Takara Bio USA

TB Green® Advantage® qPCR Premix User Manual

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I. Introduction

The TB Green Advantage qPCR Premix is a reagent supplied at 2X concentration, consisting of a full-length *Taq* polymerase with a hot-start *Taq* antibody and TB Green dye, that has been specially designed for real-time PCR using the dye intercalator method.

This product combines the high performance of our enzyme for hot-start PCR utilizing *Taq* antibody, with a newly developed buffer, to provide superior specificity, increased amplification efficiency, and superb performance in high-speed real-time PCR.

Use of the TB Green Advantage qPCR Premix enables you to carry out successful real-time PCR with high sensitivity, broad dynamic range, and accurate quantification.

This reagent is supplied together with separate tubes of ROX Reference Dye LSR and ROX Reference Dye LMP that can be used to normalize the fluorescent signal intensity between reactions for use with instruments that are equipped with this option. In one tube (LSR), ROX is provided at the optimal concentration for instruments whose excitation source is a 488 nm laser (e.g., the Applied Biosystems 7700 and 7900 instruments; in another tube (LMP), ROX is provided at the optimal concentration for instruments whose excitation source is either a lamp or an LED.

This manual provides protocols for use of the TB Green Advantage PCR Premix with the following real-time PCR instruments:

- Cepheid SmartCycler
- Applied Biosystems Instruments
    - ABI PRISM 7000/7700/7900HT Sequence Detection Systems
    - Applied Biosystems 7300/7500 Real-Time PCR Systems
    - 7500 Fast Real Time PCR System
- Roche LightCycler
- Stratagene Mx3000P

This product is expected to work on other real-time PCR instruments in a similar fashion, although it has not yet been tested on these other instruments.

A. Principle

This product employs a full-length *Taq* polymerase with hot-start antibody for the PCR reaction. PCR products are detected with TB Green dye in real-time monitoring.

1. PCR

PCR (Polymerase Chain Reaction) is a simple and powerful method to amplify a very small amount of target DNA by cycling three incubation steps at different temperatures:

1. Double-stranded target DNA is heat-denatured (denaturation step).
2. The two primers complementary to the target segment are annealed at low temperature (annealing step).
3. The annealed primers are then extended at the same, or an intermediate temperature (extension step) with a DNA polymerase.

The target copy number doubles with each cycle, and therefore, PCR can amplify DNA fragments up to 10^6-fold in a short period. Because this product utilizes an enzyme for hot-start PCR, i.e., full-length *Taq* with hot-start antibody, non-specific amplification due to mispriming prior to cycling or to primer-dimer formation can be minimized and highly specific and sensitive detection is achieved.

2. Fluorescence Detection by the Intercalator Method

This is a detection method utilizing a DNA-intercalating dye that fluoresces once it binds to double-stranded DNA (Figure 1). The most commonly used is green intercalating dye. The dye is added in the reaction system and its fluorescence is detected during amplification. When an intercalator binds to double-stranded DNA synthesized during PCR amplification, fluorescence is emitted and detected during amplification. From measurements of the fluorescence intensity, plotted versus cycle number, amplification plots can be drawn. Moreover, after cycling has been completed, a melting curve analysis can be performed to determine the melting temperature (T_m) of the amplified DNA.

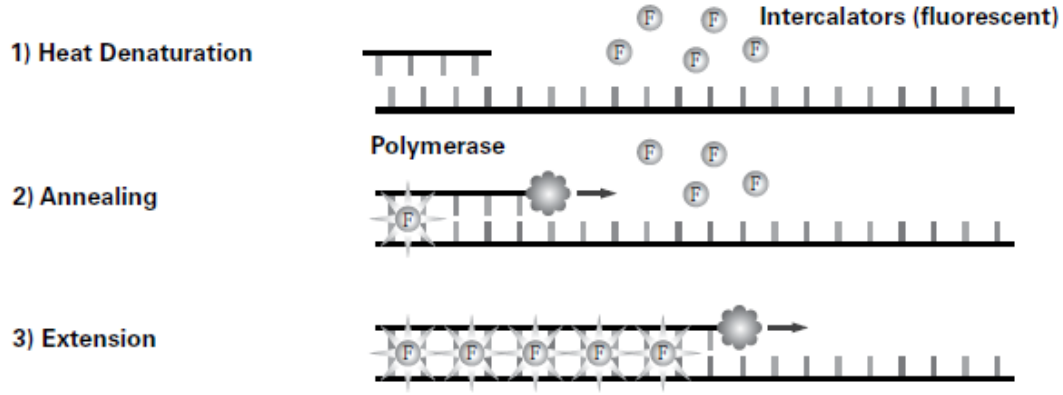

Figure 1. Fluorescence detection by the intercalator method.

II. List of Components

The TB Green Advantage qPCR Premix is available in the following two sizes:

Cat. No. 639676 (suitable for 50 PCR reactions—25 µl rxn volume)

- TB Green Advantage qPCR Premix (2X)[1] 625 µl x 4 tubes
- ROX Reference Dye LSR (50X)[2] 100 µl x 1 tube
- ROX Reference Dye LMP (50X)[2] 100 µl x 1 tube

[1]TB Green Advantage qPCR Premix contains full-length *Taq*, hot-start *Taq* antibody, dNTP mixture, Mg^{2+}, TB Green dye, and buffer.

[2] ROX Reference Dyes used for normalization of intensity by background subtraction. For the ABI PRISM 7000/7700/7900HT Sequence Detection Systems and the Applied Biosystems 7300 Real Time PCR System, the use of ROX Reference Dye LSR (50X) is recommended. For the Applied Biosystems 7500 Real Time PCR System and the Stratagene Mx3000P, the use of ROX Reference Dye LMP is recommended. The use of ROX Reference Dye LSR or LMP is optional. It is not required for use with Smart Cycler or LightCycler real-time instruments. The ROX LSR tube contains the optimal concentration of ROX for instruments whose excitation source is a 488 nm laser. The ROX LMP tube contains the optimal concentration of ROX for instruments whose excitation source is either a lamp or an LED.

III. Additional Materials Required

The following equipment, materials, and reagents are required, but not supplied.

- Thermal cycler for real-time PCR
- Reaction tube or plate for real-time PCR
- PCR-grade H_2O
- Micropipet and micropipet tips (autoclaved prior to use)

NOTE: We recommend filter tips to minimize contamination.

IV. General Considerations

A. Recommended Precautions

Please be sure to read through the following precautions prior to starting the protocol.

1. A white or yellowish precipitate may form in this product during frozen storage. This precipitate can be completely dissolved by briefly warming the tube with your hands or by placing the tube at room temperature while protecting it from light, followed by inversion of the tube several times to mix until dissolved. If product containing precipitate is used for real-time amplification of DNA, decreased signal intensity and reduced reactivity may result due to an inadequate concentration of TB Green dye and other components. USE THE REAGENT ONLY AFTER COMPLETELY RE-DISSOLVING THE PRECIPITATE to ensure a uniform concentration of components. Do not vortex. Even in the absence of a precipitate, gentle mixing of the product (avoiding air bubbles) is recommended to provide a uniform concentration of components prior to use.
2. During preparation of the reaction mixture, all the reagents should be placed on ice.
3. Avoid exposure to direct light in preparation of the PCR reaction mixture because TB Green dye is included, and it is light-sensitive.
4. For preparing and dispensing the reagents, a new disposable tip should be used to minimize contamination among samples.

B. Primer Design Guidelines

It is essential to design primers that allow good reactivity for a successful real-time PCR reaction. Please follow the guidelines in Table I to design primers that offer high amplification efficiency and minimize the formation of nonspecific reaction products.

Table I. Primer Design Guidelines

Amplified product size:	80–150 bp is recommended. (It is possible to amplify a target up to 300 bp in size.)
Primer length:	17–25 mer
GC content:	40–60% (45-55% is recommended.)
T_m:	T_m values of forward and reverse primers must not be significantly different. T_m values are calculated with the software[1,2] for the calculation of T_m values. e.g., OLIGO[1]: 63–68°C Primer 3[2]: 60–65°C
Sequence:	The sequence should not be partially rich in any base in the whole sequence. Avoid including regions that have high GC or AT content, (especially at the 3'-end). Do not include polypyrimidine (serial T/C sequence). Do not include polypurine (serial A/G sequence).
Sequence of 3' end:	The 3' terminus region should not have a high GC or AT content. We recommend that you choose a sequence with G or C at the 3' end. We recommend that you do not choose a sequence with T at the 3' end. A complementary sequence of more than 3 bases should not exist within a primer or even between primer pairs. A primer pair should not have a complementary sequence of more than two bases at each 3' end.
Specificity	Specificity of primers should be confirmed through a BLAST search.[3]

[1]OLIGO™ Primer Analysis Software (Moleculor Biology Insights, Inc.)

[2]Primer3 (http://www-genome.wi.mit.edu/ftp/distribution/software/)

[3]http://www.ncbi.nlm.nih.gov/BLAST/

V. Real-Time PCR Protocols for Specific Instruments

Be sure to read the precautions in Section IV prior to beginning a protocol. Protocols for the following types of instruments are described here.

A. Cepheid SmartCycler II System
B. Applied Biosystems instruments (various)
C. Roche LightCycler
D. Stratagene Mx3000P

Each protocol includes the following general procedures:

1. Preparation of the PCR reaction mixture
2. Initial denaturation
3. PCR reaction
   - Two-step PCR protocol (recommended first approach)
   - Three-step PCR protocol (alternative approach)
4. Melting curve analysis

For the PCR reaction, we recommend that you first try the standard two-step PCR protocol. If that protocol should not yield optimal results, then we recommend that you use a three-step PCR protocol.

For each instrument or set of instruments, the details of these general procedures are described on the following pages.

A. qPCR Protocol using the Cepheid SmartCycler II System

1. Prepare the PCR reaction mixture as indicated in Table II.

Table II. PCR Reaction Mixture (Cepheid SmartCycler II)

Reagent	Volume	Final Concentration
TB Green Advantage qPCR Premix (2X)	12.5 µl	1X
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	varies[2]
PCR-grade H_2O	9.5 µl	
Total	25 µl	

[1]The final concentration of primers can be 0.2 µM in most reactions. If you do not get good results, determine the optimal concentrations within the 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. It is recommended to apply DNA template in quantities less than 100 ng. When the reverse transcription product (cDNA) is used as a template, it should be added in less than 10% the volume of the PCR reaction mixture.

2. Start the reaction. Gently centrifuge the reaction tubes using a centrifuge designed exclusively for use with the Cepheid SmartCycler. Load the tubes into a SmartCycler II System and start the reaction. Carry out the initial denaturation at 95°C for 10–30 sec with detection off as shown in Table III.

Table III. Initial Denaturation (Cepheid SmartCycler II)

Step	Temperature	Time	Detection
Initial Denaturation[1]	95°C	10–30 sec	Off

[1]Initial denaturation requires about 30 sec when the template is a genomic DNA. Depending on your situation, this time can be extended up to 1 min. Note that denaturation for longer than 1 min may make the reaction unstable. Templates shorter than 500 bp may not require this initial denaturation *step at all.

NOTE: This product combines the high performance of full-length *Taq* and hot-start antibody for hot-start PCR. The initial denaturation step prior to PCR should take place at 95°C for 10 sec. It is not necessary to heat at 95°C for 5–15 min, as the initial denaturation required for chemically modified *Taq* polymerase is not necessary. If longer heat treatment is provided, the enzyme activity decreases and the amplification efficiency and quantification accuracy can also be adversely affected.

3. Two-step and three-step PCR protocols

   a. The standard two-step PCR protocol (denaturation and annealing/extension) outlined in Table IV is recommended. Try this protocol first, and optimize the reaction conditions if necessary.

   Step 1. Denature at 95°C for 3–5 sec with detection off.
   Step 2. Carry out combined annealing/extension at 60–66°C for 20–30 sec with detection on.

Table IV. Two-Step PCR (Cepheid SmartCycler II)

Step	Temperature	Time	Detection
Denaturation[1]	95°C	3–5 sec	Off
Annealing/Extension[2]	60–66°C	20–30 sec	On

[1]Typical amplified sizes for real-time PCR products are less than 300 bp, so denaturation at 95°C for 3–5 sec is sufficient.

[2]First attempt annealing/extension at 60°C for 20 sec. The temperature should be optimized within the range of 60–66°C if optimization is required. If the reaction does not proceed efficiently, extend the time or change the reaction to a three-step PCR.

b. If the two-step protocol still does not give optimal results (e.g., if T_m values for the primers are low), try the three-step PCR protocol (denaturation, annealing, and extension) shown in Table V.

Step 1. Denature at 95°C for 3–5 sec with detection off.
Step 2. Carry out annealing at 55–60°C for 10–20 sec with detection off.
Step 3. Carry out extension at 72°C for 6–15 sec with detection on.

Table V. Three-Step PCR (Cepheid SmartCycler II)

Step	Temperature	Time	Detection
Denaturation[1]	95°C	3–5 sec	Off
Annealing[2]	55–60°C	10–20 sec	Off
Extension[3]	72°C	6–15 sec	On

[1]Typical amplified sizes for real-time PCR are less than 300 bp, so denaturation at 95°C for 3–5 sec is sufficient.

[2]Begin with an annealing step at 55°C for 10 sec. If nonspecific amplified product is generated or if the amplification efficiency is low, these issues can be resolved by optimization of the annealing temperature. Longer annealing time may improve the amplification efficiency.

[3]When the amplified size is less than 300 bp, the extension time should be determined within the range of 6–15 sec. Longer extension times can cause nonspecific amplification.

4. For either the two-step or the three-step protocol, set the cycle number in the range of 30–45 cycles.

NOTE: The SmartCycler System has a function that terminates the reaction when amplified product is detected, and moves on to melting curve analysis. This function can allow more rapid analysis and prevent excess amplification prior to melting curve analysis. Consequently, you do not have to be precise about the cycle number you set, as long as it is within the 30-45 cycle range. This function is available with SmartCycler Software Version 2.0. Although the function is not available with SmartCycler Software Version 1.2, it can be used by upgrading the software using SmartCycler Software Version 2.0 Upgrade Kit. This is a software upgrade only, and an instrument upgrade is not necessary.

5. After the reaction is complete, perform analysis and verify the amplification curve and melting curve. Establish the standard curve when quantification is done. Refer to the SmartCycler System operation manual and to the application example in Section VI for the analysis method to be used with the SmartCycler.

B. qPCR Protocol using Applied Biosystems Instruments

This protocol may be carried out using the following instruments:

- ABI PRISM 7000/ 7700/ 7900HT Sequence Detection Systems
- Applied Biosystems 7300/ 7500 Real-Time PCR System
- Applied Biosystems 7500 Fast Real-Time PCR System

Perform the protocol as follows:

1. Prepare the PCR reaction mixture as indicated in Table VI.

Table VI. PCR Reaction Mixture (Applied Biosystems Instruments)

Reagent	Volume	Volume	Final Concentration
TB Green Advantage qPCR Premix (2X)	10 µl	25 µl	1X
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 LSR or LMP[2] (50X)	0.4 µl	1 µl	
Template	2 µl	4 µl	varies[3]
dH_2O	6.8 µl	18 µl	
Total	20 µl[4]	50 µl[4]	

[1]The final concentration of primers should be 0.2 µM in most reactions. When this concentration does not work, determine the optimal concentrations within the range of 0.1–1.0 µM.

[2]The ROX Reference Dye LSR/LMP is supplied for performing normalization of fluorescent signal intensities among wells when used with real-time PCR instruments that have this option. For ABI PRISM 7000/7700/7900HT and Applied Biosystems 7300 Real-Time PCR Systems, the use of ROX Reference Dye LSR (50X) is recommended. For Applied Biosystems 7500 Real-Time PCR System, and 7500 Fast Real-Time PCR System, the use of ROX Reference Dye LMP is recommended.

[3]Final template concentration varies depending on the copy number of target present in the template solution. Optimal amounts should be determined by preparing the dilution series. It is recommended to apply DNA template in amounts less than 100 ng per 20 µl of reaction mixture. When the reverse transcription product (cDNA) is used as a template, it should be added in less than 10% the volume of the PCR reaction mixture.

[4]A reaction volume of 50 µl is recommended for a 96-well plate, a single tube, or an 8-strip tube. A reaction volume of 20 µl is recommended for a 384-well plate or a 96-well Fast Thermal Cycler Plate.

2. Start the reaction with the initial denaturation step at 95°C for 10–30 sec with detection off, as shown in Table VII.

Table VII. Initial Denaturation (Applied Biosystems Instruments)

Step	Temperature	Time	Detection
Initial Denaturation[1]	95°C	10–30 sec	Off

[1]When using genomic DNA as a template, it is necessary to heat-denature at this step for about 30 seconds, occasionally for about 1 minute. Initial denaturation over 1 min may cause unstable monitoring results. When DNA fragments shorter than 500 bp are used as templates, the initial denaturation step may be unnecessary.

3. Two-step and three-step PCR protocols

   a. The standard two-step PCR protocol (denaturation and annealing/extension) outlined in Table VIII is recommended. Try this protocol first, and optimize the reaction conditions if necessary.

   Step 1. Denature at 95°C for 3–5 sec with detection off.
   Step 2. Carry out combined annealing/extension at 60–66°C for 30–34 sec with detection on.

Table VIII. Two-Step PCR (Applied Biosystems Instruments)

Step	Temperature	Time	Detection
Denaturation[1]	95°C	3–5 sec	Off
Annealing/Extension[2]	60–66°C	30–34 sec[3]	On

[1]Because the size of a target amplified for real-time PCR is generally shorter than 300 bp, denaturation at 95°C for 3–5 sec should be sufficient.

[2]Begin with an annealing/extension step at 60°C for 30 sec (31 sec, 34 sec). If optimization is required, the temperature should be optimized within the range of 60–66°C. If the reaction does not proceed efficiently, extend the annealing/extension time or use the three-step PCR protocol.

[3]Fluorescence detection should be 31 sec with the ABI PRISM 7000 and 7300 Real Time PCR Systems, for 34 sec with the ABI PRISM 7500 Real Time PCR System, and for 30 sec with the Applied Biosystems 7700 and 7900HT Sequence Detection Systems.

b. If the two-step protocol does not give optimal results (e.g., if T_m values for the primers are low), try the three-step PCR protocol (denaturation, annealing, and extension) shown in Table IX.

Step 1. Denature at 95°C for 3–5 sec with detection off.
Step 2. Carry out annealing at 55–60°C for 10–20 sec with detection off.
Step 3. Carry out extension step at 72°C for 30–34 sec with detection on.

Table IX. Three-Step PCR (Applied Biosystems Instruments)

Step	Temperature	Time	Detection
Denaturation[1]	95°C	3–5 sec	Off
Annealing[2]	55–60°C	10–20 sec	Off
Extension[3]	72°C	30–34 sec[3]	On

[1]Because the size of a target amplified for real-time PCR is generally shorter than 300 bp, the denaturation at 95°C for 3–10 sec should be sufficient.

[2]Begin with an annealing step at 55°C for 10 sec. If nonspecific amplified product is generated or if the amplification efficiency is low, these issues can be resolved by optimization of the annealing temperature. Longer annealing time may sometimes improve amplification efficiency.

[3]If the amplified size is less than 300 bp, perform extension for 30 sec (31 sec, 34 sec). Longer extension times can cause nonspecific amplification.

[4]Fluorescence detection time should be 31 sec with the ABI PRISM 7000 amd 7300 Real Time PCR Systems, 34 sec with the ABI PRISM 7500 Real Time PCR System and 30 sec with the Applied Biosystems 7700 and 7900HT Sequence Detection Systems.

NOTE: This product combines the high performance of full-length *Taq* and hot-start antibody for hot-start PCR. The initial denaturation step prior to PCR should take place at 95°C for 10 sec. It is not necessary to heat at 95°C for 5–15 min, as the initial denaturation required for chemically modified *Taq* polymerase is not necessary. If longer heat treatment is provided, the enzyme activity decreases and the amplification efficiency and the accuracy in quantification can also be negatively affected.

4. For either the two-step or three-step PCR, set the cycle number for 30–45 cycles.
5. After the reaction is complete, verify the amplification curve and dissociation curve. Establish the standard curve when quantification is done. For analysis procedures, please refer to the operation manual of the Applied Biosystems instrument that you used.

C. qPCR Protocol using the Roche LightCycler

1. Prepare the PCR reaction mixture as indicated in Table X.

Table X. PCR Reaction Mixture (Roche LightCycler)

Reagent	Volume	Final Concentration
TB Green Advantage qPCR Premix (2X)	10 µl	1X
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	varies[2]
dH_2O	7.2 µl	
Total	20 µl	

[1]The final concentration of primers can be 0.2 µM in most reactions. If this concentration does not work, determine the optimal concentrations within the 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. It is recommended to apply DNA template in amounts less than 100 ng. When cDNA is used as a template, it should be added in less than 10% volume of the PCR reaction mixture.

2. After gently centrifuging the LightCycler capillaries, place them in a LightCycler and start the reaction with the initial denaturation step, at 95°C for 10–30 sec with detection off, as shown in Table XI.

Table XI. Initial Denaturation (Roche LightCycler)

Step	Temperature	Time	Detection
Initial Denaturation[1]	95°C	10–30 sec	Off

[1]When using genomic DNA as a template, it is necessary to heat-denature at this step for about 30 seconds, occasionally for about 1 minute. Initial denaturation for longer than 1 min may cause unstable monitoring results. When DNA fragments shorter than 500 bp are used as templates, the initial denaturation step may be unnecessary.

3. Two-step and three-step PCR protocols

   a. The standard two-step PCR protocol (denaturation and annealing/extension) outlined in Table XII is recommended. Try this protocol first, and optimize the reaction conditions if necessary.

   Step 1. Denature at 95°C for 3–5 sec with detection off.
   Step 2. Carry out combined annealing/extension at 60–66°C for 20–30 sec with detection on.

NOTE: This product combines the high performance of full-length *Taq* and hot-start antibody. The initial denaturation step prior to PCR should take place at 95°C for 10 sec. It is not necessary to heat the PCR reactions at 95°C for 5–15 min, as would be required for the initial denaturation step if chemically modified *Taq* polymerase were used. If the heat treatment is longer than 10 sec, the enzyme activity decreases, and both the amplification efficiency and the quantification accuracy can both be adversely affected.

Table XII. Two-Step PCR

Step	Temperature	Time	Detection
Denaturation[1]	95°C	3–5 sec	Off
Annealing/Extension[2]	60–66°C	20–30 sec	On

[1]Because the size of the target amplified for real-time PCR is generally shorter than 300 bp, denaturation at 95°C for 3–5 sec is sufficient.

[2]Begin with an annealing/extension step at 60°C for 20 sec. If optimization is required, the temperature should be optimized within the range of 60–66°C. If the reaction does not proceed efficiently, extend the time or change the reaction to a three-step PCR.

   b. Three-step PCR. If the two-step protocol still does not give optimal results (e.g., if T_m values for the primers are low), try the three-step PCR protocol (denaturation, annealing, and extension) shown in Table XIII.

   Step 1. Denature at 95°C for 3–5 sec with detection off.
   Step 2. Carry out annealing at 55–60°C for 10–20 sec with detection off.
   Step 3. Carry out extension step at 72°C for 30–34 sec with detection on.

Table XIII. Three-Step PCR

Step	Temperature	Time	Detection
Denaturation[1]	95°C	3–5 sec	Off
Annealing[2]	55–60°C	10–20 sec	Off
Extension[3]	72°C	6–15 sec	On

[1]Because the size of the target amplified for real-time PCR is generally shorter than 300 bp, denaturation at 95°C for 3–10 sec is sufficient.

[2]Begin with an annealing step at 55°C for 10 sec. If nonspecific amplified product is generated or if the amplification efficiency is low, these issues can be resolved by optimization of the annealing temperature. Longer annealing times may sometimes improve amplification efficiency.

[3]If the amplified size is less than 300 bp, attempt amplification for 30 sec (31 sec, 34 sec). Longer extension times can cause nonspecific amplification.

4. For either the two-step or three-step PCR, set the cycle number for 30–45 cycles.
5. After the reaction is complete, verify the amplification curve and melting curve. Establish the standard curve when quantification is done. Refer to the LightCycler operation manual for analysis procedures.

D. qPCR Protocol using the Stratagene Mx3000P

1. Prepare the PCR reaction mixture as indicated in Table XIV.

Table XIV. PCR Reaction Mixture (Stratagene Mx3000P)

Reagent	Volume	Final Concentration
TB Green Advantage qPCR Premix (2X)	12.5 µl	1X
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 LMP[2] (50X)	0.5 µl	1X
Template (<100ng)	2 µl	varies[3]
dH_2O	9 µl	
Total	25 µl	

[1]The final concentration of primers can be 0.2 µM in most reactions. If this concentration does not work, determine the optimal concentrations within the range of 0.1–1.0 µM.

[2]For Mx3000P, the use of ROX Reference Dye LMP is recomended. ROX Reference Dye LSR is not suitable for the use with Mx3000P, because it has a higher concentration of ROX than ROX Reference Dye LMP.

[3]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. It is recommended to apply DNA template in amounts less than 100 ng. When the reverse transcription reactant (cDNA) is used as a template, it should be added in less than 10% volume of the PCR reaction mixture.

2. Start the reaction with the initial denaturation step at 95°C for 10–30 sec with detection off, as shown in Table XV.

Table XV. Initial Denaturation (Stratagene Mx3000P)

Step	Temperature	Time	Detection
Initial Denaturation[1]	95°C	10–30 sec	Off

[1]When using genomic DNA as a template, it is necessary to heat-denature at this step for 30–60 sec. Initial denaturation for longer than 1 min may cause unstable monitoring results. When DNA fragments shorter than 500 bp are used as template, the initial denaturation step may be unnecessary.

3. Two-step and three-step PCR protocols

   a. The standard two-step PCR protocol (denaturation and annealing/extension) outlined in Table XVI is recommended. Try this protocol first, and optimize the reaction conditions if necessary.

      Step 1. Denature at 95°C for 3–5 sec with detection off.
      Step 2. Carry out combined annealing/extension at 60–66°C for 20–30 sec with detection on.

Table XVI. Two-Step PCR

Step	Temperature	Time	Detection
Denaturation[1]	95°C	3–5 sec	Off
Annealing/Extension[2]	60–66°C	20–30 sec	On

[1]Because the size of the target amplified for real-time PCR is generally shorter than 300 bp, denaturation at 95°C for 3–5 sec should be sufficient.

[2]Begin with an annealing/extension step at 60°C for 20 sec. If optimization is required, the temperature should be optimized within the range of 60–66°C. If the reaction does not proceed efficiently, extend the time or change the reaction to a three-step PCR.

   b. If the two-step protocol still does not give optimal results (e.g., if T_m values for the primers are low), try the three-step PCR protocol (denaturation, annealing, and extension) shown in Table XVII.

      Step 1. Denature at 95°C for 3–5 sec with detection off.
      Step 2. Carry out annealing at 55–60°C for 10–20 sec with detection off.
      Step 3. Carry out extension step at 72°C for 6–15 sec with detection on.

Table XVII. Three-Step PCR

Step	Temperature	Time	Detection
Denaturation[1]	95°C	3–5 sec	Off
Annealing[2]	55–60°C	10–20 sec	Off
Extension[3]	72°C	6–15 sec	On

[1]Because the target size amplified for real-time PCR is generally shorter than 300 bp, the denaturation at 95°C for 3–5 sec is sufficient.

[2]Begin with an annealing step at 55°C for 10 sec. If nonspecific amplified product is generated or if the amplification efficiency is low, these issues can be resolved by optimization of the annealing temperature. Longer annealing times may sometimes improve the amplification efficiency.

[3]If the amplified size is less than 300 bp, amplify for 30 sec (31 sec, 34 sec). Longer extension times can cause nonspecific amplification.

NOTE: This product combines the high performance of full-length *Taq* and hot-start antibody. The initial denaturation step prior to PCR should be at 95°C for 10 sec. It is not necessary to heat at 95°C for 5–15 min, as would be required for the initial denaturation step if chemically modified *Taq* polymerase were used. If the heat treatment is longer than 10 sec, the enzyme activity decreases, and the amplification efficiency and the quantification accuracy can both be adversely affected.

4. For either the two-step or three-step PCR, set the cycle number for 30–45 cycles.
5. After the reaction is complete, verify the amplification curve and dissociation curve. Establish the standard curve after quantification is finished. For analysis procedures, refer to the operation manual of the Mx3000P you used.

VI. Application Example

Detection of λ DNA with TB Green dye (with Smart Cycler II System)

Detection of λ DNA (300 bp) was performed by using TB Green Advantage qPCR Premix. The dilution series of λ DNA was prepared and the standard curve was established.

1. Preparation of dilution series of λ DNA: The eight-dilution series (5 ng/µl–0.5 fg/µl) was prepared by carrying out serial tenfold dilutions of λ DNA. 2 µl of each concentration was applied to the reaction.
2. Preparation of reaction mixture and reaction: The PCR reaction was carried out according to the protocol described in Section V, Tables III and IV. A negative control was prepared by adding dH_2O instead of λ DNA. The PCR reaction conditions were set as shown below (Figure 2).

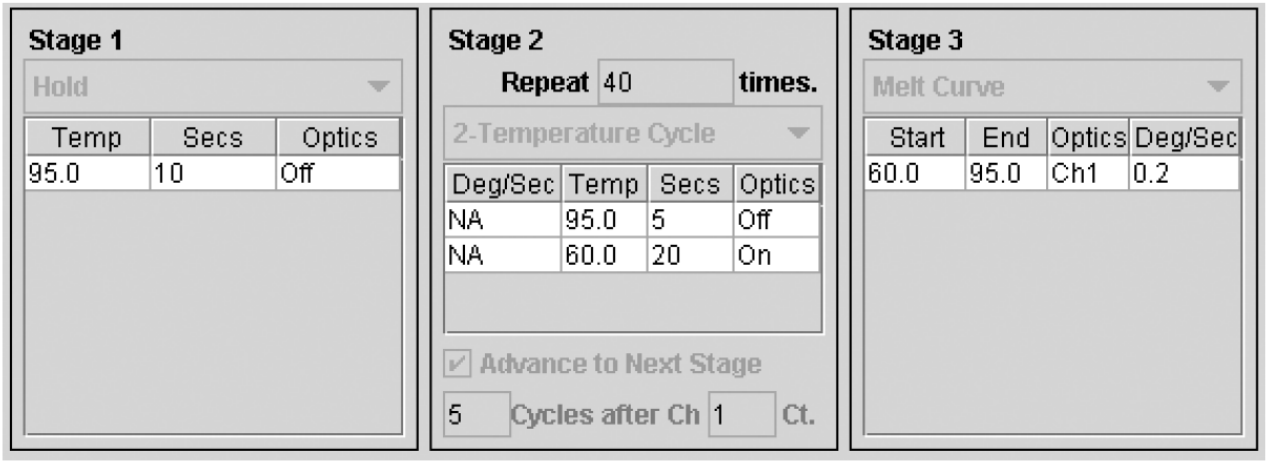

Figure 2. PCR conditions for the application example (λ DNA detection, dilution series)

3. The amplification of PCR product was monitored in real time, as shown in the amplification and melting curves below (Figures 3 & 4).

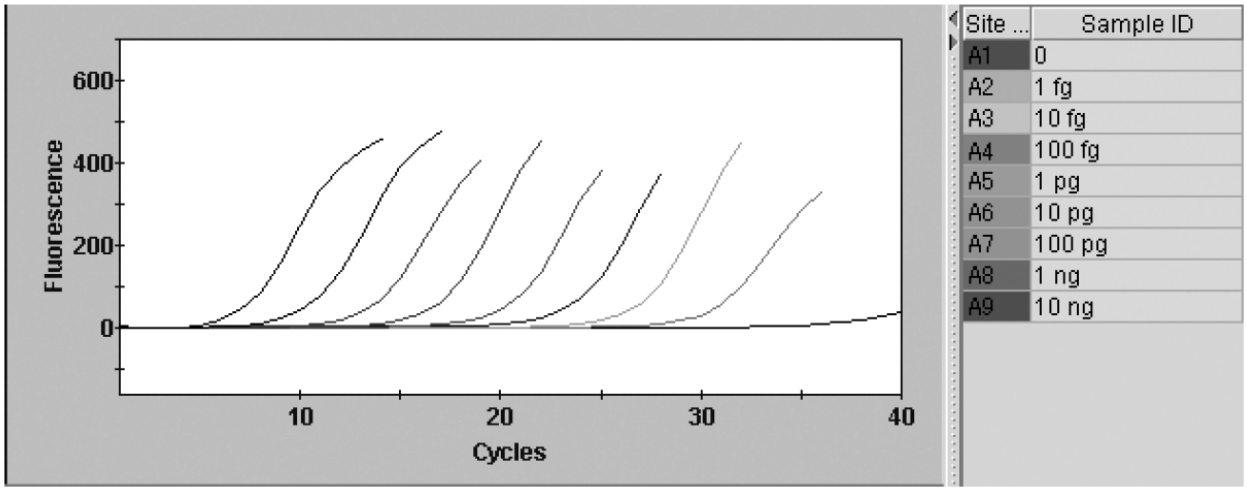

Figure 3. Amplification curves for the λ DNA PCR product.

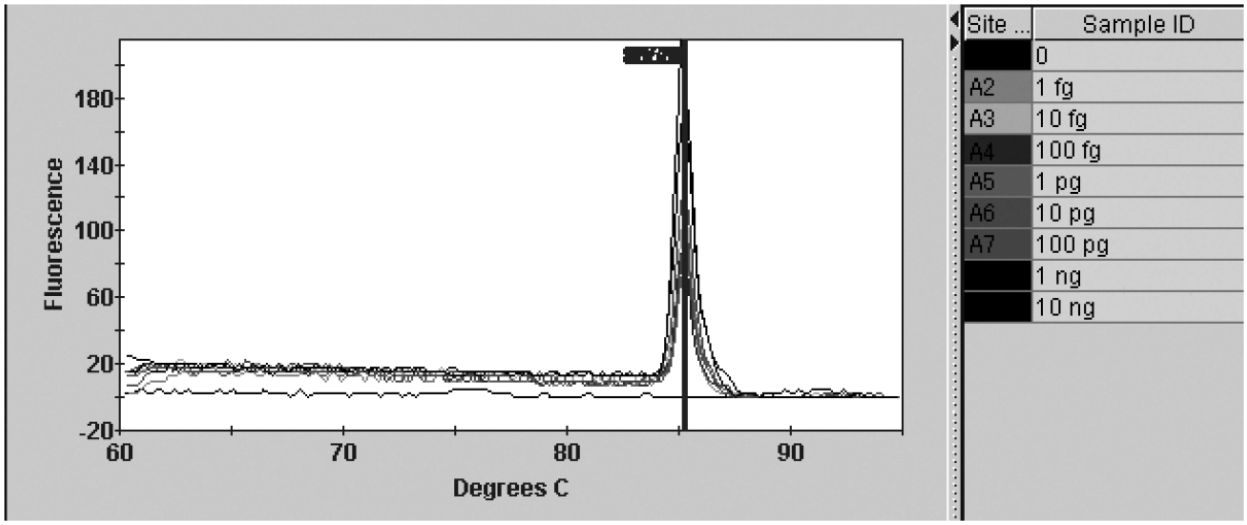

Figure 4. Melting curves for the λ DNA PCR product.

4. Establishing the standard curve: After the reaction was completed, C_t values were calculated from the amplification curves and the standard curve was established (Figure 5).

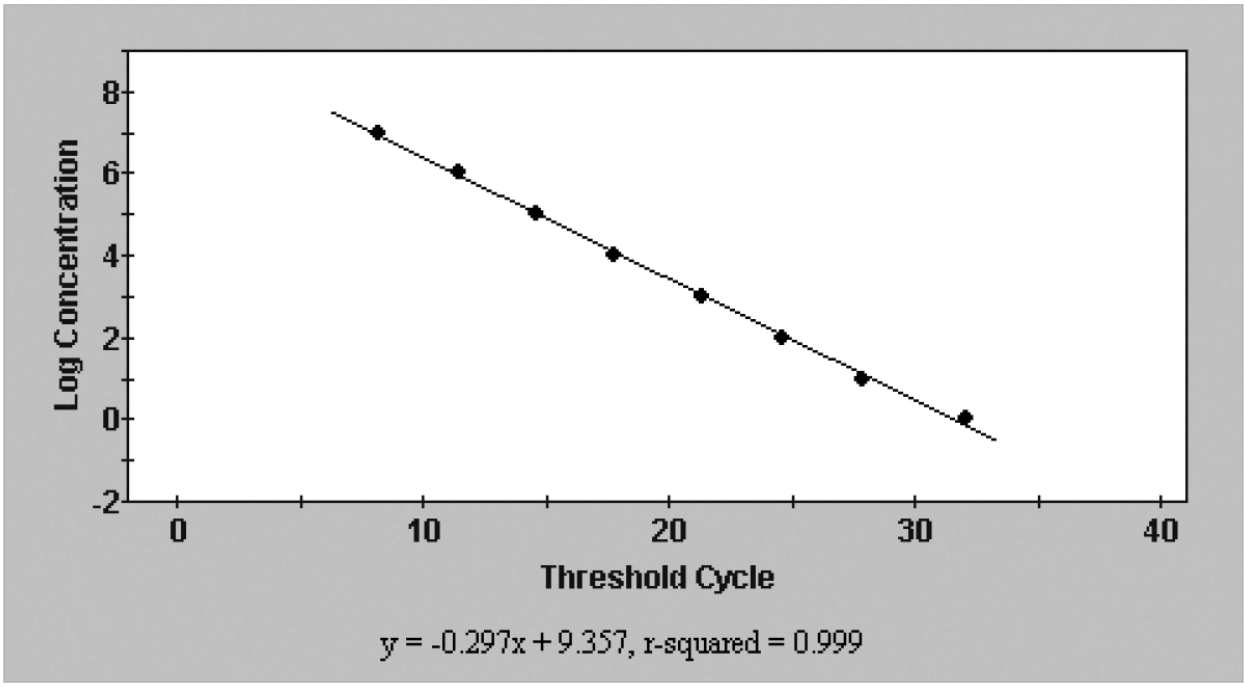

Figure 5. Standard curve for the λ DNA PCR amplification.

Detection of λ DNA was possible when just 1 fg was used as template. The melting curve shows that only single amplified product was obtained in all template amounts. In addition, the standard curve shows a high correlation, indicating accurate quantification can be achieved in the range of the applied concentrations.

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