.3-17.5 kb in size with excellent specificity and yield. The reaction times were three times shorter than those required for standard long PCR enzymes (see Table 2).



Eight Different *E. coli* Genomic DNA Targets were Amplified using SpeedSTAR™ and a Standard High Efficiency Enzyme using the Takara DICE Thermocycler. Fast Buffer I was used in lanes 1 and 2; Fast Buffer II was used in lanes 3-8.

PCR Conditions:

94°C 1 min
↓
95°C 5 sec
68°C 45 sec } 30 cycles

Reaction Mix:

SpeedSTAR™ HS (1.25 U/50 µL)	0.25 µL
dNTP mixture (2.5mM each)	4 µL
Primer 1	10-50 pmol
Primer 2	10-50 pmol
Template	< 500 ng
10X Buffer	5 µL
Sterilized distilled H ₂ O	up to 50 µL

Amplified Sizes:

M1: pHY Marker
1: 0.3 kb
2: 0.5 kb
3: 1.0 kb
4: 2.7 kb
5: 8.5 kb
6: 17.5 kb
M2: λ Hind III digest

Fragment size	Target genome	Standard PCR	SpeedSTAR™ HS Polymerase
1 kb-2 kb	<i>E. coli</i>	96 min (2-step)	45 min
4 kb- 6 kb	<i>E. coli</i>	226 min (2-step)	53 min
8 kb- 10 kb	<i>E. coli</i>	346 min (2-step)	83 min
18 kb-20 kb	<i>E. coli</i>	8 hrs 16 min (2-step)	3 hrs 29 min

Table 1: Comparison of SpeedSTAR™ and Standard High Efficiency Enzyme Reaction Times on Fragments of Varying Sizes. (2-step refers to PCR cycle conditions)

Fragment size	Target genome	Standard PCR	SpeedSTAR™ HS Polymerase
8.5 kb	Human	4 hrs 59 min (2-step)	1 hr 40 min
17.5 kb	Human	8 hrs 16 min (2-step)	3 hrs 29 min

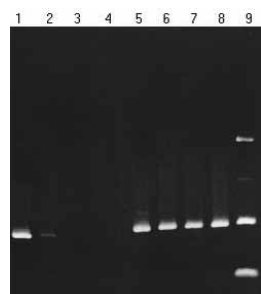
Table 2: Comparison of SpeedSTAR™ and Standard High Efficiency Enzyme Reaction Times on Large Size Human Genomic Targets. (2-step refers to PCR cycle conditions)

Application: High Performance PCR

Amplification of *Helicobacter pylori* DNA Extracted from Gastric Biopsy Specimens.*

Helicobacter pylori DNA was extracted from gastric biopsy specimens collected from patients with gastric ulcers. PCR was performed to confirm the presence of *H. pylori* and *H. pylori* NCTC11637 controls were loaded in lanes 1 and 5. This amplification was difficult because of an impure, low-abundance template which made a high yield enzyme necessary.

TaKaRa Ex Taq™ yielded abundant product with all three samples; *Taq* polymerase yielded only a small amount of product in specimen 1. In addition, this PCR method is much faster than the conventional culture method typically used for detection of *H. pylori*.



Lane contents:

1: *H. pylori* NCTC11637
2: Gastric biopsy specimen (1)
3: Gastric biopsy specimen (2)
4: Gastric biopsy specimen (3)
5: *H. pylori* NCTC11637
6: Gastric biopsy specimen (1)
7: Gastric biopsy specimen (2)
8: Gastric biopsy specimen (3)
9: Marker

TaKaRa Taq™

Ex Taq™

PCR Conditions:

94°C	30 sec	} 30 cycles
45°C	90 sec	
72°C	60 sec	
↓		
94°C	30 sec	} 10 cycles
45°C	90 sec	
72°C	90 sec	
Total	40 cycles	

Reaction Mix:

<i>TaKaRa Ex Taq™</i> or <i>TaKaRa Taq™</i> (5U/µL)	0.5 µL (2.5 U)
10X <i>Ex Taq™</i> or TaKaRa PCR Buffer	10 µL
dNTP mix	4 µL (2.5 mM each)
Primers (each)	0.2 µM
Template DNA	10 µL
Sterilized dH ₂ O	up to 100 µL

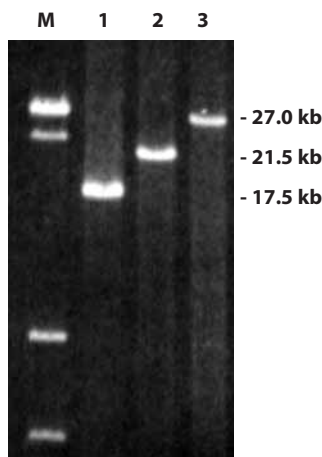
Amplification of *H. pylori* DNA Extracted from Gastric Biopsy Specimens.

*Data provided courtesy of Dr. Kurokawa, Dr. Nukina and Dr. Nakanishi, Public Health Research Institute of Kobe City.

Application: High Performance PCR

Amplification of β -globin Gene Cluster and the Human Tissue Plasminogen Activator (TPA) Gene using *TaKaRa LA Taq*TM.

Various target regions of the β -globin gene cluster and the Tissue Plasminogen Activator (TPA) gene were amplified using different primer sets. 500 ng of purified human genomic DNA was used in a 50 μ L reaction as a template for PCR with *TaKaRa LA Taq*TM DNA Polymerase. The amplified products ranged in size from 17.5–27.0 kb. Results of the amplification were separated by electrophoresis on a 0.4% SeaKem Gold Agarose gel. All bands yielded approximately equivalent amounts of product.



Amplification of Different Target Regions of the β -globin Gene Cluster and the Human TPA Gene.

PCR Conditions:

94°C	1 min	} 14 cycles
↓		
98°C	10–20 sec*	
68°C	20 min	} 16 cycles
↓		
98°C	10–20 sec	
68°C	20 min + 15 sec./cycle**	} 16 cycles
↓		
72°C	10 min	

*The denaturation conditions were based upon thermal cycler used, tubes, and type of PCR. High GC content made these fragments difficult templates to amplify.

** Autosegment extension Autosegment extension was used because of the length of the target.

Amplified Sizes:

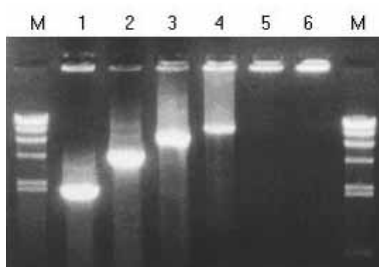
M: λ -Hind III digest
1: 17.5 kb (β -globin)
2: 21.5 kb (β -globin)
3: 27.0 kb (TPA)

Reaction Mix:

Human Genomic DNA (500 ng)	1 μ L
10X LA PCR Buffer II (Mg ²⁺ Plus)	5 μ L
dNTP Mix (2.5 mM each)	8 μ L
Primers (10 pmol/ μ L)	1 μ L each
<i>TaKaRa LA Taq</i> TM (5U/ μ L)	0.5 μ L
Sterilized dH ₂ O	up to 50 μ L

Heat Treated Cell Lysates Obtained from *E. coli* Cells Generate Fragments up to 10 kb with *TaKaRa LA Taq*TM.

To test the ability of *TaKaRa LA Taq*TM to amplify large fragments from difficult templates, an *E. coli* cell culture (37°C overnight in L-broth) was heat-treated at 98°C for 2 minutes, with 2 μ L of this lysate used as a template in a 50 μ L *LA Taq*TM PCR reaction. This amplification yielded a significant number of large fragments, up to 10 kb. This demonstrates *LA Taq*TM's robustness in amplifying large DNA fragments from impure templates.



Heat Treated Cell Lysates Obtained from *E. coli* Cells Generate Fragments up to 10 kb.

PCR Conditions:

94°C	1 min	} 30 cycles
↓		
98°C	10 sec	
68°C	15 min	} 30 cycles

Reaction Mix:

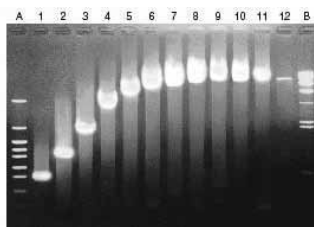
Heat-treated <i>E. coli</i> cells	2 μ L
10X LA PCR Buffer II (Mg ²⁺ Plus)	5 μ L
dNTP Mix (2.5 mM each)	8 μ L
Primers (20 pmol/ μ L each)	0.5 μ L
<i>TaKaRa LA Taq</i> TM (5U/ μ L)	0.5 μ L
Sterile dH ₂ O	up to 50 μ L

Amplified Sizes:

M: λ -Hind III digest
1: 2 kb
2: 4 kb
3: 6 kb
4: 10 kb
5: 20 kb
6: 30 kb
M: λ -Hind III digest

Amplification of λ DNA of Varying Lengths with *TaKaRa LA Taq*TM.

The ability of *TaKaRa LA Taq*TM to amplify DNA fragments from 0.5–35.0 kb in size using different primer sets was tested. *LA Taq*TM DNA Polymerase successfully amplified all the fragments and generated high product yields, even with very long fragments.



Amplification of Various Template Lengths of λ DNA from 0.5–35.0 kb.

PCR Conditions:

Lane 1-3:

94°C	1 min	} 30 cycles
↓		
98°C	5 sec	
68°C	5 min	
↓		
72°C	10 min	

Lane 4-12:

94°C	1 min	} 30 cycles
↓		
98°C	5 sec	
68°C	5 min	
↓		
72°C	10 min	

Amplified Sizes:

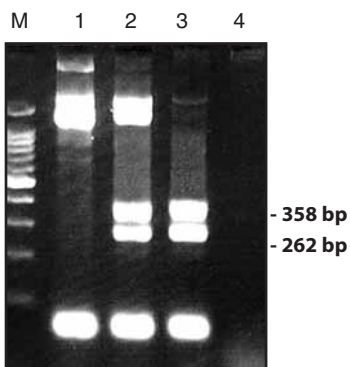
A:	pHY Marker
1:	0.5 kb
2:	1 kb
3:	2 kb
4:	4 kb
5:	6 kb
6:	8 kb
7:	10 kb
8:	12 kb
9:	15 kb
10:	20 kb
11:	28 kb
12:	35 kb
B:	λ -Hind III marker

Reaction Mix:

<i>TaKaRa LA Taq</i> TM (5 U/ μ L)	0.5 μ L
10X LA PCR Buffer II (Mg ²⁺ Plus)	5.0 μ L
dNTP Mix (2.5 mM each)	8.0 μ L
Template	10 pg
Primers	0.2 μ M each
Sterile dH ₂ O	up to 50 μ L

Amplification of a Huntington's Disease Gene (High-GC Content) using *TaKaRa LA Taq*TM.

*TaKaRa LA Taq*TM with GC Buffers was used in the amplification of two GC-rich portions of a Huntington's Disease gene (IT 15 CAG repeat). The fragments were amplified using *LA Taq*TM with either LA Buffer II (lane 1), GC Buffer I (lane 2), or GC Buffer II (lane 3). The GC content of the 262 bp fragment is 73%; the GC content of the 358 bp product is 71.5%. Amplification products obtained using a GC Kit from Company A, (reaction performed according to manufacturer's protocols), are shown in lane 4. Lane M contains a 100 bp molecular weight ladder.



Amplification of a Huntington's Disease Gene using *TaKaRa LA Taq*TM with GC buffers.

PCR Conditions (LA PCR Kit, Version 2.1)

94°C	1 min	} 30 cycles
↓		
94°C	30 sec	
60°C	30 sec	
72°C	1 min	
↓		
72°C	5 min	

PCR Conditions (GC kit from Company A)

94°C	1 min	} 30 cycles
↓		
94°C	30 sec	
68°C	3 min	
↓		
68°C	3 min	

Lane Contents:

M:	100 bp DNA ladder
1:	<i>LA Taq</i> TM with 10X LA PCR Buffer II
2:	<i>LA Taq</i> TM with 2X GC Buffer I
3:	<i>LA Taq</i> TM with 2X GC Buffer II
4:	GC kit from Company A

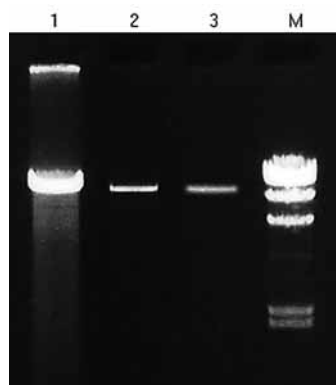
Reaction Mix:

Template	100 ng
2X GC Buffer I or II	25 μ L
dNTP Mix (2.5 mM each)	8 μ L
Primers	0.2 μ M each
<i>TaKaRa LA Taq</i> TM (5U/ μ L)	0.5 μ L
Sterile dH ₂ O	up to 50 μ L

Application: High Performance PCR

Amplification of a 21.5 kb Human Genomic DNA Fragment using *TaKaRa LA Taq*[™].

The efficiency of *TaKaRa LA Taq*[™] in amplification of a 21.5 kb genomic DNA fragment was measured at various template concentrations. Product generated even at the 5 ng level demonstrated the excellent sensitivity of *LA Taq*[™] DNA Polymerase in amplification of large, complex templates.



Amplification of a 21.5 kb Human Genomic DNA Fragment using *LA Taq*[™] and Various Amounts of Template DNA.

PCR Conditions

94°C	1 min	} 30 cycles
↓		
98°C	10 sec	
68°C	15 min	
↓		
72°C	10 min	

Template Concentration:

1:	500 ng
2:	50 ng
3:	5 ng
M:	λ-Hind III marker

Reaction Mix:

10X LA PCR Buffer II (Mg ²⁺ plus)	5 μL
dNTP Mix (2.5 mM each)	8 μL
Primer 1	0.2 μM
Primer 2	0.2 μM
<i>TaKaRa LA Taq</i> [™] (5 U/μL)	0.5 μL
Template	5–500 ng
Sterile dH ₂ O	up to 50 μL

High Performance Products Summary

SpeedSTAR[™] HS DNA Polymerase (TAK RR070)*

SpeedSTAR[™] HS DNA Polymerase is a convenient, efficient DNA polymerase specially optimized for fast PCR. Extension times of as little as 10 sec/kb are possible (compared to 60 sec/kb with general enzymes), dramatically reducing total reaction times. SpeedSTAR[™] reactions can be performed using standard PCR instrumentations, eliminating the requirement for special equipment. SpeedSTAR[™]'s robust two-buffer system facilitates efficient amplification of varying size fragments (up to 20 kb) with less optimization than other polymerases. In addition, the hot-start formulation provides convenience and reduced background.

TaKaRa Ex Taq[™] (TAK RR001)*

TaKaRa Ex Taq[™] DNA Polymerase combines the proven performance of *TaKaRa Taq*[™] DNA Polymerase with an efficient 3'→5' exonuclease activity for unsurpassed PCR performance. *Ex Taq*[™] DNA Polymerase is a high yield-high sensitivity enzyme which gives improved and more reproducible results in both routine PCR and high performance PCR applications.

TaKaRa LA Taq[™] (TAK RR002)*

TaKaRa LA Taq[™] is a mixture of *Taq* Polymerase with a proof-

reading polymerase optimized for amplification of long DNA templates. Using *LA Taq*[™], routine extensions to 20 kb, and up to 48 kb on some templates are possible, with less optimization than other long PCR systems. Because of the presence of the proofreading polymerase, the fidelity is better than that of *Taq* Polymerase alone.

TaKaRa LA Taq[™] with GC buffers (TAK RR02AG)*

TaKaRa LA Taq[™] with GC buffers is a version of *LA Taq*[™] supplied with two optimized buffers specifically designed to amplify DNA templates with high-GC content or a significant secondary structure.

LA PCR Kit, Version 2.1 (TAK RR013)*

The LA PCR Kit includes all the reagents necessary for amplification of large DNA templates, including *TaKaRa LA Taq*[™], 4 buffers (2 *LA Taq*[™] buffers, 2 GC buffer formulations), dNTP mixture, control template and primers (2 sets - one for normal templates one for GC rich templates) and a molecular weight marker. This kit can be used to optimize the amplification conditions of any long DNA fragment.

For complete licensing information see page 56.

*Fidelity is dependent upon many factors including template sequence, magnesium and dNTP concentrations, and may need to be empirically determined for your template.

Hot Start PCR

Importance of Primer Design

Primer design is one of the most important aspects of successful PCR. PCR primers, 20–30 bases in length, should be designed such that they have complete or very high sequence similarity to the desired target fragment to be amplified. However, even with well designed primers, amplification of unwanted or “secondary” products is possible, especially when the reaction mixture is assembled at room temperature. This problem most typically occurs when genomic DNA is used as the template and is due to mispriming (i.e. recognition of incorrect template binding sites due to partial sequence similarity between the designed primers and other regions of the genomic sequence). Assembly of reaction mixtures at room temperatures promotes incorrect binding of primers to undesired low T_m (melting temperature) genomic sequences. Additionally, *Taq* polymerase, which retains some activity at these temperatures, is able to initiate and extend DNA synthesis from a duplex molecule creating incorrect substrates for future rounds of amplification.

Hot Start Technology Introduced

Mispriming can be avoided by employing a Hot Start technology when assembling a PCR reaction. This can be as simple as waiting to add the *Taq* polymerase until after the Initial Denaturation Step. However, this is typically not convenient and increases the risk of contamination. The three most common strategies for Hot Start are sequestration, chemical modification and antibody-mediated methods

Sequestration methods involve separating a required reaction component until after the initial denaturing step. USBiologicals uses a protein which binds to all available primers for sequestration. Wax bead methods use a small hollow wax bead filled with *Taq* enzyme. During the Initial Denaturation Step of cycling, the

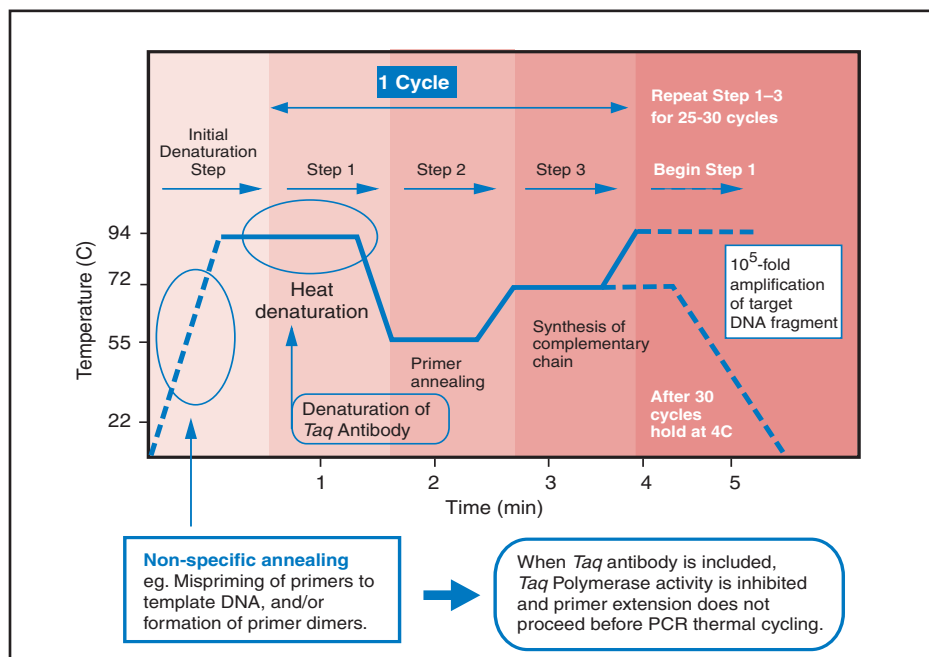
wax melts (~80°C), and the enzyme is allowed to mix with the rest of the reaction components. Although effective, the beads require cool temperature storage or refrigeration (to prevent softening of the wax) and researchers have found them to be somewhat expensive.

Chemically-modified *Taq* Hot Start methods use a *Taq* polymerase which has been modified with the addition of a heat-labile blocking group to a specific amino acid of the enzyme. The addition of a heat-labile group inactivates the enzyme at room temperatures. Incubation at 95°C for 15 minutes results in removal of the group and activation of the enzyme. One disadvantage with this method is that a long pre-incubation step is needed prior to cycling in order to activate the enzyme.

The third Hot Start Technology method, **antibody-mediated Hot Start**, relies on a *Taq* antibody which is bound to *Taq* DNA polymerase. The antibody-bound *Taq* complex is inactive until the Initial Denaturation Step, when the antibody is heat-denatured, releasing it from the enzyme and restoring full activity to *Taq* (see figure below). The antibody-mediated Hot Start method has proven to be an effective and inexpensive means of eliminating secondary PCR products, and does not require special storage precautions or added time-consuming incubation steps as do the previous two Hot Start methods.

Takara uses antibody-mediated Hot Start Technology for all of its Hot Start PCR products. Hot start enzymes are available for Routine PCR (*Taq* Hot Start, *Ex Taq*™ Hot Start), High Performance PCR (*Ex Taq*™ Hot Start, *LA Taq*™ Hot Start), and Real Time PCR (SYBR® Premix *Ex Taq*™ (Perfect Real Time)), Premix *Ex Taq*™ (Perfect Real Time), PrimeSTAR® HS DNA Polymerase and SpeedSTAR™ HS DNA Polymerase.

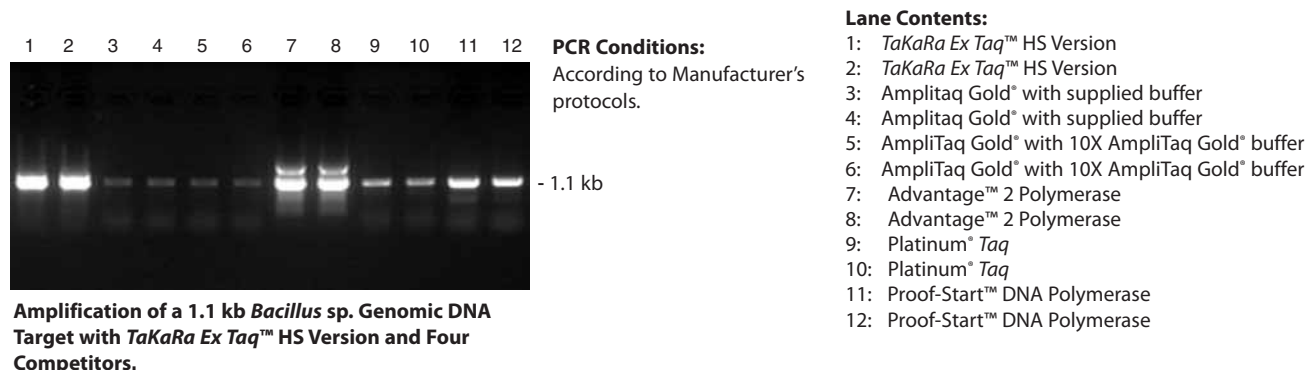
Profile of Hot Start PCR Reaction



Application: Hot Start PCR

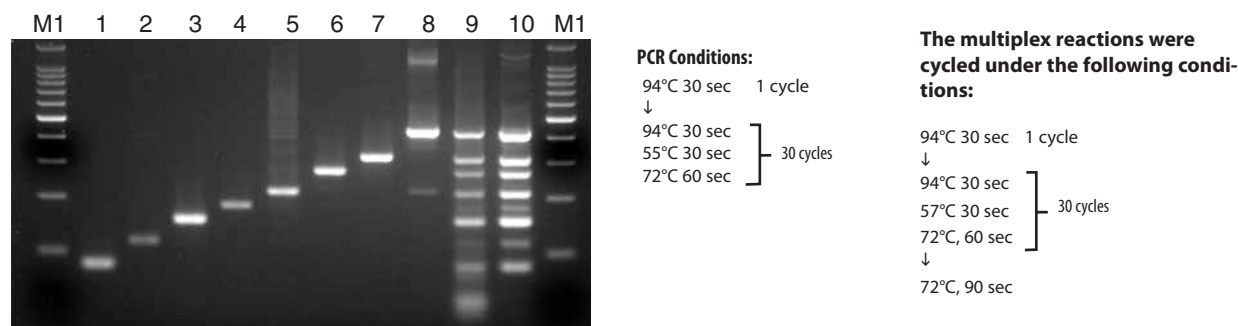
Comparison of *TaKaRa Ex Taq*™ Hot Start version Four Competing Hot Start Enzymes in Amplification of a 1.1 kb *Bacillus sp.* genomic DNA target.

The performance of *TaKaRa Ex Taq*™ Hot Start Version was compared against four competitor's enzymes in amplification of a *Bacillus sp.* target. *Ex Taq*™ Hot Start shows high specificity, no non-specific bands and high yield of the targeted product.



Application: Multiplex PCR

PCR reactions were performed using *TaKaRa Taq* or *Taq* HS to amplify a human genomic DNA template with eight different primer pairs, each specific for a target ranging from 84 to 432 bp in size. Lanes 1-8 contain individual reactions for each primer pair amplified using *TaKaRa HS Taq*. Lanes 9 and 10 include multiplex PCR reactions containing all eight primer pairs in a single tube, amplified with either *Taq* (lane 9) or *Taq* HS DNA Polymerase (lane 10). The results show that multiplex PCR using *Taq* HS results in target amplification efficiencies equivalent to that of separate (single target) amplification reactions. In addition, *Taq* HS demonstrates superior efficiency and specificity over standard *Taq* Polymerase in this multiplex PCR application.



Hot Start Products Summary

TaKaRa Taq™ Hot Start Version (TAK R007)

TaKaRa Taq™ HS Version offers the same reliable performance of *TaKaRa Taq*™ with the added benefit of Hot Start Technology.

TaKaRa Ex Taq™ Hot Start Version (TAK RR006)

Ex Taq™ HS DNA Polymerase offers the same high performance as the original *Ex Taq*™ Polymerase including high yield, excellent sensitivity and reliable results, along with the advantages of Hot-Start: lower background, increased specificity and room temperature reaction assembly.

TaKaRa LA Taq™ Hot Start Version (TAK RR042)

LA Taq™ Hot-Start DNA Polymerase consists of *LA Taq*™ DNA Polymerase plus a monoclonal *Taq* antibody bound to the polymerase. It retains all of the high performance features of *LA Taq*™ and, because the enzyme is sequestered by the antibody until the first denaturation step, it also provides increased reaction specificity and reduced background.

Also see SpeedSTAR™ HS (page 28) and PrimeSTAR® HS (page 22).

For complete licensing information see page 56.

Reverse Transcriptase PCR (RT-PCR)

Nuclear Genes

Nuclear genes have a complex structure consisting of coding (exon) and noncoding (intron) regions. Consequently, transcribed mRNA must be processed prior to translation to remove the noncoding regions from its genes. mRNA splicing, the mechanism by which noncoding introns are removed from the sequence and exon ends are joined together, takes place in the cell nucleus. This splicing process results in the creation of a continuous mRNA reading frame which encodes a full length functional protein. The spliced molecule is then exported into the cell cytoplasm, and its coding sequence is eventually translated into protein by cytoplasmic ribosomes.

RT-PCR Defined

Many gene expression studies preferentially analyze cDNA (i.e. complementary DNA, DNA derived from reverse transcription of an mRNA transcript that has undergone RNA splicing) rather than mRNA. Two advantages of using cDNA for analyses include the greater stability of cDNA over mRNA (mRNA is susceptible to RNase degradation) and the continuous reading frame sequence offered by the cDNA. Reverse transcription, the process by which RNA sequence is converted into DNA sequence, is accomplished by the enzyme reverse transcriptase. RT-PCR (reverse transcription PCR) synthesis of cDNA is a PCR amplification method that employs both reverse transcriptase and a thermostable polymerase to synthesize millions of copies of a cDNA sequence beginning from an mRNA transcript. In this procedure, the first PCR cycle (cycle 1, also called first strand synthesis) involves reverse transcription of an mRNA transcript into a cDNA template using a reverse transcriptase. In subsequent rounds of PCR cycling (cycles 2–30), the thermostable polymerase is used to amplify the cDNA template, creating millions of copies of the target cDNA molecule for study.

Different reverse transcriptases and DNA PCR polymerases are available for use in the RT-PCR process. Two common RT enzymes used for first strand synthesis are AMV (Avian Myeloblastosis Virus) Reverse Transcriptase and MMLV (Moloney Murine Leukemia Virus) Reverse Transcriptase. Both of these enzymes require a primer to initiate synthesis.

AMV Reverse Transcriptase is an RNA-dependent DNA polymerase that will synthesize a complementary DNA strand from a single-stranded RNA template in the presence of a primer. This enzyme possesses multiple activities in addition to its reverse transcriptase activity, including DNA-dependent DNA polymerase activity (which can be inhibited by Actinomycin D), RNase H activity (which results in degradation of the RNA strand of an RNA:DNA duplex) and unwinding activity. Also, the enzyme lacks 3'→5' exonuclease activity. AMV Reverse Transcriptase is particularly suited for reverse transcription of fragments containing secondary structure due to its high 42°C optimum temperature for activity. However, one drawback to this enzyme is its relatively high level of RNase H activity, which can limit the ultimate length and total yield of cDNA to be synthesized.

MMLV Reverse Transcriptase, like AMV Reverse Transcriptase, is

also an RNA-dependent DNA polymerase useful for first strand cDNA synthesis. This transcriptase also includes a DNA-dependent DNA polymerase activity and a weak RNase H activity. Further, the enzyme lacks 3'→5' exonuclease activity and has a lower optimum temperature for activity (37°C) than AMV Reverse Transcriptase. Because the RNase H activity of MMLV Reverse Transcriptase is much lower than that of AMV Reverse Transcriptase, MMLV Reverse Transcriptase is particularly recommended for synthesis of long cDNA fragments.

In addition to these two enzymes, Takara's *BcaBEST*™ RNA PCR Kit Version 1.1, offers a third reverse transcriptase, *BcaBEST*™ polymerase. This transcriptase has a 65°C optimum temperature for activity, offering superior reverse transcriptase activity for templates containing a very high degree of secondary structure (higher temperatures result in more efficient denaturing of bonds). Once first strand synthesis is completed, use of a high fidelity-high yield thermostable DNA polymerase then provides subsequent PCR amplification of the target gene. It is important to stress that the enzyme used for amplification should possess high fidelity, since reduced error rates are desirable for products that will either be sequenced or used in gene expression studies. Takara's RNA PCR Kit Version 3.0 includes improved fidelity *Ex Taq*™ Hot Start DNA polymerase for this purpose. *Ex Taq*™ Hot Start is an antibody-mediated hot start enzyme specially formulated to reduce unwanted amplification products due to mispriming, minimize reaction optimization and provide high yields of target genes with fidelity that is greater than regular *Taq* Polymerase. For applications where longer cDNA fragments (up to 12 kb) must be obtained, the RNA LA PCR Kit (AMV), Version 1.1, offers *TaKaRa LA Taq*™ DNA polymerase, a work-horse enzyme for very long PCR and with better fidelity than regular *Taq*. For real time RT-PCR, Takara's Real Time One Step RNA PCR Kit, Version 2.0, is supplied with a convenient 2X buffer, Reverse Transcriptase XL (AMV) and *TaKaRa Ex Taq*™ Hot Start for reverse transcription and amplification as well as ROX reference dye for normalization of real time signal intensity by background subtraction.

RT-PCR is a powerful process that has greatly enhanced gene expression analysis studies. Takara offers different RT-PCR kits that are well suited to handle various RT amplification needs.

RNA Purification

RT-PCR requires high quality poly A or Total RNA as a template, Takara carries a simple and quick RNA extraction kit for isolation of highly pure Total RNA from mammalian tissues, plants and cultured cells. The FastPure™ RNA Kit (TAK 9190) allows isolation of RNA without laborious and time-consuming organic extractions or ethanol precipitations by using a polymer filter with a high affinity for nucleic acids and centrifugation. Total RNA can be prepared with higher yield and purity than the standard methods.

Reverse Transcriptase PCR (RT-PCR)

RT-PCR Product Summary

FastPure™ RNA Kit (TAK 9190)

The FastPure™ RNA Kit is a simple and quick extraction kit for isolation of highly pure total RNA from cultured cells and mammalian tissues via centrifugation. This kit allows isolation of RNA without laborious and time-consuming organic extractions or ethanol precipitations. In addition, the polymer membrane used is more efficient at RNA extraction than conventional glass fiber filters, and total RNA can be prepared with higher yield and purity than standard methods.

RNA PCR Kit (AMV), Ver. 3.0 (TAK RR019)

Allows both RT and PCR reactions to be conducted in a single tube using AMV RT XL and *TaKaRa Ex Taq*™ Hot Start Version. The supplied Oligo-dT Adaptor Primer is constructed to have M13 primer M4 sequences at the 5' side of the dT region. This arrangement allows efficient amplification of unknown 3' termini using 3'-RACE.

One-Step RNA PCR Kit (AMV) (TAK RR024)

Allows reverse transcription of RNA to cDNA and subsequent amplification in a single tube without adding reagents during the protocol. This kit, using AMV RT XL and AMV-optimized *Taq*, gives results equivalent to those obtained with two-step RNA PCR protocols, while minimizing pipetting errors and the risk of contamination.

Real Time One Step RNA PCR Kit (TAK RR026)

Real time RT-PCR (synthesis of cDNA from total RNA or mRNA using reverse transcriptase, and subsequent monitoring of the cDNA amplification products) is an essential tool for RNA analysis, since it allows analysis of even tiny amounts of RNA. Takara's Real Time One Step RNA PCR Kit reaction is performed in a single tube, and real-time monitoring of the amplification process is performed using either SYBR® Green I or *TaqMan*® probes.

RNA LA PCR Kit, Ver. 1.1 (TAK RR012)

Designed to perform longer and more accurate RT-PCR reactions in a single tube using AMV RT XL and *LA Taq*™ DNA Polymerase. cDNAs of up to 12 kb can be synthesized with this kit. The supplied Oligo-dT-Adaptor Primer is constructed to have M13 primer M4 sequences at the 5' side of the dT region, allowing efficient amplification of 3' termini using 3'-RACE.

BcaBEST™ RNA PCR Kit, Ver. 1.1 (TAK RR023)

Utilizes the high optimum reverse transcription temperature of *BcaBEST*™ Polymerase (65°C) enabling cDNA synthesis from GC-rich templates or RNA having high secondary structure. The subsequent cDNA synthesis can be performed in the same tube using *Bca*-Optimized *Taq* Polymerase, which utilizes long and accurate PCR technology.

For complete licensing information see page 56.

PCR Cloning

PCR Cloning

One of the most common applications for PCR fragments is cloning into a plasmid vector for sequencing, storage or protein expression. Generally, PCR products contain either blunt or single-base 3' A overhang ends, generated because of a terminal transferase-like action of *Taq* polymerase. Traditional restriction fragment cloning techniques rely on the creation of "sticky ended" DNA overhangs, typically between 2–4 bases long, which enhance ligation reactions by creating a small amount of complementarity between the insert and vector termini. Although restriction sites can be incorporated into PCR primers allowing standard sticky-ended cloning, this method increases the cost of the reaction (because longer primers must be synthesized), and also introduces potential problems related to mispriming, poor enzyme cleavage at DNA ends and unanticipated internal product cleavage (particularly when amplifying a product of unknown sequence).

Most commonly, PCR products are cloned via blunt-ended cloning or by a variation of traditional cloning called TA-cloning. TA-cloning takes advantage of a special cloning vector, called a T-vector, which possesses a short 3' T overhang, thus making it "sticky" to the 3' A overhang of a PCR product. These can be purchased or created via incubation of blunt-ended vectors with *Taq* polymerase (many protocols are available).

Ligation of blunt-ended PCR products into plasmid vectors can be more difficult because of the complete lack of overhang ends on the insert and vector molecules. Accordingly, longer ligation reactions at lower temperatures are recommended for blunt-ended ligations (i.e. overnight incubations at 16°C can enhance the success of blunt-ended ligations). Note that these ligations can also benefit from the use of 5' dephosphorylated vectors. Dephosphorylation of vectors, using bacterial alkaline phosphatase (BAP) or calf intestinal phosphatase (CIP), prevents vectors from self-ligating, thus increasing the opportunity for insertion of a fragment.

For more information on general cloning see Sambrook, Fritsch and Maniatis' *Molecular Cloning, A Laboratory Manual* by Cold Spring Harbor Laboratory Press.

Choosing a Vector

The four most important factors to consider when choosing a vector to be used in a ligation reaction include: 1) the size of the DNA insert; 2) the purpose for cloning; 3) the multiple cloning site (MCS); and, 4) the antibiotic selection marker(s) required.

Insert size is the first consideration to be made when cloning. For routine cloning (i.e. cloning of fragments from 0.1–8 kb), plasmids are the vectors of choice. Most plasmid vectors range in size from 2.6–5.5 kb, and normally accept inserts which are approximately matched in size or smaller than the size of the vector. Based upon an average plasmid vector size of 3.2 kb, it becomes increasingly more difficult to successfully clone a fragment as the DNA insert size increases above 5 kb. Previously, these fragments would generally have required digestion into two smaller fragments before cloning. However, Takara's DNA Ligation Kit LONG is specially formulated for excellent performance in ligation of fragments 10 kb or larger.

If the **purpose of cloning** is for basic long-term storage of the insert, then use of a general cloning vector, such as one of the

pUC plasmid series, will be sufficient. If sequence analysis of the insert is the goal, then a vector which contains sequencing primer sites must be considered. If the purpose of cloning is to express the gene in a bacterial system and obtain recombinant protein, then a vector which will provide a strong promoter is desirable. Some vectors, such as Takara's pCold vector series, offer unique promoters for gene expression. The pCold vector series each contain the *cspA* promoter for gene expression, which selectively allows expression of the target gene with subsequent protein synthesis at cool (15°C) temperatures, resulting in high yields (up to a 60% maximum of total intracellular protein) of the target protein.

The third vector consideration for blunt ends is the **choice of restriction enzyme sites** that are contained within the MCS. Most commonly used vectors contain at least one blunt-ended cloning site in the MCS, although more obscure vectors may have limited site selection, and may need further modification before use. (For TA cloning considerations, see previous section).

Finally, **choice of a selection marker** (i.e. antibiotic resistance gene) contained by the vector must be made in order to identify and retrieve ligated DNA once transformed into competent *E. coli* cells.

Common antibiotic resistance genes include *amp^r* (ampicillin), *tet^r* (tetracycline), and *cm^r* (chloramphenicol). Usually the choice of a selection marker is not critical unless you plan to express more than one target gene in your *E. coli* expression system. In this case, it is important that each plasmid carry a different selection marker so that transformation with each plasmid can be verified.

Takara has four ligation kits to suit any DNA ligation need. DNA Ligation Kit LONG is specially optimized for difficult long ligations even with blunt ends. It also provides excellent performance on smaller fragments. The DNA Ligation Kit, Version 2.1 provides simple ligation reactions for circular sticky-ended plasmids in 30 minutes at 16°C or 5 minutes at 25°C. The third ligation kit, the DNA Ligation Kit, Version 1.0, is recommended for linear ligations such as λ DNA concatenations as well as circular plasmid ligations. The DNA Ligation Kit, Mighty Mix has a single premix solution that offers quick, high efficiency ligation reactions (even for blunt-ended and TA-cloning reactions), in 30 minutes at 16°C or 5 minutes at 25°C.

Blunt-Ended Cloning Protocol:

For amplifications that yield blunt end products. For products with unknown ends use Klenow Fragment to fill them in.

A protocol is provided below:

Use a Total Reaction Volume of 50 μ L

Set the Reaction up as follows:	
DNA plasmid(final conc 100 μ g)	X μ L
DNA Fragment(final conc 400ng)	X μ L
10X ligase buffer	5 μ L
10mM ATP	5 μ L
DNA Ligase	2.5 μ L
ddH ₂ O	up to 50 μ L
Total	50 μ L

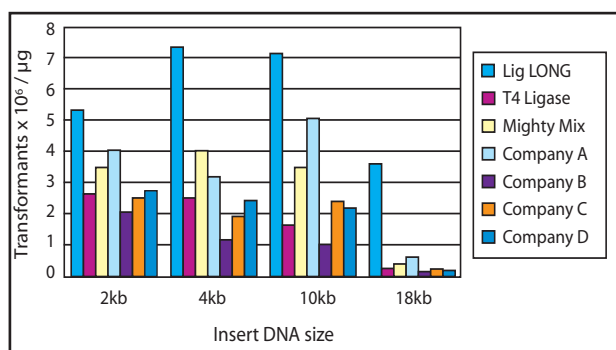
Gently tap the tube several times to mix but do not vortex.

Incubate at 16° C for 6-8 hrs.

Note: Do not forget to do a negative control of vector only.

Application: DNA Ligation LONG

Comparison of Ligation Efficiency of the DNA Ligation Kit LONG and Several Competing DNA Ligation Kits.



Comparison of Ligation Efficiency with Various DNA Ligation Kits.

Hind III-digested DNA fragments of varying sizes (2 kb, 4 kb, 10 kb and 18 kb) were ligated into the cloning vector pUC118/*Hind* III/BAP using the Ligation Kit LONG (Lig LONG) and several other commercially available DNA ligation kits. Ligation products were transformed into *E. coli* DH5 α cells and grown overnight on LB-amp plates at 37°C.

PCR Cloning Product Summary

DNA Ligation Kit LONG (TAK 6024)

The DNA ligation Kit LONG is a powerful tool for cloning DNA fragments from 2 kb to over 10 kb in length. The kit contains an optimized ligase/buffer system which enables ligation of long fragments without difficult techniques and special expertise. It is especially well-suited for the construction of BAC libraries.

DNA Ligation Kit, Version 2.1 (TAK 6022)

The DNA Ligation Kit, Version 2.1 provides simple ligation reaction for circular sticky-ended plasmids in 30 minutes at 16°C or 5 minutes at 25°C. The kit uses a single ligation solution which allows low volume ligation in instances where DNA amounts may be limiting. Furthermore, transformation efficiency can be improved by the addition of the Transformation Enhancer Solution to the ligation reaction mixture before transformation into competent cells.

DNA Ligation Kit, Version 1.0 (TAK 6021)

The DNA Ligation Kit, Version 1.0 is recommended for linear ligations such as λ DNA concatenations, as well as circular plasmid ligations. The kit is composed of two ligation solutions, rather than a single solution.

DNA Ligation Kit, Mighty Mix (TAK 6023)

The DNA Ligation Kit, Mighty Mix is a single premix solution that offers efficient, fast, one-solution ligation reactions, particularly for blunt-ended and TA-cloning reactions (30 minutes at 16°C or 5 minutes at 25°C). The 2X Mighty Mix solution allows small ligation reaction volumes (10 μ L). The reaction mix can be used directly in transformations and sufficient reagent is supplied for 75–150 ligation reactions.

PCR Related Products

dNTP Mixture (TAK 4030)

The purity and quality of deoxynucleotide triphosphates (dNTPs) are vital to the success of demanding applications such as PCR and RT-PCR. Takara's dNTPs are >98% pure and are quality-tested in a variety of applications.

RACE Core Set, 5'-Full (TAK 6122)

The RACE Core Set, 5' Full uses inverse PCR to amplify an unknown 5' end of a cDNA. The kit contains all the reagents needed for reverse transcription, degradation of the DNA-RNA hybrid and circularization of single-stranded DNA.

RACE Core Set, 3'-Full (TAK 6121)

The RACE Core Set, 3' Full uses a specially designed Oligo dT Adaptor Primer for efficient synthesis from the 3'-end of poly(A) RNA.

LA PCR *in vitro* Cloning Kit (TAK RR015)

The LA PCR *in vitro* Cloning Kit facilitates rapid and specific amplification of an unknown region of target DNA from only one known end of the region. Use of

LA Technology allows amplification of long fragments. No library construction or screening is required.

LA PCR *in vitro* Mutagenesis Kit (TAK RR016)

The LA PCR *in vitro* Mutagenesis Kit provides a simple and easy way to introduce site-specific mutations into DNA via PCR. The use of *TaKaRa* LA Taq™ and LA Buffer II allows generation of mutants in longer fragments. No repeated bacterial transformations are required.

PCR Mycoplasma Detection Set (TAK 6601)

Designed for rapid, sensitive and specific detection of mycoplasma via nested PCR, the PCR Mycoplasma Detection Set allows sensitive and specific detection of a wide spectrum of *Mycoplasma* species as well as one common *Ureaplasma* species.

One Shot Insert Check PCR Mix (TAK RR010A)

The One Shot Insert Check PCR Mix allows fast and simple confirmation of PCR inserts via PCR. The premixed 2X PCR Solution contains all of the necessary

reagents including PCR enzyme, dNTPs, specialized buffer and M13 primers dispensed into 0.2 mL tubes. The primers are compatible with a variety of common vectors, and the specialized mix allows a <1 kb target to be amplified in 20–45 minutes.

DNA-OFF™ (TAK 9036)

DNA-OFF™ is a non-alkaline, non-corrosive and non-carcinogenic cleansing solution to eliminate DNA contamination at PCR workstations. This contamination may result in DNA amplification artifacts.

RNase-OFF™ (TAK 9037)

RNase-OFF™ is a non-alkaline, non-corrosive and non-carcinogenic cleansing solution that is highly active against RNase contamination. RNase-OFF™ is stable and heat resistant and is ready-to-use for eliminating RNase from any surface, including the interior of microcentrifuge tubes.

Appendix I: Frequently Asked Questions

TaKaRa Ex Taq™ and LA Taq™ FAQ

What are the compositions of 10X Ex Taq™ and 10X LA Taq™ Buffers?

The 10X Ex Taq™ and the 10X LA Taq™ Buffers are proprietary and optimized for high amplification yield and larger fragment size. The magnesium concentration is 20 mM in the 10X Ex Taq™ Buffer and 25 mM in the 10X LA Taq™ Buffer. Because the optimal Mg^{2+} concentration in a reaction may be affected by variations in the reaction mix (including concentration of dNTPs), template-primer concentrations and chelating agents carried along with template DNA, Mg^{2+} -free buffer versions of both polymerases are available for optimization of your PCR reaction.

What cautions should I use in handling PCR buffers?

Repeated freeze-thawing of magnesium-containing solutions (like the 10X buffers) may result in the formation of a fine precipitate. This precipitate can reduce the effective concentration of Mg^{2+} in the PCR reaction, thereby impairing performance. We recommend thawing the 10X buffers at room temperature, warming gently to 37°C for 2–3 minutes and briefly vortexing to ensure a uniform suspension. Vortex buffer before first and subsequent uses.

Can TaKaRa Ex Taq™ or LA Taq™ DNA Polymerase be used to amplify GC-rich templates or those with large amounts of secondary structure?

TaKaRa Ex Taq™ can be used for amplification of GC-rich templates or those with large amounts of secondary structure by supplementing the PCR reaction mixture with DMSO, at a final concentration of up to 5% DMSO. Two GC Buffers have been developed for use with TaKaRa LA Taq™ and GC-rich or high secondary structure templates, and are available in the LA PCR Kit, Version 2.1 and with the LA Taq™ with GC Buffers. GC Buffer I is for amplification of longer targets, whereas GC Buffer II works best for the amplification of shorter GC-rich targets. Takara recommends GC Buffer I first, and GC Buffer II if satisfactory amplification is not seen with GC Buffer I.

When amplifying a 262 bp fragment (73% GC content) and a 358 bp fragment (71.5% GC content) with LA Taq™ with GC buffers, the suggested reaction conditions are**:

94°C	1 min	1 cycle
94°C	30 sec	} 30 cycles
60°C	30 sec	
72°C	1 min	
72°C	5 min	1 cycle

**See application page 27 for further information.

What is touchdown PCR?

Touchdown PCR was originally intended to simplify the process of determining optimal primer annealing temperatures. During the initial cycles of touchdown PCR, annealing takes place at

approximately 15°C above the calculated T_m . In subsequent cycles, the annealing temperature is gradually reduced by 1–2°C until it has reached approximately 5°C below the calculated T_m . Many thermal cyclers have a gradient temperature function which allows touchdown PCR to be performed in a single reaction.

What is autosegment extension (auto-extend cycles), and when should it be used?

Autosegment extension is a technique used to increase the yield of products over 10 kb in length and is used to compensate for death or depletion of reagents. At the 15th (half the total number of cycles) and subsequent cycles, the extension time is extended by 15 seconds for each cycle, allowing for a significant increase in amplification efficiency in long PCR.

Are TaKaRa Taq™, Ex Taq™ and LA Taq™ LD (low DNA) enzymes?

TaKaRa Taq™, Ex Taq™ and LA Taq™ are LD enzymes—(≤ 10 fg DNA), confirmed by nested PCR of the Ori region of *E. coli* genomic DNA (See page 10).

What are the compositions of TaKaRa Ex Taq™ and LA Taq™ Premixes?

Premix Taq (Ex Taq™ Version) is a 2X mixture with an enzyme concentration of 0.05 U/ μ L and dNTP concentration of 0.4 mM for each nucleotide, with a final dNTP concentration of 0.2 mM each. The One Shot LA Taq™ Polymerase Premix has a 0.1 U/ μ L concentration of TaKaRa LA Taq™ and a dNTP concentration of 0.8 mM for each nucleotide. The final dNTP concentration in a 50 μ L reaction is 0.4 mM for each nucleotide. Customers performing high-throughput experiments find the premixes more convenient, because they reduce the number of pipetting steps. Fewer pipetting steps reduce the probability of error, decrease user-to-user variation and minimize the risk of contamination.

Can template quality affect amplification results?

Yes. Successful amplification requires intact and highly purified template, particularly with longer DNA (>5 kb). Performing an additional phenol/chloroform extraction, ethanol (EtOH) precipitation or using "hot start" technology often resolves problems related to template quality.

Can Takara's Polymerases be used for combinatorial or multiplex PCR?

TaKaRa Taq™ Hot Start Version can be used for both combinatorial and multiplex PCR. Combinatorial and multiplex PCR are very similar techniques. Multiplex PCR uses one template (usually genomic DNA), and several sets of primers in the same reaction. Combinatorial PCR uses several templates and several primer sets in the same reaction.

Appendix I: Frequently Asked Questions

SYBR® Premix Ex Taq™ FAQ

How many reactions (points) are recommended for a typical standard curve?

Generally 5 or 6 reactions (5 or 6 points) are used to establish the standard curve, plus dH₂O for a negative control. Takara has used cDNAs which corresponded to 1 pg, 10 pg, 100 pg, 1 ng, 10 ng and 100 ng of mouse liver total RNA, respectively (and dH₂O for negative control). If possible, establish the standard curve within a Ct range of ~15–35 (See page 15).

The Ct (threshold cycle) is the number of cycles at which fluorescence intensity is measurable above background levels (threshold line) and is set in the exponential amplification phase to allow the most accurate reading.

How do I determine the number of qPCR reactions for my experiments? For example, if I have two different cell lines and want to characterize three different genes in each?

For each of the 3 genes, a standard curve (composed of 7 data points, for example) plus 2 experimental samples that are run in triplicate, are performed. Therefore, 3 (triplicate) x (7 pts + 2 samples) x 3 (genes) = 81 reactions are required for 3 genes. One package of SYBR® Premix Ex Taq™ (Perfect Real Time) contains sufficient reagent for 200 reactions (50 µl reaction).

What target size is optimal for real-time?

A size range of 80–150 bp is generally recommended for qPCR amplification, although sizes up to 300 bp are possible.

Can the SYBR® Premix Ex Taq™ solution precipitate? Is there a good way to resuspend it?

A greenish-yellow precipitate can sometimes be observed in SYBR® Premix Ex Taq™ when stored at –20°C. When this occurs, dissolve the precipitate completely by mixing the Premix gently after letting the tube stand at room temperature for several minutes (protected from light), or by warming with your hands. **Do not vortex!** We have verified that this product shows good performance after the precipitate is dissolved completely.

What is the composition of SYBR® Premix Ex Taq™?

The Premix contains *Takara Ex Taq*™ Hot Start Version, buffer, dNTP mix, Mg²⁺ and SYBR® Green I. The Mg²⁺ and SYBR® Green I concentrations are proprietary.

What is the purpose of the ROX™ reference dye included with the SYBR® Premix Ex Taq™?

ROX™ (Carboxy-X-Rhodamine) is a convenient internal reference standard for use in normalizing signals due to non-PCR related fluorescence fluctuations that occur either between wells or over time.

Please note that two types of ROX™ Reference Dye (Original Version ROX™ and ROX™ II) are supplied with this product. For normalization when using ABI PRISM® 7000/7700/7900HT and

Applied Biosystems 7300 Real-Time PCR System, please use the Original Version ROX™. For normalization when using Applied Biosystems 7500 Real-Time PCR System, please use ROX™ II Reference Dye.

Can you mix the ROX™ Reference Dyes and SYBR® Premix Ex Taq™ to help avoid pipetting errors?

The ROX™ Reference Dye I can be premixed. Add 40 µl of ROX™ to 1 ml of the SYBR® Premix Ex Taq™ and store at –4°C (protect from light). Use this solution within one month for best performance.

The ROX™ Reference Dye II should not be premixed prior to reaction assembly.

DNA Ligation Kits FAQ

What are the recommended conditions for ligation of a large circular plasmid with a comparatively small DNA insert?

Use DNA Ligation Kit LONG. The DNA ligation Kit LONG is a powerful tool for cloning DNA fragments from 2 kb to over 10 kb in length. The kit contains an optimized ligase/buffer system which enables ligation of long fragments without difficult techniques and special expertise. It is especially well-suited for the construction of BAC libraries.

How can I improve my ligation efficiencies when performing a blunt-ended ligation reaction?

To improve the efficiency of blunt-ended ligations, please follow the suggestions below:

- The use of BAP (bacterial alkaline phosphatase) vs. CIAP (Calf intestinal alkaline phosphatase) is recommended for dephosphorylation of the vector. Dephosphorylation with CIAP may be insufficient.
- If a gel-purified insert DNA is used for ligation, then DNA clean-up by EtOH precipitation is recommended prior to ligation.
- Recommended molar ratio is vector:insert = 1:5–10.
- Takara's DNA Ligation Kit, Mighty Mix and Version 2.1 kit are generally able to accomplish blunt-ended ligations at 16°C for 30 minutes. However, extended time (overnight incubation) may be necessary for more difficult ligations. Incubation at room temperature may inhibit the circularization of DNA.

How can I clean up ligated DNA in order to digest it with restriction enzymes?

To perform a restriction enzyme digestion using the ligated DNA, we strongly recommend cleaning the ligated DNA via EtOH precipitation in order to avoid inhibition of the digestion reaction by the ligation solution.

In general, how can I improve transformation efficiencies with my ligated DNA?

If it is necessary to improve transformation efficiencies, we suggest trying the following:

Appendix I: Frequently Asked Questions

- For particularly difficult ligations, extend the ligation reaction time to overnight at 16°C to improve the number of ligated molecules.
- Add Solution III from Version 2.1 or NaCl to a final conc. 500 mM into the ligation mixture prior to transformation. If a large volume of ligation mixture is needed for transformation or when performing electroporation, EtOH precipitation is recommended for cleanup of the DNA.

SpeedSTAR™ HS DNA Polymerase FAQ

I am observing smearing of my PCR product after agarose gel electrophoresis. What might be the problem?

Usually smearing of PCR product is observed when PCR conditions are not optimal. Try modifying your PCR cycling conditions using one or more of the following suggestions:

Extension time: An extension time that is too long may cause nonspecific priming. Refer to the following guideline.

2-step PCR: 10–20 sec/kb

3-step PCR: 5–10 sec/kb

Annealing temperature: Raise the temperature in increments of 2°C. Use 2-step PCR.

Template DNA: Use an appropriate amount of DNA. Excess template DNA increases the likelihood of non specific priming.

Primer: Reduce the primer amount.

I observed little or no PCR product band on my agarose gel. How can I generate more PCR product?

Usually low or no PCR product yield is observed when PCR conditions are not optimal. Try modifying your PCR cycling conditions using one or more of the following suggestions:

Extension time: Set the extension time at 20 sec/kb.

Annealing temperature: Lower the temperature in decrements of 2°C. Use 3-step PCR.

Template DNA: Repurify template DNA. For long amplifications, intact or minimally damaged DNA should be used.

Primer: Redesign primers. Or, increase the primer amount.

What is the recommended amount of template DNA needed in a SpeedSTAR™ reaction?

The proper amount of template DNA to be used in a SpeedSTAR™ reaction varies with the DNA source. Excess template can result in non-specific amplification or smearing.

Refer to the following for the recommended amount of template for a 50 µL PCR:

Human genomic DNA	5 ng–500 ng
<i>E.coli</i> genomic DNA	50 pg–100 ng
λ DNA	0.5 ng–2.5 ng
Plasmid	10 pg–1 ng

What type of PCR product ends does SpeedSTAR™ generate?

Eighty percent of the PCR products amplified with SpeedSTAR™

HS DNA Polymerase have one A added at the 3'-termini. Therefore, PCR products can be directly used for cloning into a T-vector. In addition, it is possible to clone the product into a blunt-end vector after blunting and phosphorylation of the end.

PrimeSTAR® HS DNA Polymerase FAQ

Can PrimeSTAR® HS reactions use the same PCR cycling conditions which are used with *Taq* Polymerase?

PrimeSTAR® HS cannot use the same PCR cycling conditions used with *Taq* Polymerase. Since the characteristics of this enzyme are very different from those of *Taq* Polymerase, Takara strongly recommends following the conditions described in the PrimeSTAR® HS product protocol.

Takara recommends the following initial cycle protocol for primers with a T_m of >55°C:

Denaturing step, 98°C, 10 sec	} 30 cycles
Annealing step 55°C, 5 sec.	
Extension step, 72°C, 1 min/kb	
If $T_m < 55^\circ\text{C}$, annealing step = 15 sec.	

What is the basis of PrimeSTAR® HS's antibody mediated Hot Start Technology?

PrimeSTAR® HS's Hot Start Technology uses a single monoclonal antibody which blocks both PrimeSTAR®'s polymerase and nuclease activities.

What is the advantage offered by Takara's measurement of PrimeSTAR® HS's fidelity by sequence analysis?

A simple comparison of the fidelity rates available for different PCR enzymes is not possible due to the variety of different fidelity measurement methods used by different manufacturers. Takara has determined PrimeSTAR®'s error rate based upon genotype, that is, the error rate as determined by actual sequence analysis. The method Takara used to obtain their fidelity data follows: Eight arbitrarily selected GC-rich regions were amplified with PrimeSTAR® HS and other enzymes using the *Thermus thermophilus* HB8 genomic DNA as a template. Each PCR product (approx. 500 bp each) was cloned into a suitable plasmid. For each different DNA region cloned, multiple clones were picked and subjected to sequence analysis. Sequence analysis results of DNA fragments amplified using PrimeSTAR® HS demonstrated only 15 mismatched bases per 480,000 total bases. This data confirms PrimeSTAR® HS's extremely high fidelity, with a calculated error frequency of only 0.0031%. Sequencing analysis is determined to be one of the most accurate ways to determine the fidelity of an enzyme. Sequence analysis can detect silent and lethal mutations which are not detected using traditional error rate methods.

What is the composition of PrimeSTAR® HS Buffer (Mg^{2+})?

The PrimeSTAR® HS Buffer composition is proprietary.

Appendix I: Frequently Asked Questions

Why does PrimeSTAR® HS use a 5X concentration buffer?

A 5X concentration buffer was determined to provide the best optimization for this reaction system.

How does PrimeSTAR® HS differ from KOD DNA Polymerase (Hot Start)?

PrimeSTAR® HS provides higher fidelity than KOD Hot Start while offering the same level of amplification efficiency.

What is the source of PrimeSTAR® HS DNA polymerase? Is it a cloned enzyme?

PrimeSTAR® HS is a recombinant enzyme that is expressed in *E. coli*. It was derived from a proprietary thermostable bacterial strain chosen by Takara after studying various strains that were identified as producing high fidelity enzymes. PrimeSTAR® HS was not obtained from the same bacterial strain that was used to produce KOD (*Pfx*).

Are PrimeSTAR® HS PCR products suitable for TA cloning?

PCR products cannot be used directly for TA cloning. The termini are blunt-ended due to the 3'→5' exonuclease activity of this enzyme. PrimeSTAR® HS PCR products should be used for blunt-end cloning. Takara recommends use of a dephosphorylated vector and phosphorylated PCR products. Products can be enzymatically phosphorylated or made using PCR primers possessing phosphoric acid residues at their 5' termini.

e2TAK™ DNA Polymerase FAQ

What are the recommended annealing conditions for e2TAK™ DNA polymerase?

Because e2TAK™ DNA polymerase possesses very high priming efficiency, set the annealing time at 5 sec. or 15 sec. Longer annealing times can cause smearing.

If $T_m > 55^\circ\text{C}$, annealing time is 5 sec.

If $T_m \leq 55^\circ\text{C}$, annealing time is 15 sec.

Calculation formula of T_m value:

- $T_m (^\circ\text{C}) = 2 (\text{NA} + \text{NT}) + 4 (\text{NC} + \text{NG}) - 5$
 T_m should be calculated with above method only for a primer of less than 25 bases. When the primer is longer than 25 bases, the annealing time should be set at 5 sec.

Does Takara recommend a 3 Step PCR or a 2-Step PCR for e2TAK™ amplifications?

A 3-step PCR protocol is generally recommended.

When is a 2-Step PCR protocol recommended for e2TAK™?

Better results could be obtained with a 2-step PCR protocol using a long or GC-rich primer.

When should a longer annealing time be used?

If the primer is short (<25 mer) and/or has high AT content, the 15 second annealing time may give better results.

What is the recommended template amount?

Please refer to the following:

Human genomic DNA	5 ng - 100 ng (<100 ng)
<i>E. coli</i> genomic DNA	100 pg - 100 ng
λ DNA	10 pg - 10 ng
Plasmid	100 pg - 1 ng

Are the PCR products produced with e2TAK™ sticky or blunt-ended?

The PCR products obtained using e2TAK™ will possess **blunt-ends**. Thus, obtained PCR products can be directly cloned into blunt-end vectors. However, direct TA cloning is not possible.

Can e2TAK™ be used for colony PCR?

We do not recommend this product for direct colony PCR. However, dilution of the heat extracted sample may allow amplification.

How can I improve my cDNA template results?

When poor yield is obtained from a cDNA template, results may be improved by decreasing the amount of template or lengthening the extension time.

What is the composition of the 5X e2TAK™ Buffer?

The composition of the 5X e2TAK™ Buffer is proprietary.

Can the e2TAK™ denaturing temperature be set at 98°C?

A 98°C denaturing temperature is not required, but can be used for 10 sec and a 94°C denaturing temperature can be used for 30 sec. e2TAK™ possesses high priming efficiency, therefore a short annealing time (5 or 15 seconds) will allow high specificity amplification. If a longer annealing performed is tried, i.e. 30 sec, the PCR products will likely smear.

What is the fidelity of e2TAK™?

The fidelity of this enzyme has not been determined.

What is the half life of e2TAK™?

The half life of this enzyme in the PCR reaction mixture (template, primers) at 98°C is about 4 hours.

Can e2TAK™ be used with common PCR additives, such as DMSO, glycerol, BSA, or betaine?

BSA: The buffer for this enzyme contains BSA, so Takara does not recommend adding more BSA.

Glycerol: The addition of glycerol has not been tested.

DMSO, Betaine: Preliminary experiments, indicate this enzyme can be used with these additives. However, we cannot provide detailed recommendations at present.

Appendix II: PCR Nomenclature (for regular PCR and qPCR)

Assembly PCR Assembly PCR is the completely artificial synthesis of long gene products by performing PCR on a pool of long oligonucleotides with short overlapping segments. The oligonucleotides alternate between sense and antisense directions, and the overlapping segments serve to order the PCR fragments so that they selectively produce their final product.

Asymmetric PCR Asymmetric PCR is used to preferentially amplify one strand of the original DNA more than the other. It finds use in some types of sequencing and hybridization probing where having only one of the two complementary strands is ideal. PCR is carried out as usual, but with a great excess of the primers for the chosen strand. Due to the slow (arithmetic) amplification later in the reaction after the limiting primer has been used up, extra cycles of PCR are required. A recent modification on this process, known as Linear-After-The-Exponential-PCR (LATE-PCR), uses a limiting primer with a higher melting temperature (T_m) than the excess primer to maintain reaction efficiency as the limiting primer concentration decreases mid-reaction.

Baseline A linear function subtracted from the data to eliminate background signal.

Colony PCR Bacterial clones (*E. coli*) can be screened for the correct ligation products. Selected colonies are picked with a sterile toothpick from an agarose plate and dabbed into the master mix or sterile water. Primers (and the master mix) are added—the PCR protocol has to be started with an extended time at 95°C.

Dynamic Range The linear range of fluorescent signal (from the lowest to the highest in the experiment) that can be detected without saturating the system. A wide dynamic range in a real-time system confers the ability to detect samples with high and low copy number in the same run.

Multiplex-PCR The use of multiple, unique primer sets within a single PCR reaction to produce amplicons of varying sizes specific to different DNA sequences. By targeting multiple genes at once, additional information may be elicited from a single test run that otherwise would require several times the reagents and technician time to perform. Annealing temperatures for each of the primer sets must be optimized to work correctly within a single reaction and amplicon sizes should be separated by enough difference in final base pair length to form distinct bands via gel electrophoresis.

Methylation Specific PCR Methylation Specific PCR (MSP) is used to detect methylation of CpG islands in genomic DNA. DNA is first treated with sodium bisulfite, which converts unmethylated cytosine bases to uracil, which is recognized by PCR primers as thymine. Two PCR reactions are then carried out on the modified DNA, using primer sets identical except at any CpG islands within the primer sequences. At these points, one primer set recognizes DNA with cytosines to amplify methylated DNA, and one set recognizes DNA with uracil or thymine to amplify unmethylated DNA. MSP using qPCR can also be performed to obtain quantitative rather than qualitative information about methylation.

Nested PCR Nested PCR is intended to reduce the contaminations in products due to the amplification of unexpected primer binding sites. Two sets of primers are used in two successive PCR runs, the second set intended to amplify a secondary target within the first run product. This is very successful, but requires more detailed knowledge of the sequences involved.

Qualitative Detection Allows one to determine the presence or absence of template of interest based on either Ct values or endpoint fluorescence.

Quantitative PCR Analysis Allows PCR product measurement and monitoring of the PCR reaction in a closed-tube system by measuring fluorescence intensity during each amplification cycle. Methods for both RNA and DNA are available to determine mRNA signal levels and/or DNA gene quantification. Quantitative PCR analysis software uses absolute standard curves, relative standard curves or comparative methods for data analysis.

Quencher A compound used in qPCR experiments that absorbs the energy of the reporter dye in its excited state. The quencher can emit its own fluorescent signal (e.g. TAMRA) or emit no fluorescent signal (e.g., DABCYL, BHQ)

Real-Time Experiments Experiments that monitor and report the accumulation of PCR product by measuring fluorescence intensity at each cycle while the amplification reaction progresses. Data is collected at the end of each melt/elongation cycle of the thermal cycling, and is available for analysis by Mx3000P software while the run is in progress.

Reference Dye Dye used in real-time experiments for normalization of the fluorescence signal of the reporter fluorophore. The reference dye fluoresces at a constant level during the reaction. ROX™ is commonly used as a reference dye.

Reporter Dye The fluorescent dye used to monitor PCR product accumulation in a qPCR experiment. This can be attached to a probe (such as with TaqMan or Molecular Beacons) or free in solution (such as SYBR® Green I). Also known as the fluorophore.

Sensitivity of Detection The level at which a given assay is able to detect low copy numbers. This is important when working with samples that have low expression levels.

Standard Curve The qPCR Standard Curve is a correlation plot generated by running a series of standards of known template concentration and then plotting the known starting quantities against the measured Ct values. The range of concentrations run should span the expected unknown concentration range. On the X-axis, the concentration measured for each standard is plotted in log scale. On the Y-axis the Ct (threshold cycle) correlating to each standard is plotted. A best-fit curve is generated by the software, and the data is displayed for each individual dye or multiple dyes used in the experiment on the same graph. In the absolute quantitation method, Ct values for unknown samples are compared to the Standard Curve plot to determine the starting concentration of template in the unknown wells.

Threshold Cycle (Ct) The PCR cycle at which fluorescence measured by the instrument is determined to be at a statistically significant level above the background signal. The threshold cycle is inversely proportional to the log of the initial copy number.

Touchdown PCR Touchdown PCR is a variant of PCR that reduces non-specific primer annealing by more gradually lowering the annealing temperature between cycles. As higher temperatures give greater specificity for primer binding, primers anneal first as the temperature passes through the zone of greatest specificity.

Inverse PCR Inverse PCR is a method used to allow PCR when only one internal sequence is known. This is especially useful in identifying flanking sequences to various genomic inserts. This involves a series of digestions and self ligation before cutting by an endonuclease, resulting in known sequences at either end of the unknown sequence.

RT-PCR RT-PCR (Reverse Transcription PCR) is the method used to amplify, isolate or identify a known sequence from a cell or tissues RNA library. Essentially normal PCR preceded by transcription by Reverse transcriptase (to convert the RNA to cDNA) this is widely used in expression mapping, determining when and where certain genes are expressed.

RACE-PCR Rapid amplification of cDNA ends.

Appendix III: Troubleshooting for *Endpoint PCR*

Non-specific bands on gel	
Template concentration is inappropriate	Use appropriate template concentrations. For a 50 μ L PCR reaction, recommended concentrations are: human genomic DNA = 0.1–1 μ g; <i>E.coli</i> genomic DNA = 10–100 ng; λ phage DNA = 0.5–2.5 ng; plasmid DNA = 10–100 ng.
Damaged template DNA	Minimize damage to template DNA by avoiding vortexing, heat treatment, strong UV, shearing or ultra sonication.
Denaturation time is too short	Optimize the denaturation time in increments of 5 seconds
Denaturation temperature is too low	Optimize the temperature in increments of 0.5°C
Annealing temperature is too low	Raise the temperature in increments of 2°C
Extension time is too short	Lengthen the extension time in increments of 1 minute
Cycle number is too high	Reduce the number of cycles in decrements of 2 cycles
Primer design is not appropriate to amplify the target sequence	Design primers with high specificity to the target DNA
Primer concentration is too high	Decrease the primer concentration in decrements of 0.1 μ M
Non-specific annealing of primers due to room temperature set up	Use Hot Start DNA polymerase
Contaminating DNA in reaction	Decontaminate work area and pipette. Use a dedicated pipette for PCR only. Use aerosol barrier tips and wear gloves.
Mg ²⁺ concentration inappropriate	Optimize Mg ²⁺ concentration in 0.5 mM increments (for Mg ²⁺ free buffer)
Template contains high GC region or high secondary structure	Use <i>TaKaRa LA Taq™</i> with GC buffer (TAK RR02AG) or try addition of an enhancing reagent (See page 5)

e2TAK™ and PrimeSTAR® require the following annealing times and temperatures:

Annealing temperature:

Initially, use 5 sec at 55°C.

Annealing time:

When T_m value* is $\geq 55^\circ\text{C}$: 5 sec.

When T_m value* is $< 55^\circ\text{C}$: 15 sec.

Appendix III: Troubleshooting for *Endpoint PCR*

No or poor amplification yield	
Enzyme concentration is too low	Increase the enzyme amount in increments of 0.5 U
Denaturation time is too short	Lengthen the denaturation in increments of 5 seconds
Denaturation temperature is low	Raise the temperature in increments of 0.5°C
Extension time is too short	Increase the extension time in increments of 1 minute
Cycle number is too low	Increase the number of cycles in increments of 2 cycles
Template concentration is too low	Increase the template amount in increments of 20% of the previously used amount
Template degraded/dirty	Reclean the DNA using ETOH precipitation, examine template quality via gel electrophoresis, re-prepare template if necessary.
Enzyme inactive	Use fresh enzyme
dNTP's degraded	Use fresh dNTP's; store frozen aliquots and avoid freeze-thaws
Primers not matched	Rethink and resynthesize the primers
Annealing temperature is too high	Lower the temperature in decrements of 2°C
Annealing time is too short	Increase annealing time incrementally
Problem with thermocycler operation or program	Run positive control with every reaction
Mg ²⁺ concentration inappropriate	Optimize Mg ²⁺ concentration in 0.5 mM increments (for Mg ²⁺ free buffer)
Template contains high GC region or high secondary structure	Use <i>TaKaRa LA Taq</i> [™] with GC buffers (TAK RR02AG) or try addition of an enhancing reagent (See page 5)

Appendix III: Troubleshooting for *Endpoint PCR*

Diffuse smearing within lane on a gel	
Concentration of primers is too high	Reduce the primer amount in decrements of 0.1 μ M
Primers are not well designed for the target sequence	Increase the specificity of the primers by changing the complimentary region of the template, within 20–30 bases
Enzyme concentration is too high	Reduce the enzyme amount in decrements of 0.5 U
Cycle number is too high	Reduce the number of cycles in decrements of 2 cycles
Annealing temperature is too low	Raise the temperature in increments of 2°C
Non-specific annealing of primers due to room temperature set up	Use Hot Start DNA Polymerase
Extension time is inappropriate	Set time to 0.5–1 min/kb
Denaturation is not complete	Optimize denaturation conditions by extending the time in increments of 5 sec., raising the temperature in increments of 0.5°C, or adding an enhancing reagent (See page 5)
Template concentration is too high	Reduce the template amount in decrements of 20% of the previously used amount
Mg ²⁺ concentration inappropriate	Optimize Mg ²⁺ concentration in 0.5 mM increments (for Mg ²⁺ free buffer)
Contaminating DNA in reaction	Decontaminate work area and pipette. Use a dedicated pipette for PCR only. Use aerosol resistant tips and wear gloves.

e2TAK™ and PrimeSTAR® require the following annealing times and temperatures:

Annealing temperature:

Initially, use 5 sec at 55°C.

Annealing time:

When T_m value* is $\geq 55^\circ\text{C}$: 5 sec.

When T_m value* is $< 55^\circ\text{C}$: 15 sec.

Appendix III: Troubleshooting for *Real Time PCR (qPCR)*

Because of the time and effort that goes into doing a qPCR experiment, care must be taken in getting the correct parameters for each step. These are troubleshooting issues that could be encountered in any qPCR experiment. Consult the instrument manual for detection troubleshooting issues or probe issues.

No C _T value (No or poor amplification)	
No curves seen after data analysis	Verify if the PCR worked by running a gel
One or more reagents not added	Redo experiment assuring that reagents are added
Run on a gel: no product seen on an agarose gel	Check cycling parameters
Annealing temperature or time incorrect	Check the temperature and optimize the annealing temperature by changing the temperature in 2°C increments. Annealing times are as written in the protocol.
Extension time is inappropriate	Extension should be increased for longer amplicons. Amplicons ideally should be between 80–200 bp.
Primer design	Primer dimers may be present. Run gel to determine if there are primer dimers.
Primer concentration incorrect	If incorrect, do a titration of the primer starting at 50 nM to 300 nM
Primers are not working	Primers have been degraded or incorrectly designed or synthesized
Detection step incorrect	Detection step taken at wrong step. Must be taken at the annealing step. Consult the instrument manual for further information.
Amplicon too long	Amplicons ideally should be between 80–200 bp. Amplicons over 300 bp should not be amplified using qPCR.
Cycle number insufficient	Cycle number should be 35–40 cycles. More cycles than the 35–40 may cause an increase in background.
“Dirty” or degraded template	Purify the DNA before using it in a qPCR experiment. Reclean the DNA using ETOH precipitation and check on gel. Use fresh stock of DNA for each experiment.
Insufficient template concentration	Template concentration should be 100–500 ng. If higher or lower, readjust.
Mg ²⁺ concentration inappropriate	Use the Mg ²⁺ supplied in the mix. Add additional Mg ²⁺ if necessary up to 6 mM.
Probe design issues	Consult manufacturer of probe fluor.

Appendix III: Troubleshooting for *Real Time PCR (qPCR)*

Irregular or Wavy data lines	
Machine malfunction	Lamp problem or mirror misalignment. Check instrument manual.
Cycle number is too high	Reduce the number of cycles to 35–40
Detection step is incorrect	Detection step taken at wrong step. Must be taken at the annealing step.
No reference dye used	Some instruments require ROX™ reference dye or fluorescein as a reference dye for normalization. Check your instrument to see if it requires a reference dye.
Reaction volume is insufficient	Most qPCR instruments are set to read volumes of at least 15 µL

Amplification in the No Template Control	
Presence of primer dimers	Primer dimers are normally seen in the 72°C–79°C on the melt curve. Their presence may require a redesign of the primers and/or adjustment to the annealing temperature by decreasing or titrating the primer concentration. To confirm primer dimers, run a gel of product. If confirmed, the ratio of template concentration to primer concentration will need to be adjusted.
DNA Polymerase contamination	Most <i>Taq</i> polymerases on the market are recombinant polymerases and may have some contaminating <i>E. coli</i> . If contamination persists, check the homology of your target with the <i>E. coli</i> genome.
Reagent or tip contamination	Repeat the experiment with new reagents and plasticware

Non-specific amplification detected in Melt Curve	
AT-rich subdomains	Shoulders on the melt curve could be caused by these regions. Run product on gel to confirm.
Primer concentration inappropriate	Detection of primer dimers could mean the concentration of the primers is incorrect. Decrease primer concentration in increments of 0.1 µM.
Detection step temperature incorrect	Set the temperature to ~3°C below the T_m of the PCR product, but above the T_m of the primer dimers
Annealing temperature is too low	Raise the temperature in 2°C increments
Mispriming or non-specific probe binding	Primer and probe design may need to be redesigned
Template contamination	Re-purify the template. If doing a qRT-PCR, treat the RNA template with a recombinant DNase I.

Appendix III: Troubleshooting for *Real Time PCR (qPCR)*

Late Ct value (low sensitivity)	
Primer concentration incorrect	Primer may need to be titrated to increase primer concentration which will increase sensitivity, but may also increase non-specific amplification. Primer concentration is between 50 nM–250 nM.
Presence of primer dimers	Primer dimers are normally seen in the 72°C–79°C on the melt curve. Their presence may require a redesign of the primers and/or adjustment to the annealing temperature by decreasing or titrating the primer concentration. To confirm primer dimers, run a gel of product. If confirmed, the ratio of template concentration to primer concentration will need to be adjusted.
Annealing temperature or time incorrect	Check the temperature and optimize the annealing temperature by changing the temperature in 2°C increments. Annealing times are as written in the protocol.
Extension time is inappropriate	Extension should be increased for longer amplicons. Amplicons ideally should be between 80–200 bp.
Evaporation of sample	Use correct seal for the microplate. Avoid the outer row/column if seals are of poor quality.
No template added	Assure that all reagents including DNA template have been correctly added and repeat experiment
Primer-Probe ratio is incorrect	Use matrices in Table 1 and Table 2 below
Probe bleached from being left out in light	When probes are received, they should be aliquoted to avoid this problem. Aliquot and store at –20°C in the dark.
Probe may be hydrolyzed	When probes are dissolved in an acid solution the fluorophores can hydrolyze, generating a low fluorescence signal and high background. Resuspend the probes in TE buffer at pH 8.0.

No linearity in the Ct values of a dilution series	
Secondary structures in probes	When a dilution series is performed with probe and target, a 2X dilution series should yield a 1 cycle change in the Ct value between each dilution. If a 10X dilution series is performed, a change in Ct value should be 3.2 cycles between each dilution. Secondary structures in the probes will cause gaps in these values. At the point where these gaps occur, the target DNA amount is no longer in excess or balanced with the amount of probe. Less efficient detection is caused by the intra-probe binding and the target-probe binding competing for the probes. Redesign of the probes is necessary.

Table 1. Primer Optimization Matrix

	Forward Primer		
Reverse Primer	50 nM	300 nM	900 nM
50 nM	50/50	300/50	900/50
300 nM	50/300	300/300	900/300
900 nM	50/900	300/900	900/900

Primer optimization should be done before beginning experimentation. The tables to the left contain a matrix of primer concentrations that can be tested with either the SYBR® Green I detection method or the probe detection method. For SYBR® Green I, lower concentrations of primers are used to avoid primer-dimer formation. Primer concentrations ranging from 50–300 nM should be tested. For Probe chemistries, a larger range of primer concentrations should be tested, 50–900 nM.

Table 2. Primer-Probe Optimization Matrix

	Probe		
	50 nM	125 nM	250 nM
Optimized Primer Pair	50/optimized primers	125/optimized primers	250/optimized primers

Appendix III: Troubleshooting for *Real Time PCR (qPCR)*

High Background	
SYBR® Detection Method (SYBR® Premix <i>Ex Taq</i> ™ (Perfect Real Time))	
SYBR® Green concentration is too high	An easy way to avoid this is to use a premix like Takara SYBR® Premix <i>Ex Taq</i> (Perfect Real Time). From a 10,000X stock solution of SYBR® Green, do a 1:3000 dilution and add 1.25 µL to 25 µL reaction.
"Dirty" template	Purify the DNA before using in a qPCR experiment. Reclean the DNA using ETOH precipitation and check on gel. Use fresh stock of DNA for each experiment.
Template concentration is incorrect	Template concentration should be 100–500 ng. If higher or lower, readjust.
Mg ²⁺ concentration inappropriate	Use the Mg ²⁺ supplied in the mix. Add additional Mg ²⁺ if necessary, up to 6 mM.
Probe Detection Method (Premix <i>Ex Taq</i> ™ (Perfect Real Time))	
Insufficient quenching	Quencher doesn't fit to dye. (See the reporter dye/quencher table on page 18). The quencher may be too far from dye. Redesign of probe may be needed.
Probe concentration may be too high	Titrate the probe to find a good concentration (See Table 2. Page 45)
Probe is degraded	Use fresh probe each experiment
Free dye in your probe	Clean up the probe
Low ΔRn (change in reporter fluorescence)	
Annealing temperature or time incorrect	Check the temperature and optimize the annealing temperature by changing the temperature in 2°C increments. Annealing times are as written in the protocol.
Extension time is inappropriate	Extension should be increased for longer amplicons. Amplicons ideally should be between 80–200 bp.
Extension temperature too low	Increase temperature in 2°C increments
Primer concentration incorrect	If incorrect, do a titration of the primer starting at 50 nM to 300 nM
Primer-Probe ratio is incorrect	Use matrices in Table 1 and Table 2 on page 45
Probe bleached from being left out in light	When probes are received, they should be aliquoted to avoid this problem. Aliquot and store at –20°C in the dark.
Probe may be hydrolyzed	When probes are dissolved in an acid solution, the fluorophores can hydrolyze, generating a low fluorescence signal and high background. Resuspend the probes in TE buffer at pH 8.0.
Cycle number insufficient	Cycle number should be 35–40 cycles. More cycles will cause an increase in background.
Mg ²⁺ concentration inappropriate	Use the Mg ²⁺ supplied in the mix. Add additional Mg ²⁺ if necessary, up to 6 mM.

Appendix IV: PCR Protocols

TaKaRa Ex Taq™:

Below is Takara's general reaction and cycling recommendations for *TaKaRa Ex Taq*™ DNA Polymerase. This procedure can easily be modified to meet your amplification requirements for specialized applications or challenging or problematic templates.

Standard Protocol for TaKaRa Ex Taq™

Reagent	Volume	Final Conc.
10X Ex Taq™ Buffer (Mg ²⁺ plus)	5.0 µL	1X
dNTP Mixture (2.5 mM each)	4.0 µL	200 µM
Primer 1 [20 pmol/µL]	0.5 µL	0.2 µM
Primer 2 [20 pmol/µL]	0.5 µL	0.2 µM
TaKaRa Ex Taq™	0.25 µL	1.25 U/50 µL
Purified genomic DNA	1.0 µL	500 ng/50 µL
dH ₂ O	38.75 µL	
Total	50.0 µL	

PCR Conditions for TaKaRa Ex Taq™

Simple cycling		
94°C	1 min	
↓		
94°C	30 sec	} 30 cycles
55°C*	30 sec	
72°C	0.5–1 min/kb	
↓		
72°C	2 min (final extension)	

* The annealing temperature should be optimized for each primer pair.

TaKaRa LA Taq™:

Below is Takara's general reaction and cycling recommendations for *TaKaRa LA Taq*™ DNA Polymerase. This procedure can easily be modified to meet your amplification requirements for specialized applications or challenging or problematic templates.

Standard Protocol for TaKaRa LA Taq™

Reagent	Volume	Final Conc.
10X LA PCR Buffer II (Mg ²⁺ free)	5.0 µL	1X
MgCl ₂ (25 mM)	5.0 µL	2.5 mM
dNTP Mixture (2.5 mM each)	8.0 µL	400 µM
Primer 1 [20 pmol/µL]	0.5 µL	0.2 µM
Primer 2 [20 pmol/µL]	0.5 µL	0.2 µM
TaKaRa LA Taq™	0.5 µL	2.5 U/50 µL
Purified genomic DNA	1.0 µL	500 ng/50 µL
dH ₂ O	29.5 µL	
Total	50.0 µL	

Autosegment extension**

94°C	1 min	
↓		
94°C	30 sec*	} 14 cycles
68°C	15 min	
↓		
94°C	30 sec	} 16 cycles
68°C	15 min	
+ 15 sec/cycle**		
↓		
72°C	10 min	

*The denaturation conditions were based on thermal cycler used, tubes and type of PCR.

**Autosegment extension: At the 15th cycle and following, the extension time should be extended by 15 seconds each cycle. Autosegment extension is generally used when amplifying DNA fragments greater than 15 kb.

2-step PCR for TaKaRa LA Taq™

Fragment size 0.5–2 kb	Fragment size 4–35 kb
94°C 1 min	94°C 1 min
↓	↓
98°C 5 sec	98°C 5 sec
68°C 5 min	68°C 15 min
↓	↓
72°C 10 min	72°C 10 min

3-step PCR for TaKaRa LA Taq™

Simple cycling		
94°C	1 min	
↓		
94°C	30 sec	} 30 cycles
60°C	30 sec	
↓		
72°C	1 min/kb	
↓		
72°C	5 min	

Appendix IV: PCR Protocols

PrimeSTAR® HS DNA Polymerase:

Below is Takara's general reaction and cycling recommendations for PrimeSTAR® HS DNA Polymerase. This procedure can easily be modified to meet your amplification requirements for specialized applications or challenging or problematic templates.

Standard Protocol for PrimeSTAR® HS DNA Polymerase

Reagent	Volume	Final Conc.
5x PrimeSTAR® Buffer (Mg2+ Plus)	10 µL	1X
dNTP Mixture (2.5 mM each)	4 µL	200 µM each
Primer 1	10 ~ 15 pmol	0.2 ~ 0.3 µM
Primer 2	10 ~ 15 pmol	0.2 ~ 0.3 µM
Template	<200 ng	
PrimeSTAR® HS DNA Polymerase (2.5 units/µL)	0.5 µL	1.25U/50 µL
Sterilized Distilled Water	up to 50 µL	

PCR Conditions for PrimeSTAR® HS

3-step PCR Method (0.5 - 6 kb)	} 30 cycles
98°C 10 sec	
55°C 5 sec or 15 sec	
72°C 1 min/kb	
2-step PCR Method (7.5 - 8.5 kb)	} 30 cycles
98°C 10 sec	
68°C 1 min/kb	

SpeedSTAR™ DNA Polymerase:

Below is Takara's general reaction and cycling recommendations for SpeedSTAR™ HS DNA Polymerase. This procedure can easily be modified to meet your amplification requirements for specialized applications or challenging or problematic templates.

Standard Protocol for SpeedSTAR™ DNA Polymerase

Reagent	Volume	Final Conc.
SpeedSTAR™ HS DNA Polymerase (5 U/µL)	0.25 µL	1.25 units/50 µL
dNTP Mixture (2.5 mM each)	4 µL	200 µM
Primer 1	10-50 pmol	0.2 µM ~ 1 µM
Primer 2	10-50 pmol	0.2 µM ~ 1 µM
Template	< 500 ng	
10 x Fast Buffer I or II	5 mL	1X
Sterilized distilled water	up to 50 mL	

2-step or 3-step PCR for SpeedSTAR™

2-step PCR

Target: 4 or 6 kb (with Fast Buffer I or II)

95°C, 5 sec	} 30 cycles
65°C, 10 sec(or up to 20 sec)/kb	

Target: longer than 4 or 6 kb (with Fast Buffer II)

98°C, 5 sec	} 30 cycles
68°C, 10 sec(or up to 20 sec)/kb	

3-step PCR (with either Fast Buffer I or II)

95°C, 5 sec	} 30 cycles
55°C, 10-15 sec	
72°C, 5-10 sec/kb	

NOTE: Efficient amplification can be achieved by varying the temperature of each step, depending on an amplified size.

e2TAK™ DNA Polymerase:

Below is Takara's general reaction protocol and cycling recommendations for e2TAK™ DNA Polymerase. This procedure can easily be modified to meet your general amplification requirements.

Standard Protocol for e2TAK™ DNA Polymerase

Reagent	Volume
e2TAK™ DNA Polymerase	0.5 µL
5X e2TAK™ Buffer	10 µL
dNTP Mixture (2.5 mM each)	4 µL
Template DNA	< 100 ng
Primer 1	0.2-0.3 µM (final conc.)
Primer 2	0.2-0.3 µM (final conc.)
dH ₂ O	up to 50 µL

PCR Conditions for e2TAK™ DNA Polymerase

PCR cycling

3-step PCR Method	} 30 cycles
98°C 10 sec	
55°C 5 sec (or 15 sec)	
72°C 1 min/kb	
or 2-step PCR Method	} 30 cycles
98°C 10 sec	
68°C 1 min/kb	

Appendix IV: PCR Protocols for Real Time Applications

SYBR® Premix Ex Taq™ (Perfect Real Time):

Below are Takara's general protocols for SYBR® Premix Ex Taq™ (Perfect Real Time) on three different Real Time PCR instruments. These can easily be modified to meet your assay requirements.

FOR CYCLING CONDITIONS (SEE PAGE 15)

Protocol using Smart Cycler® II System

Reagent	Volume	Final Conc.
SYBR® Premix Ex Taq™ (2 X)	12.5 µL	1 X
PCR Forward Primer (10 µM)	0.5 µL	0.2 µM* ¹
PCR Reverse Primer (10 µM)	0.5 µL	0.2 µM* ¹
Template (<100 ng)	2 µL* ²	
dH ₂ O	9.5 µL	
Total	25 µL	

Protocol using ABI PRISM® 7000/7700/7900HT or Applied Biosystems 7300/7500 Real Time PCR Systems

Reagents	Volume	Volume	Final Conc.
SYBR® Premix Ex Taq™ (2 X)	10 µL	25 µL	1 X
PCR Forward Primer (10 µM)	0.4 µL	1 µL	0.2 µM* ¹
PCR Reverse Primer (10 µM)	0.4 µL	1 µL	0.2 µM* ¹
ROX™ Reference Dye or Dye II* ³ (50X)	0.4 µL	1 µL	1 X
Template	2 µL* ²	4 µL	
dH ₂ O	6.8 µL	18 µL	
Total	20 µL* ⁴	50 µL* ⁴	

Protocol using Stratagene Mx 3000P

Reagent	Volume	Final Conc.
SYBR® Premix Ex Taq™ (2 X)	12.5 µL	1 X
PCR Forward Primer (10 µM)	0.5 µL	0.2 µM* ¹
PCR Reverse Primer (10 µM)	0.5 µL	0.2 µM* ¹
ROX™ Reference Dye II* ³	0.5 µL	1 X
Template (<100 ng)	2 µL* ²	
dH ₂ O	9 µL	
Total	25 µL	

Protocol using Roche LightCycler®

Reagent	Volume	Final Conc.
SYBR® Premix Ex Taq™ (2 X)	10 µL	1 X
PCR Forward Primer (10 µM)	0.4 µL	0.2 µM* ¹
PCR Reverse Primer (10 µM)	0.4 µL	0.2 µM* ¹
Template (<100 ng)	2 µL* ²	
dH ₂ O	7.2 µL	
Total	20 µL	

*¹ In most reactions a primer concentration of 0.2 µM is optimal. This may need to be optimized within a range of 0.1–1.0 µM.

*² Final template concentration varies depending on the copy number of target present in the template solution. The optimal amount should be determined by preparing a dilution series. We recommend using <100 ng of DNA template for a 20 or 25 µL reaction. When cDNA, direct from an RT reaction, is used as a template, it should be <10 % volume of the PCR reaction mixture.

*³ The ROX™ Reference Dye/Dye II is supplied for performing normalization of fluorescent signal intensities within wells when used with real time PCR instruments which have this option. For ABI PRISM® 7000/7700/7900HT and Applied Biosystems 7300 Real-Time PCR Systems, the use of ROX™ Reference Dye (50X) is recommended. For the Applied Biosystems 7500 Real-Time PCR System, use of ROX Reference Dye II is recommended. The use of ROX™ Reference Dye or Dye II is optional, and not required when using Smart Cycler® and LightCycler® real time instruments.

*⁴ The 50 µL reaction volume is for use with 96-well plates, single tubes and 8-strip tubes. The 20 µL reaction volume is for a 384-well plate.

Appendix V: Technical Fact sheet for Takara's Premium PCR Enzymes

TaKaRa Ex Taq™

- The half life of TaKaRa Ex Taq™ at:
95°C = 35 min
97.5°C = 7 min
- Temperature range for extension = 60–72°C.
- The extension speed of Ex Taq™ = 1–2 kb/min.
- Ex Taq™ has weak 5'→3' activity and no strand displacement activity.
- 80% of Ex Taq™ PCR products contain 3'-A overhangs and can be cloned into T-Vectors.
- Does not incorporate dUTP or dITP.

Amount of DNA template per 50 µL reaction:

Human Genomic DNA	0.1–1 mg
<i>E. coli</i> Genomic	10–100 ng
λ Phage DNA	0.5–2.5 ng
Plasmid DNA	10–100 pg

TaKaRa LA Taq™

- The half life of TaKaRa LA Taq™ at:
95°C = 35 min
97.5°C = 7 min
- Temperature range for extension = 60–72°C.
- The extension speed of LA Taq™ = 1–2 kb/min.
- LA Taq™ has weak 5'→3' activity and no strand displacement activity.
- 80% of LA Taq™ PCR products contain 3'-A overhangs and can be cloned into T-Vectors.
- Does not incorporate dUTP or dITP.

Amount of DNA template per 50 µL reaction:

Human Genomic DNA	0.1–1 ng
<i>E. coli</i> Genomic	10–100 ng
λ Phage DNA	0.5–2.5 ng
Plasmid DNA	10–100 pg

PrimeSTAR® DNA Polymerase

- The half life of PrimeSTAR® at:
98°C = 50 min
- Temperature range for extension = 60–72°C.
- The extension speed of PrimeSTAR® = 1–2 kb/min.
- All products are blunt ended.
- Does not incorporate dUTP or dITP.

Amount of DNA template per 50 µL reaction:

Human Genomic DNA	5–200 ng
<i>E. coli</i> Genomic	100 pg–100 ng
λ Phage DNA	10 pg–10 ng
Plasmid DNA	10 pg–1 ng

SpeedSTAR™ DNA Polymerase

- The half life of SpeedSTAR™ at:
95°C = 35 min
97.5°C = 7 min.
- Temperature range for extension = 60–72°C.
- The extension speed of SpeedSTAR™ = 6 kb/min.
- 80% of SpeedSTAR™ PCR products contain 3'-A overhangs and can be cloned into T-Vectors.
- Does not incorporate dUTP or dITP.

Amount of DNA template per 50 µL reaction:

Human Genomic DNA	5–100 ng
<i>E. coli</i> Genomic	50 pg–100 ng
λ Phage DNA	0.5–2.5 ng
Plasmid DNA	10 pg–1 ng

Common Nucleic Acid Conversions

A₂₆₀ unit conversions

Double-stranded DNA: 50 µg per 1 A₂₆₀ unit

Sheared or single-stranded DNA and RNA:
40 µg per 1 A₂₆₀ unit

Oligonucleotides: 25 µg per 1 A₂₆₀ unit

Bases of nucleic acid x Mass of nucleic acid

1 kb double-stranded DNA (Na⁺) = 6.6 x 10⁵ Da

1 kb single-stranded DNA (Na⁺) = 3.3 x 10⁵ Da

1 kb single-stranded RNA (Na⁺) = 3.4 x 10⁵ Da

1 megadalton double-stranded DNA (Na⁺) = 1.52 kb

average mass of dNMP = 330 Da

average mass of dNMP base pair = 660 Da

Mass of nucleic acid x Moles of nucleic acid

1 µg of a 1 kb DNA fragment = 1.5 pmol

1 µg of a 1 kb DNA fragment = 0.3 pmol ends

1 µg of pUC18 or pUC19 DNA (2,686 bp) = 0.5 pmol

1 µg of pBR322 DNA (4,361 bp) = 0.35 pmol

1 µg of λ DNA (48,502 bp) = 0.33 pmol

Moles of nucleic acid x Mass of nucleic acid

1 pmol of 1,000 bp DNA = 0.66 µg

1 pmol of pUC18 or pUC19 DNA (2,686 bp) = 1.77 µg

1 pmol of pBR322 DNA (4,361 bp) = 2.88 µg

1 pmol of λ DNA (48,502) = 32.01 µg

Miscellaneous

Molecular weight of a double-stranded DNA molecule =
(# of base pairs) x (660 Da/base pair)

1 µg/mL of nucleic acid = 3.0 µM phosphate

Moles of ends of a double-stranded DNA molecule =
(2) x (grams DNA)/(molecular weight of DNA in Da)

Moles of ends generated by restriction digestion:

Circular DNA molecule = (2) x (moles DNA) x (number of sites)

Linear DNA molecule =

(2) x (moles DNA) x (number of sites) + [(2) x (moles DNA)]

Appendix VI: References

Selected References - Takara DNA Polymerases:

SYBR® Premix Ex Taq™ (Perfect Real Time)

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Takara Taq™ Hot Start Version

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Guide to TaKaRa

Polymerase*	Amplification Efficiency	Product Size λ DNA Recommended/Max	Product size Human Genomic DNA Recommended/Max	Fidelity	Proofreading Activity	Specificity
PrimeSTAR® HS*	+++	up to 20 kb	up to 8.5 kb	10 X <i>Taq</i> [#]	Yes	++++
PrimeSTAR® HS with GC Buffers	+++	up to 10 kb	up to 5 kb	10 X <i>Taq</i> [#]	Yes	++++
PrimeSTAR® HS, Premix	+++	up to 10 kb	up to 5 kb	10 X <i>Taq</i> [#]	Yes	++++
SpeedSTAR™ HS	+++	20 kb/30 kb	10 kb/ 20 kb	4.5 X <i>Taq</i> ^{**}	Yes	++++
<i>Ex Taq</i> ^{TM*}	++++	20 kb/30 kb	10 kb/20 kb	4.5 X <i>Taq</i> ^{**}	Yes	++
Premix <i>Ex Taq</i> TM	++++	20 kb/30 kb	10 kb/20 kb	4.5 X <i>Taq</i> ^{**}	Yes	++
<i>Ex Taq</i> TM HS*	++++	20 kb/30 kb	10 kb/20 kb	4.5 X <i>Taq</i> ^{**}	Yes	++++
<i>Ex Taq</i> TM HS, Premix	++++	20 kb/30 kb	10 kb/20 kb	4.5 X <i>Taq</i> ^{**}	Yes	++++
Premix <i>Ex Taq</i> ^{TM*} (Perfect Real Time)	++++	—	—	4.5 X <i>Taq</i> ^{**}	Yes	++++
SYBR® Premix <i>Ex Taq</i> ^{TM*} (Perfect Real Time)	++++	—	—	4.5 X <i>Taq</i> ^{**}	Yes	++++
<i>LA Taq</i> ^{TM*}	+++	35 kb/48 kb	20 kb/30 kb	6.5 X <i>Taq</i> ^{**}	Yes	++
<i>LA Taq</i> TM w/GC Buffers	+++	35 kb/48 kb§	(20 kb/30 kb)§	(6.5 X <i>Taq</i>) ^{‡**}	Yes	++
LA PCR Kit, V.2.1	+++	35 kb/48 kb	20 kb/30 kb	6.5 X <i>Taq</i> ^{**}	Yes	++
One-Shot LA PCR Mix	+++	35 kb/48 kb	20 kb/30 kb	6.5 X <i>Taq</i> ^{**}	Yes	++
<i>LA Taq</i> TM HS	+++	35 kb/48 kb	20 kb/30 kb	6.5 X <i>Taq</i> ^{**}	Yes	++++
<i>e2TAK</i> TM DNA Polymerase*	++	up to 10 kb	up to 8 kb	1 X <i>Taq</i> ^{**}	Yes	++
<i>Taq</i> [*]	++	6 kb/12 kb	2 kb/4 kb	1 X <i>Taq</i> ^{**}	No	++
Premix <i>Taq</i>	++	6 kb/12 kb	2 kb/4 kb	1 X <i>Taq</i> ^{**}	No	++
<i>Taq</i> HS*	++	6 kb/12 kb	2 kb/4 kb	1 X <i>Taq</i> ^{**}	No	++++
<i>Taq</i> HS, Premix	++	6 kb/12 kb	2 kb/4 kb	1 X <i>Taq</i> ^{**}	No	++++

* Free Sample Available

Unit Definition

One unit is the amount of enzyme that will incorporate 10 nmol of dNTP into acid-insoluble products in 30 min. at 74°C with activated salmon sperm DNA as the template-primer.

Purity

Nicking activity, endonuclease, and exonuclease activity were not detected after the incubation of 0.6 µg of double-stranded supercoiled pBR322 DNA, 0.6 µg of λ DNA, or 0.6 µg of λ-*Hind* III digest with 10 units of enzyme for 1 hour at 74°C.

PCR Polymerases

Convenience	GC-Rich Templates	Hot-Start PCR	Real Time PCR (QPCR)	Low DNA Enzyme	Processing Speed	Guidelines for Length of Primers	Terminal Transferase Activity (3'-A overhang)
++	++++	++++	—	≤ 10 fg	1-2 kb/min	20-30 bp	No (blunt end)
++	++++	++++	—	≤ 10 fg	1-2 kb/min	20-30 bp	No (blunt end)
++++	++++	++++	—	≤ 10 fg	1-2 kb/min	20-30 bp	No (blunt end)
++	+	++++	—	≤ 10 fg	6 kb/min	20-30 bp	Yes
++	+	—	—	≤ 10 fg	1-2 kb/min	20-30 bp	Yes
++++	+	—	—	≤ 10 fg	1-2 kb/min	20-30 bp	Yes
++	+	++++	++	≤ 10 fg	1-2 kb/min	20-30 bp	Yes
++++	+	++++	++	≤ 10 fg	1-2 kb/min	20-30 bp	Yes
++++	+	++++	++++	≤ 10 fg	—	17-25 bp	Yes
++++	+	++++	++++	≤ 10 fg	—	17-25 bp	Yes
++	+	—	—	≤ 10 fg	1-2 kb/min	20-30 bp	Yes ⁺
++	++++	—	—	≤ 10 fg	1-2 kb/min	20-30 bp	Yes ⁺
++	++++	—	—	≤ 10 fg	1-2 kb/min	20-30 bp	Yes ⁺
++++	+	—	—	≤ 10 fg	1-2 kb/min	20-30 bp	Yes ⁺
++	+	++++	—	≤ 10 fg	1-2 kb/min	20-30 bp	Yes ⁺
++	++	—	—	≤ 10 fg	1 kb/min	20-30 bp	No (blunt end)
++	+	—	—	≤ 10 fg	1 kb/min	20-30 bp	Yes
++++	+	—	—	≤ 10 fg	1 kb/min	20-30 bp	Yes
++	+	++++	+++	≤ 10 fg	1 kb/min	20-30 bp	Yes
++++	+	++++	+++	≤ 10 fg	1 kb/min	20-30 bp	Yes

* All of Takara's PCR polymerases are provided with dNTPs and buffer.

+ T-vector cloning efficiency diminishes as the length of the PCR product to be cloned increases above 5 kb.

§ When used with GC Buffer I.

‡ When amplifying GC-rich templates, the fidelity is reduced.

** All fidelity determined by using the Kunkel method.

Fidelity determined by direct sequencing.

For more information, see our website at www.takarabio.com

Appendix VIII: Technical Article

SYBR® Green I: A sensitive, cost-effective detection method for real-time PCR

By Ken Doyle, PhD (Technical Writer)*

Introduction

Real-time PCR, a variation of the original PCR process, is a quantitative method to study the amount of products synthesized during the early (exponential) stages of an amplification reaction. During this stage of PCR, the amount of product corresponds to the amount of initial template present. 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, real-time technology has advanced considerably, with the use of specialized instruments designed to detect the light emitted by amplified, fluorescently labeled DNA molecules.

Real-Time PCR Detection Methods

The technology 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.

The key to successful real-time PCR lies in the detection method used. A variety of probe-based methods are available today, in which fluorescently labeled oligonucleotides are used to detect specific target sequences in PCR products. These probes offer high sensitivity; however, a specific probe must be designed for each target being examined. In addition, the design and synthesis of suitable fluorescently labeled probes can be challenging and expensive.

An alternative to probe-based methods is the use of fluorescent dyes that bind double-stranded DNA (dsDNA) regardless of sequence. Ideally, such a dye should fulfill three criteria: i) it should

be stable under the conditions used for PCR; ii) it should not inhibit amplification; and iii) it should exhibit little or no fluorescence in the unbound state and strong fluorescence in the bound state. Additional requirements for DNA-binding fluorescent dyes include uniform (non-specific) binding and a large linear detection range.

DNA-binding dyes are comparatively easy to use, and are ideally suited for researchers who are new to real-time PCR. They can also be used for initial screening of relative gene expression levels in quantitative RT-PCR, validation screening in high-throughput applications, or other real-time techniques where specific detection of target sequences is not required.

Characteristics and Applications of SYBR® Green I

SYBR® Green I is a fluorescent 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 fluorescence.

In its simplest form, this method of real-time PCR is performed by adding a small amount of SYBR® Green I to the reaction mixture prior to thermal 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 measured by the real-time PCR instrument.

SYBR® Green I has been used to quantitate low-copy number transcripts, with excellent results

*Loquent: Technical, Medical and Scientific Communications

down to 10 copies of template per reaction; single copies of template were also detected under optimal conditions. SYBR® Green I has also been used as a sensitive and accurate detection method for examining genetic mutations in clinical diagnostic studies, as an alternative to conventional DNA quantitation techniques. In studies that compared SYBR® Green I to 5'-exonuclease and hybridization probe-based methods, it was found to possess comparable sensitivity, with linear detection over 7 orders of magnitude.

Takara's Optimized Premix for Real-Time PCR

Takara's SYBR® Premix *Ex Taq*™ system is a convenient (2X) premix consisting of Takara's high-fidelity, high-performance *Ex Taq*™ Hot Start DNA Polymerase, SYBR® Green I and a newly formulated real-time PCR buffer that provides superior specificity and increased amplification efficiency compared to conventional *Taq* DNA polymerase. The premix uses antibody-mediated hot start technology to prevent non-specific amplification due to mispriming and/or formation of primer dimers during reaction assembly. The *Taq* antibody-polymerase complex is denatured in the first cycling step, releasing the polymerase and allowing DNA synthesis to proceed.

Two ROX reference dyes are also supplied as

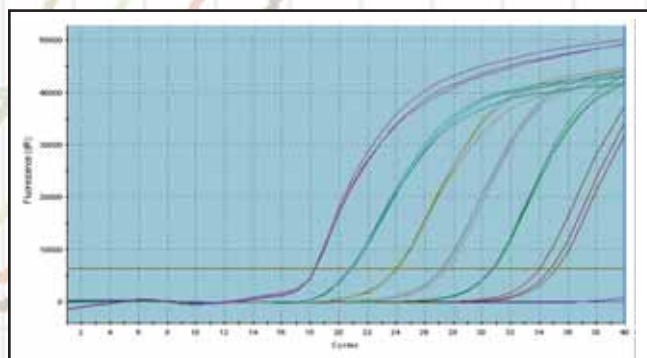


Figure 1: SYBR® Premix *Ex Taq*™ (Perfect Real Time) Amplification Curve using a MX3000P® (Stratagene)

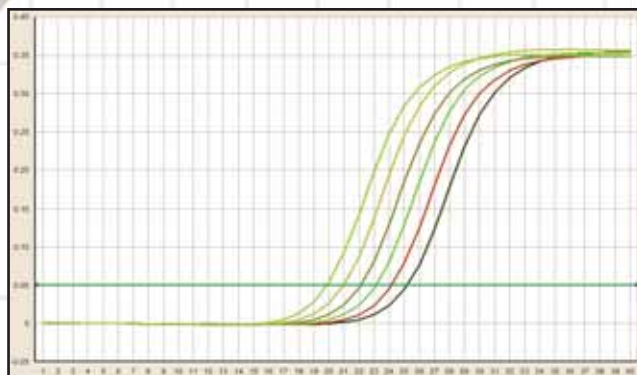


Figure 2: Accurate detection of 2-fold difference, using SYBR® Premix *Ex Taq*™ with an Applied Biosystems 7500 Real Time System.

separate components. These serve as convenient internal reference standards for use in normalizing signals due to non-PCR-related fluctuations in fluorescence intensity that may occur either among wells or over time in different instruments.

The Takara premix performs well using popular real-time PCR instruments, including the SmartCycler (Cepheid), ABI 7500 (Applied Biosystems), and MX3000P (Stratagene) (Figure 1). Further, a comparison of Takara's SYBR® Premix *Ex Taq*™ enzyme with other suppliers demonstrates superior amplification efficiency and reaction specificity using three popular real-time PCR instruments (Figure 2).

In conclusion, SYBR® Green I is an inexpensive, easy-to-use, and highly sensitive detection method for real-time PCR. When used with Takara's SYBR® Premix *Ex Taq*™ system, SYBR® Green I provides real-time PCR results that are comparable or superior to those from other manufacturers.

Higuchi, R. et al. (1993) *Bio/Technology* 11:1026-1030.

Skeidsvoll, J. and Ueland, P. M. (1995) *Anal Biochem* 231:359-365.

Morrison, T. B. et al. (1998) *BioTechniques* 24:954-962.

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Newby, D. T. et al. (2003) *Appl Envir Microbiol* 69:4753-4759.

Appendix IX: Licensing

PCR Limited Use Label License for Takara PCR Enzymes and RNA PCR Products

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Use of this product is covered by one or more of the following US patents and corresponding patent claims outside the US: 5,079,352, 5,789,224, 5,618,711, 6,127,155 and claims outside the US corresponding to US Patent No. 4,889,818. The purchase of this product includes a limited, non-transferable immunity from suit under the foregoing patent claims for using only this amount of product for the purchaser's own internal research. No right under any other patent claim (such as the patented 5' Nuclease Process claims in US Patents Nos. 5,210,015 and 5,487,972), no right to perform any patented method, and no right to perform commercial services of any kind, including without limitation reporting the results of purchaser's activities for a fee or other commercial consideration, is conveyed expressly, by implication, or by estoppel. This product is for research use only. Diagnostic uses under Roche patents require a separate license from Roche. Further information on purchasing licenses may be obtained by contacting the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA.

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5,079,352, 5,789,224, 5,618,711, 6,127,155, 5,677,152, 5,773,258, 5,407,800, 5,322,770, 5,310,652, 5,210,015, 5,487,972, and claims outside the US corresponding to US Patent No. 4,889,818. The purchase of this product includes a limited, non-transferable immunity from suit under the foregoing patent claims for using only this amount of product for the purchaser's own internal research. Separate purchase of a Licensed Probe would convey rights under the applicable claims of US Patents Nos. 5,538,848, 5,723,591, 5,876,930, 6,030,787, 6,258,569, 5,804,375 (claims 1-12 only), and 6,214,979, and corresponding claims outside the United States. No right under any other patent claim and no right to perform commercial services of any kind, including without limitation reporting the results of purchaser's activities for a fee or other commercial consideration, is conveyed expressly, by implication, or by estoppel. This product is for research use only. Diagnostic uses under Roche patents require a separate license from Roche. Further information on purchasing licenses may be obtained from the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA.

Limited Use Label License [L1] : One Step RNA PCR / One Step RT-PCR

Use of this product is licensed from bioMerieux, is covered by US Patent 5,817,465 and equivalents, and is for Research Use Only.

Limited Use Label License [L11] : SYBR® Green I

This product is covered by the claims of U.S. Patent No. 5,436,134 and 5,658,751 and their foreign counterpart patent claims. Takara PCR products containing SYBR® Green I are sold under license from Molecular Probes Inc. only for the usage in Real-time PCR for internal research purpose. These products are not to be used for the purpose such as; providing medical, diagnostic, or any other testing, analysis or screening services or providing clinical information or clinical analysis in return for compensations.

Limited Use Label License [L15] : Hot Start PCR

Licensed under U.S. Patent No. 5,338,671 and 5,587,287, and corresponding patents in other countries.

Limited Use Label License [M21] : Bca BEST™ DNA Polymerase

This product is covered by the claims of U.S. Patent Nos. 5,436,326 and 5,753,482 and their foreign counterpart patent claims.

Limited Use Label License [M57] : LA Technology

This product is covered by the claims 6-16 of U.S. Patent No. 5,436,149 and its foreign counterpart patent claims.

Takara Product Name

Takara Ex Taq™, Ex Taq™ Premix, Ex Taq™ HS, Ex Taq™ HS, Premix
Takara LA Taq™, LA Taq™ with GC Buffers, LA PCR Kit, Ver. 21, One-Shot LA PCR Mix, LA Taq™ HS
Takara Taq™, Premix Taq, Taq HS, Taq HS, Premix
SYBR Premix Ex Taq™ (Perfect Real Time)
Premix Ex Taq™ (Perfect Real Time)
PrimeSTAR® HS DNA Polymerases, PrimeSTAR® HS with GC Buffers, PrimeSTAR® HS, Premix
SpeedSTAR™ HS DNA Polymerase
All Takara Hot Start PCR Enzymes
RNA PCR Kit, Ver. 3.0
One-Step RNA PCR Kit
Real Time One Step RNA PCR Kit
RNA LA PCR Kit
BcaBest™ RNA PCR Kit, Ver. 1.1

License Numbers

[P1] [M57]
[P1] [M57]
[P1] [M57]
[P5] [L11][L15] [M57]
[P7] [L15] [M57]
[P1] [L15] [M57]
[P1] [L15] [M57]
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[P1] [L15] [M57]
[P1] [L1] [M57]
[P1] [L1] [L15] [M57]
[P1] [M57]
[P1] [M21] [M57]

Ordering Information

TaKaRa

page 10

TaKaRa Ex Taq™

TAK RR001A	250 units
TAK RR001B	1,000 units
TAK RR001C	3,000 units

TaKaRa Ex Taq™ (Mg²⁺-free Buffer)

TAK RR01AM	250 units
TAK RR01BM	1,000 units
TAK RR01CM	3,000 units

TaKaRa Taq™

TAK R001A	250 units
TAK R001B	1,000 units
TAK R001C	3,000 units

TaKaRa Taq™ (Mg²⁺-free Buffer)

TAK R001AM	250 units
TAK R001BM	1,000 units
TAK R001CM	3,000 units

e2TAK™ DNA Polymerase

TAK RF001A	200 reactions
TAK RF001B	1,000 reactions
TAK RF001C	3,000 reactions

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SYBR® Premix Ex Taq™ (Perfect Real Time)

TAK RR041A	200 reactions
TAK RR041B	400 reactions

Premix Ex Taq™ (Perfect Real Time)

TAK RR039A	200 reactions
TAK RR039B	400 reactions

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PrimeSTAR® HS DNA Polymerase

TAK RR010A	250 units
TAK RR010B	1,000 units

PrimeSTAR® HS with GC buffer

TAK RR044A	250 units
TAK RR044B	1,000 units

PrimeSTAR® HS DNA Polymerase, Premix

TAK R040A	100 reactions
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SpeedSTAR™ HS DNA Polymerase

TAK RR070A	250 units
TAK RR070B	1,000 units

TaKaRa Ex Taq™ (See page 10)

TaKaRa Ex Taq™ (Mg²⁺-free Buffer) (See page 10)

TaKaRa LA Taq™ (Trial Size)

TAK RR002T	50 reactions
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TaKaRa LA Taq™

TAK RR002M	250 units
TAK RR002B	1000 units
TAK RR002C	3,000 units

TaKaRa LA Taq™ Supplement (Mg²⁺-free Buffer)

TAK RR002A	125 units
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TaKaRa LA Taq™ (with GC Buffers)

TAK RR02AG	125 units
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One Shot LA PCR Mix

TAK RR004	24 reactions
-----------	--------------

LA PCR Amplification Kit, Version 2.1

TAK RR013A	50 reactions
TAK RR013B	100 reactions

page 30

TaKaRa Ex Taq™ Hot Start Version

TAK RR006A	250 units
TAK RR006B	1,000 units

TaKaRa Ex Taq™ Hot Start Version, Premix

TAK RR030A	100 reactions
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TaKaRa Taq Hot Start Version

TAK R007A	250 units
TAK R007B	1,000 units

TaKaRa Taq Hot Start Version, Premix

TAK R028A	100 reactions
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TaKaRa LA Taq™ Hot Start Version

TAK RR042A	125 units
TAK RR002B	500 units

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FastPure™ RNA Kit

TAK 9190	50 reactions
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RNA PCR Kit (AMV), Version 3.0

TAK RR019A	100 reactions
TAK RR019B	200 reactions

Real Time One Step RNA PCR Kit

TAK RR026A	100 reactions
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One Step RNA PCR Kit (AMV)

TAK RR024A	50 reactions
TAK RR024B	100 reactions

RNA LA PCR Kit, Version 1.1

TAK RR012A	50 reactions
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BcaBEST™ RNA PCR, Version 1.1

TAK RR023A	100 reactions
TAK RR023B	200 reactions

page 34

DNA Ligation Kit, Version 2.1

TAK 6022	75 reactions
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DNA Ligation Kit, Version 1.0

TAK 6021	50 reactions
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DNA Ligation Kit, Mighty Mix

TAK 6023	75-100 reactions
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DNA Ligation Kit, LONG

TAK 6024	50 reactions
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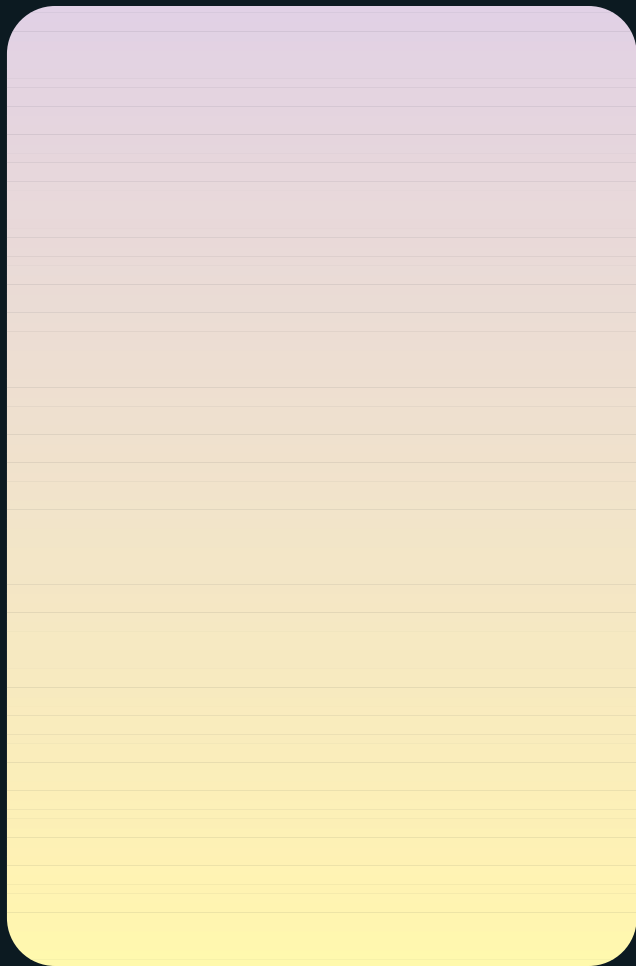
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