Cat. # RR820A

For Research Use

TaKaRa

TB Green® *Premix Ex Taq*™ II (Tli RNaseH Plus)

Product Manual

v202112Da

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I. Description

TB Green *Premix Ex Taq* II (Tli RNaseH Plus) is a reagent specifically designed for intercalator-based real-time PCR using TB Green for detection. The premix is supplied as a convenient, ready-to-use 2X concentrate and contains TB Green at a concentration appropriate for real-time monitoring. Simply add primers, template, and sterile purified water to perform intercalator-based real-time PCR.
The 2X premix also contains Tli RNaseH, a heat-resistant RNase H, which minimizes PCR inhibition due to residual mRNA when using cDNA as the template.

This product contains a modified buffer with higher reaction specificity than TB Green *Premix Ex Taq* (Tli RNaseH Plus) (Cat. #RR420A/B). The inhibition of non-specific amplification, which can interfere with quantification, allows accurate measurement over a wide dynamic range. The combination of this buffer and *TaKaRa Ex Taq*® HS, an efficient hot-start PCR enzyme that uses an anti-*Taq* antibody, allows highly sensitive, reproducible, and reliable real-time PCR amplification and analysis.

II. Principle

This product includes *TaKaRa Ex Taq* HS DNA polymerase for PCR amplification. PCR amplification products may be monitored in real time using TB Green as an intercalator.

1. PCR

PCR is a technique used to amplify a target sequence from a minute amount of DNA. By repeating cycles of denaturation, primer annealing, and elongation, the target gene fragment may be quickly amplified using DNA polymerase.

This product uses *TaKaRa Ex Taq* HS, a hot-start PCR enzyme that prevents non-specific amplification from mispriming or primer-dimer formation during reaction mixture preparation or other pre-cycling steps, allowing highly sensitive detection.

2. Fluorescence Detection - Intercalator Method

This method involves the addition of an intercalating agent (TB Green) that fluoresces when bound to double-stranded DNA in the reaction mixture.

Monitoring this fluorescence enables the detection of amplified DNA, quantitative determination of target DNA, and determination of DNA composition by melting curve analysis.

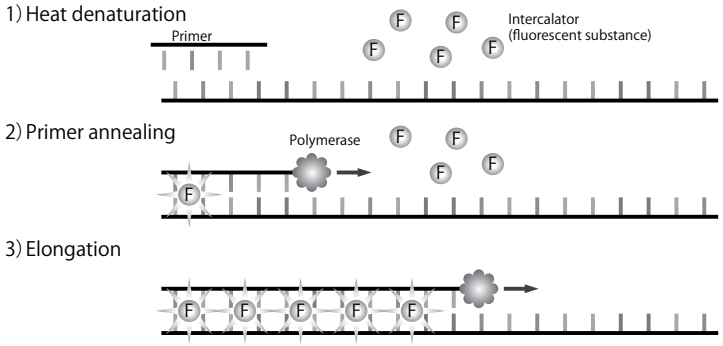


Figure 1. Fluorescent intercalator detection method.

III. Components (200 reactions, 50 μl per reaction)

TB Green *Premix Ex Taq* II (Tli RNaseH Plus) (2X)＊1	1 ml x 5
ROX Reference Dye (50X)＊2	200 μl
ROX Reference Dye II (50X)＊2	200 μl

＊1 Contains *TaKaRa Ex Taq* HS DNA polymerase, dNTP mixture, Mg^{2+}, Tli RNaseH, and TB Green.

＊2 ROX Reference Dye and ROX Reference Dye II are intended for use with instruments that correct for between-well fluorescent signal, such as the real-time PCR devices by Applied Biosystems .

◆ Use ROX Reference Dye:
- Applied Biosystems 7300 Real-Time PCR System (Thermo Fisher Scientific)
- Applied Biosystems StepOnePlus Real-Time PCR System (Thermo Fisher Scientific)

◆ Use ROX Reference Dye II:
- Applied Biosystems 7500/7500 Fast Real-Time PCR System (Thermo Fisher Scientific)

◆ Do not use either reference dye:
- Thermal Cycler Dice™ Real Time System series (Cat. #TP950*3 etc., TP700/TP900: discontinued)
- LightCycler/LightCycler 480 System (Roche Diagnostics)
- CFX96 Real-Time PCR Detection System (Bio-Rad)

＊3 Not available in all geographic locations. Check for availability in your area.

IV. Storage

Store at 4℃ (stable for up to 6 months.)

Always protect from light and avoid contamination.

For long-term storage, store at -20℃. Store thawed or opened product at 4℃ and use within 6 months.

V. Materials Required but not Provided

1. Reagents

- PCR primers
  Guidelines for real-time PCR primer design are found in Section IX.1.
- Sterile purified water

2. Materials

- Real-time PCR reaction tubes or plates designed specifically for the qPCR instrument used
- Micropipettes and tips (sterile filter tips)
- Gene amplification system for real-time PCR (authorized instruments)

  Compatible instruments include:
  - Thermal Cycler Dice Real Time System III (Cat. #TP950/TP970/TP980/TP990)*
    Thermal Cycler Dice Real Time System *II* (Cat. #TP900/TP960: discontinued)
    Thermal Cycler Dice Real Time System *Lite* (Cat. #TP700/TP760: discontinued)

    * Not available in all geographic locations. Check for availability in your area.
  - Applied Biosystems 7300, 7500, or 7500 Fast Real-Time PCR Systems, StepOnePlus Real-Time PCR System (Thermo Fisher Scientific)
  - LightCycler/LightCycler 480 System (Roche Diagnostics)
  - CFX96 Real-Time PCR Detection System (Bio-Rad)

Note: TB Green *Premix Ex Taq* (Tli RNaseH Plus) (Cat. #RR420A/B) or TB Green Fast qPCR Mix (Cat. #RR430A/B) is recommended when using the Smart Cycler System/Smart Cycler II System (Cepheid).

VI. Precautions

Read these precautions before use and follow them carefully.

1. Prior to use, make sure the reagent is evenly mixed by gently inverting several times without creating bubbles. Uneven reagent mixture will result in inadequate reactivity.
   - Do not mix by vortexing.
   - When stored at -20℃, TB Green *Premix Ex Taq* II (Tli RNaseH Plus) may develop a white to pale yellow precipitate. Gently hand-warm and allow to stand protected from light at room temperature briefly, then invert several times to dissolve the precipitate completely.
   - The presence of precipitate is indicative of uneven reagent composition; make sure the reagent is evenly mixed before use.
2. Place reagents on ice immediately after thawing, and them keep on ice while preparing the reaction mixture.
3. This product contains TB Green. Avoid exposure to bright light while preparing the reaction mixture.
4. While preparing or dispensing reaction mixtures, use sterile, disposable tips to avoid contamination between samples.

VII. Protocol

Note: Please follow the procedures in the manual provided with each respective instrument.

【For the Applied Biosystems 7300, 7500, and 7500 Fast Real-Time PCR Systems and the StepOnePlus Real-Time PCR Systems】

A. Prepare the PCR mixture shown below. To account for pipetting error, make a master mix with at least 10% more than the total volume needed for the total number of reactions.

<Per reaction>

Reagent	Volume	Volume	Final conc.
TB Green *Premix Ex Taq* II (Tli RNaseH Plus) (2X)	10 μl	25 μl	1X
PCR Forward Primer (10 μM)	0.8 μl	2 μl	0.4 μM*1
PCR Reverse Primer (10 μM)	0.8 μl	2 μl	0.4 μM*1
ROX Reference Dye (50X) or Dye II (50X)*2	0.4 μl	1 μl	1X
Template*3	2 μl	4 μl	
Sterile purified water	6 μl	16 μl	
Total	20 μl*4	50 μl*4	

*1 A final primer concentration of 0.4 μM is likely to yield good results. However, if there is insufficient reactivity, use a primer concentration between 0.2 and 1.0 μM.

*2 The dye concentration in the ROX Reference Dye II (50X) is lower than in the Reference Dye (50X).

- Use ROX Reference Dye II (50X) when performing analyses with Applied Biosystems 7500 or 7500 Fast Real-Time PCR Systems.
- Use ROX Reference Dye (50X) when using StepOnePlus or Applied Biosystems 7300 Real-Time PCR Systems.

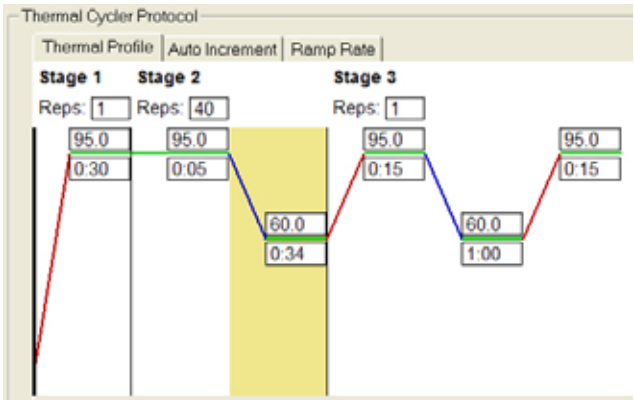
*3 The optimal quantity varies depending on the number of target copies present in the template solution. Make serial dilutions to determine the appropriate template amount and use no more than 100 ng of DNA template per 20 μl. Furthermore, if cDNA (RT reaction mixture) is used as the template, the volume of the RT reaction mixture should not exceed 10% of the PCR mixture.

*4 Prepare in accordance with the recommended volume for each instrument.

B. Start the reaction.

The recommended shuttle PCR standard protocol is described below. Try this protocol first and optimize PCR conditions as necessary. Perform 3-step PCR when using primers with low Tm values or when shuttle PCR is not feasible. To further optimize PCR conditions, see section VIII. Optimization.

1) Applied Biosystems 7300/7500 and StepOnePlus Real-Time PCR System



Stage 1: Initial Denaturation
Reps: 1
95℃ 30 sec
Stage 2: PCR
Reps: 40
95℃ 5 sec
60℃ 30 - 34 sec *
Dissociation Stage

* With StepOnePlus, set to 30 sec; with 7300, set to 31 sec; and with 7500, set to 34 sec.

Figure 2. Shuttle PCR standard protocol.

2) Applied Biosystems 7500 Fast Real-Time PCR System

Shuttle PCR standard protocol

Holding Stage
Number of Cycle: 1
95℃ 30 sec
Cycling Stage
Number of Cycles: 40
95℃ 3 sec
60℃ 30 sec
Melt Curve Stage

Note:
TaKaRa Ex Taq HS DNA polymerase is a hot-start PCR enzyme with an anti-*Taq* antibody that inhibits polymerase activity. The initial denaturation step prior to PCR should be 95℃ for 30 sec. Longer heat treatment may decrease enzyme activity and affect amplification efficiency and quantification.

C. After the reaction is complete, check the amplification and melting curves and plot a standard curve if quantification will be performed.

Refer to the instrument's instruction manual for specific analysis methods.

【 For the LightCycler/LightCycler 480 System 】

A. Prepare the PCR mixture shown below. To account for pipetting error, make a master mix with at least 10% more than the total volume needed for the total number of reactions.

<Per reaction>

Reagent	Volume	Final conc.
TB Green *Premix Ex Taq* II (Tli RNaseH Plus) (2X)	10 μl	1X
PCR Forward Primer (10 μM)	0.8 μl	0.4 μM＊1
PCR Reverse Primer (10 μM)	0.8 μl	0.4 μM＊1
Template (<100 ng)＊2	2 μl	
Sterile purified water	6.4 μl	
Total	20 μl	

＊1 A final primer concentration of 0.4 μM is likely to yield good results. However, if there is insufficient reactivity, use a primer concentration between 0.1 and 1.0 μM.

＊2 The optimal quantity varies depending on the number of target copies present in the template solution. Make serial dilutions to determine the appropriate template amount and use no more than 100 ng of DNA template per 20 μl. Furthermore, if cDNA (RT reaction mixture) is used as the template, the volume of the RT reaction mixture should not exceed 10% of the PCR mixture.

B. Start the reaction.

The shuttle PCR standard protocol is recommended for PCR. Try this protocol first and optimize PCR conditions as necessary. Perform 3-step PCR when using primers with low Tm values or when shuttle PCR is not feasible. To further optimize PCR conditions, please see section VIII. Optimization.

<LightCycler>

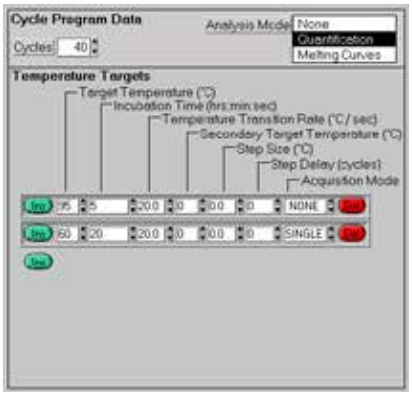


Figure 3. Shuttle PCR standard protocol

Stage 1: Initial Denaturation
95℃ 30 sec 20℃/sec
1 cycle

Stage 2: PCR (See figure on the left)
95℃ 5 sec 20℃/sec
60℃ 20 sec 20℃/sec
40 cycles

Stage 3: Melt Curve Analysis
95℃ 0 sec 20℃/sec
65℃ 15 sec 20℃/sec
95℃ 0 sec 0.1℃/sec

<LightCycler 480 System>

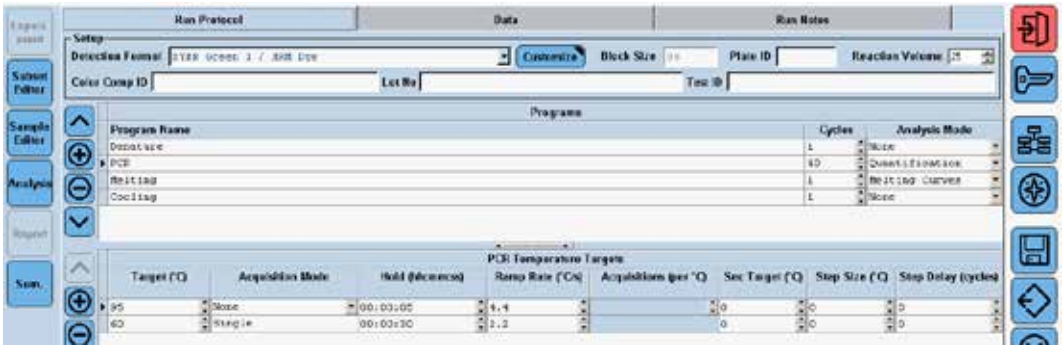


Figure 4. Shuttle PCR standard protocol

Initial Denaturation
95℃ 30 sec (Ramp Rate 4.4℃/sec)
1 cycle

PCR
Analysis Mode: Quantification
95℃ 5 sec (Ramp Rate 4.4℃/sec)
60℃ 30 sec (Ramp Rate 2.2℃/sec, Acquisition Mode: Single)
40 cycles

Melting
Analysis Mode: Melting Curves
95℃ 5 sec (Ramp Rate 4.4℃/sec)
60℃ 1 min (Ramp Rate 2.2℃/sec)
95℃ (Ramp Rate 0.11℃/sec, Acquisition Mode: Continuous, Acquisitions: 5 per ℃)
1 cycle

Cooling
50℃ 30 sec (Ramp Rate 2.2℃/sec)
1 cycle

Note:
TaKaRa Ex Taq HS DNA polymerase is a hot-start PCR enzyme with an anti-*Taq* antibody that inhibits polymerase activity. The initial denaturation step prior to PCR should be 95℃ for 30 sec. Longer heat treatment may decrease enzyme activity and affect amplification efficiency and quantification.

C. After the reaction is complete, check the amplification and melting curves and plot a standard curve if quantification will be performed.

Refer to the instrument's instruction manual for specific analysis methods.

【 For the CFX96 Real-Time PCR Detection System 】

A. Prepare the PCR mixture shown below. To account for pipetting error, make a master mix with at least 10% more than the total volume needed for the total number of reactions.

<Per reaction>

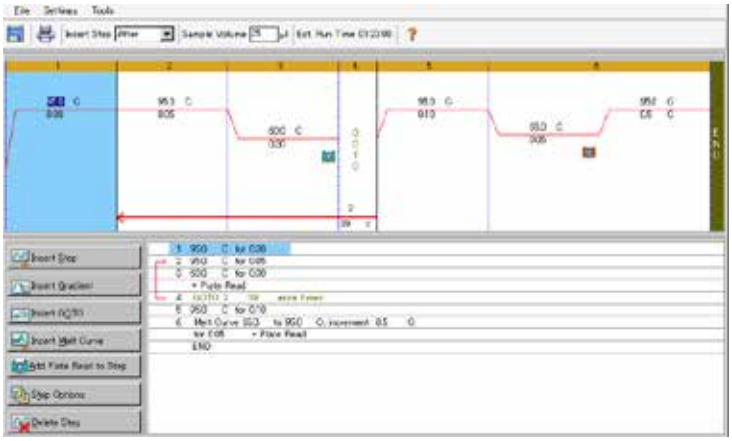
Reagent	Volume	Final conc.
TB Green *Premix Ex Taq* II (Tli RNaseH Plus) (2X)	12.5 μl	1X
PCR Forward Primer (10 μM)	1 μl	0.4 μM*1
PCR Reverse Primer (10 μM)	1 μl	0.4 μM*1
Template (<100 ng)*2	2 μl	
Sterile purified water	8.5 μl	
Total	25 μl	

*1 A final primer concentration of 0.4 μM is likely to yield good results. However, if there is insufficient reactivity, use a primer concentration between 0.2 and 1.0 μM.

*2 The optimal quantity varies depending on the number of target copies present in the template solution. Make serial dilutions to determine the appropriate template amount and use no more than 100 ng of DNA template per 25 μl. Furthermore, if cDNA (RT reaction mixture) is used as the template, the volume of the RT reaction mixture should not exceed 10% of the PCR mixture.

B. Start the reaction.

The shuttle PCR standard protocol is recommended; try this protocol first and optimize PCR conditions as necessary. Perform 3-step PCR when using primers with low Tm values or when shuttle PCR is not feasible. For guidelines on optimizing PCR conditions, please refer to Section VIII.



Sample volume: 25 μl

Step 1: Initial Denaturation
95℃ 30 sec

Step 2: PCR
GOTO: 39 (40 cycles)
95℃ 5 sec
60℃ 30 sec

Step 3: Melt Curve

Figure 5. Shuttle PCR standard protocol

Note:
TaKaRa Ex Taq HS DNA polymerase is a hot-start PCR enzyme with an anti-*Taq* antibody that inhibits polymerase activity. The initial denaturation step prior to PCR should be 95℃ for 30 sec. Longer heat treatment may decrease enzyme activity and affect amplification efficiency and quantification.

C. After the reaction is complete, check the amplification and melting curves and plot a standard curve if quantification will be performed.

Refer to the instrument's instruction manual for specific analysis methods.

【 For the Thermal Cycler Dice Real Time System *series* 】

A. Prepare the PCR mixture shown below. To account for pipetting error, make at a master mix with least 10% more than the total volume needed for the total number of reactions.

<Per reaction>

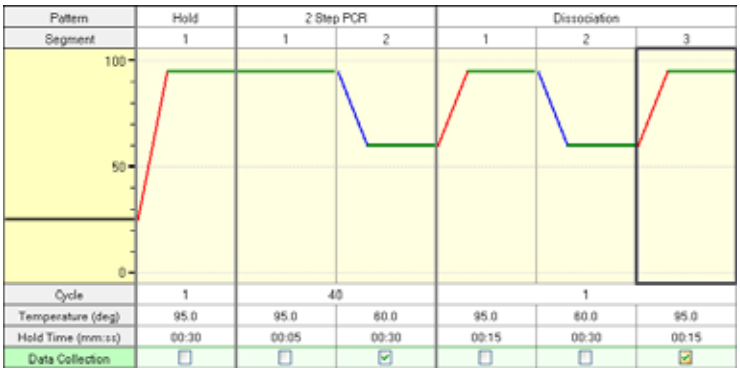
Reagent	Volume	Final conc.
TB Green *Premix Ex Taq* II (Tli RNaseH Plus) (2X)	12.5 μl	1X
PCR Forward Primer (10 μM)	1.0 μl	0.4 μM＊1
PCR Reverse Primer (10 μM)	1.0 μl	0.4 μM＊1
Template (<100 ng)＊2	2.0 μl	
Sterile purified water	8.5 μl	
Total	25 μl＊3	

＊1 A final primer concentration of 0.4 μM is likely to yield good results. However, if there is insufficient reactivity, use a primer concentration between 0.1 and 1.0 μM.

＊2 The optimal quantity varies depending on the number of target copies present in the template solution. Make serial dilutions to determine the appropriate template amount and use no more than 100 ng of DNA template per 25 μl. Furthermore, if cDNA (RT reaction mixture) is used as the template, the volume of the RT reaction mixture should not exceed 10% of the PCR mixture.

＊3 The recommended reaction volume is 25 μl.

B. Start the reaction.
The shuttle PCR standard protocol is recommended; try this protocol first and optimize PCR conditions as necessary. Perform a 3-step PCR when using primers with low Tm values or when shuttle PCR is not feasible. To optimize PCR conditions, please refer to Section VIII.



Hold (Initial Denaturation)
Cycle: 1
95℃ 30 sec

2-Step PCR
Cycles: 40
95℃ 5 sec
60℃ 30 sec

Dissociation

Figure 6. Shuttle PCR standard protocol.

Note:
TaKaRa Ex Taq HS DNA polymerase is a hot-start PCR enzyme with an anti-*Taq* antibody that inhibits polymerase activity. The initial denaturation step prior to PCR should be 95℃ for 30 sec. Longer heat treatment may decrease enzyme activity and affect amplification efficiency and quantification.

C. After the reaction is complete, check the amplification and melting curves and plot a standard curve if quantification will be performed.
Refer to the instrument's instruction manual for specific analysis methods.

VIII. Optimization

If the recommended conditions (shuttle PCR standard protocol) do not provide sufficient reactivity, follow the procedures below to optimize primer concentration and PCR conditions. Depending on the reaction system, switching to a different real-time PCR reagent from the Perfect Real Time series (Cat. #RR420A/B, RR430A/B, RR091A/B*) may greatly improve the results.

Select PCR conditions based on comprehensive analysis of reaction specificity and amplification efficiency. Using conditions that balance these two aspects will allow accurate measurement over a wide range of concentrations.

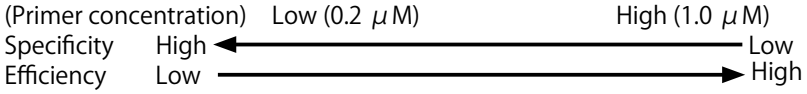
* Not available in all geographic locations. Check for availability in your area.

○ System with a high reaction specificity
- Using a negative, no-template control, non-specific amplification (e.g., primer dimers) does not occur.
- Non-specific amplification products (those other than the target product) are not generated.

○ System with a high amplification efficiency
- Amplification product is detected at earlier cycles (lower Ct value).
- PCR amplification efficiency is high (near the theoretical value of 100%).

1. Evaluation of primer concentration

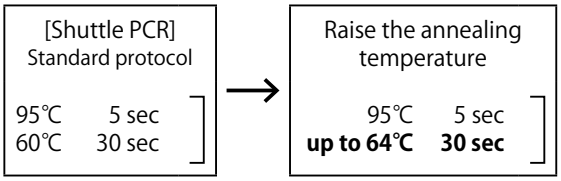
The relationship between primer concentration, reaction specificity, and amplification efficiency is illustrated below. Reducing primer concentration raises reaction specificity whereas increasing the primer concentration raises amplification efficiency.



2. Evaluation of PCR conditions

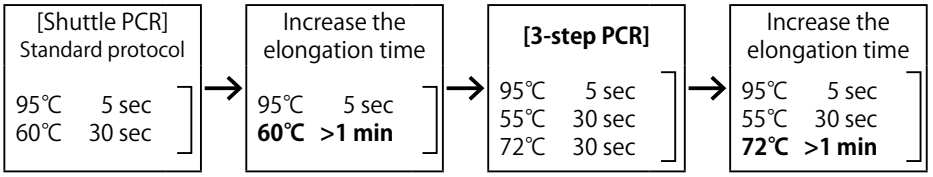
○ To improve reaction specificity

Raising the annealing temperature may improve reaction specificity. Perform optimization while checking amplification efficiency.



○ To improve amplification efficiency

Increasing the elongation time or switching to 3-step PCR may improve amplification efficiency. Perform optimization using the steps below.



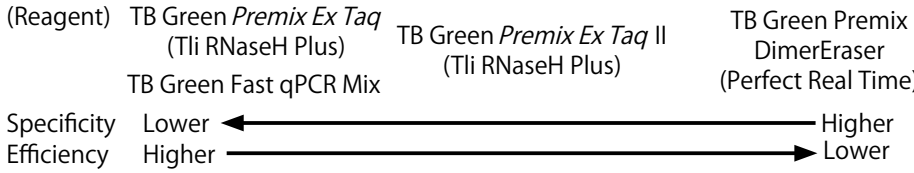
○ Initial denaturation

Generally, 95℃ for 30 sec is sufficient for initial denaturation, even for difficult to denature templates such as circular plasmids and genomic DNA. This procedure may be extended to 1 - 2 min at 95℃ depending on the template. However, prolonging this step may inactivate the enzyme. Therefore, it is recommended to avoid initial denaturation steps >2 min.

3. Relationship between reagent and reactivity

Takara Bio supplies several different reagents for intercalator-based real-time PCR analysis using TB Green. The relationship between reaction specificity and amplification efficiency for these reagents is described below.

TB Green *Premix Ex Taq* (Tli RNaseH Plus) (Cat. #RR420A/B) and TB Green Fast qPCR Mix (Cat. #RR430A/B) provides high amplification efficiency. TB Green *Premix Ex Taq* II (Tli RNaseH Plus) (Cat. #RR820A/B) and TB Green Premix DimerEraser™ (Perfect Real Time) (Cat. #RR091A/B)＊ have greater specificity.



＊ Not available in all geographic locations. Check for availability in your area.

IX. Appendix

1. Primer design

Designing primers with good reactivity is critical to successful real-time PCR. Follow the guidelines below to design primers that yield high amplification efficiency without non-specific amplification.

RT-PCR primers designed and synthesized using these guidelines are compatible with the shuttle PCR standard protocol (Section VII.).

■ Amplification product

Amplification size	The optimal size is 80 - 150 bp (amplification up to 300 bp is possible).

■ Primer

Length	17 - 25 mer
GC content	40 - 60% (preferably 45 - 55%)
Tm	Make sure the Tm values for the forward primer and the reverse primer do not differ greatly. Use software to determine Tm values. OLIGO＊1 : 63 - 68℃ Primer3 : 60 - 65℃
Sequence	Make sure there are no overall base sequence biases. Avoid having any GC-rich or AT-rich regions in the sequence (especially at the 3′ end). Avoid having consecutive T/C pairings (polypyrimidine). Avoid having consecutive A/G pairings (polypurine).
3′ end sequence	Avoid having GC-rich or AT-rich regions at the 3′ end. It is preferable to have a G or C as the terminal base at the 3′ end. Avoid a primer design with T as the terminal base at the 3′ end.
Complementation	Avoid having any complementary sequences of three bases or more within a primer and between primers. Avoid having any complementary sequences of two bases or more at a primer's 3' end.
Specificity	Verify primer specificity using a BLAST search.＊2

＊1 OLIGO Primer Analysis Software (Molecular Biology Insights, Inc.)

＊2 https://blast.ncbi.nlm.nih.gov/Blast.cgi

2. Preparing templates for real-time RT-PCR

When preparing cDNA templates for real-time RT-PCR, the following products are recommended:

- PrimeScript™ RT reagent Kit (Perfect Real Time) (Cat. #RR037A/B)
- PrimeScript RT Master Mix (Perfect Real Time) (Cat. #RR036A/B)
- PrimeScript RT Reagent Kit with gDNA Eraser (Perfect Real Time) (Cat. #RR047A/B)

When used in combination with this kit, these products provide reliable results. Refer to the product's user manual for RT reaction conditions.

A. Prepare PCR mixtures according to the following procedure.
(When using Thermal Cycler Dice Real Time System *II*: discontinued)

Prepare the following components in volumes slightly more than that needed for the required number of tubes and dispense 22.5 - 24 μl.

<Per reaction>

Reagent	Volume	Final conc.
TB Green *Premix Ex Taq* II (2X)	12.5 μl	1X
PCR Forward Primer (10 μM)	1.0 μl	0.4 μM
PCR Reverse Primer (10 μM)	1.0 μl	0.4 μM
Sterile purified water	x μl	
Total	22.5 - 24 μl	

B. Add 1.0 - 2.5 μl of the RT reaction mixture to each of the microtubes containing aliquots of the PCR reaction mixture.

Note: Add no more than 2.5 μl of the RT reaction mixture to the PCR reaction mixture.

[Experimental example]

Human TBP mRNA was detected by real-time RT-PCR. cDNA equivalent to 1 pg - 100 ng of total RNA was used as the template, with sterile purified water as the negative control.

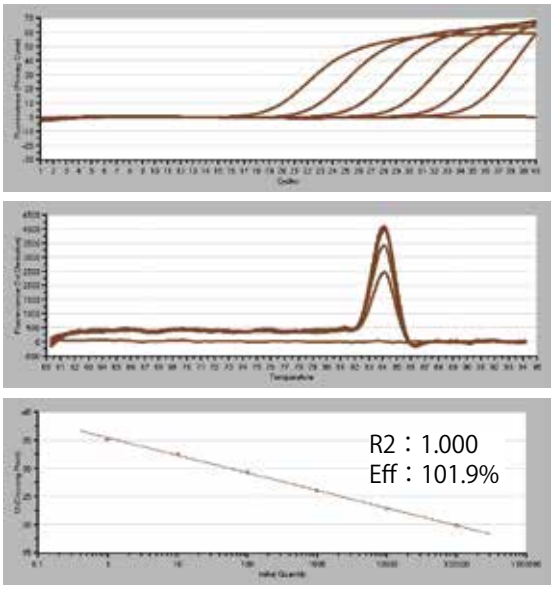


Figure 7. Detection of human TBP mRNA by real-time RT-PCR.

X. Related Products

TB Green® *Premix Ex Taq*™ (Tli RNaseH Plus) (Cat. #RR420A/B/L/W/LR/WR)
TB Green® Fast qPCR Mix (Cat. #RR430A/B)
TB Green® Premix DimerEraser™ (Perfect Real Time) (Cat. #RR091A/B)*
TB Green® *Premix Ex Taq*™ GC (Perfect Real Time) (Cat. #RR071A/B)*
PrimeScript™ RT reagent Kit (Perfect Real Time) (Cat. #RR037A/B)
PrimeScript™ RT Master Mix (Perfect Real Time) (Cat. #RR036A/B)
PrimeScript™ RT Reagent Kit with gDNA Eraser (Perfect Real Time) (Cat. #RR047A/B)
Thermal Cycler Dice™ Real Time System III (Cat. #TP950/TP970/TP980/TP990)*

* Not available in all geographic locations. Check for availability in your area.