Cat. # RR42LR

For Research Use

TaKaRa

TB Green® *Premix Ex Taq*™ (Tli RNaseH Plus), ROX plus

Product Manual

v202112Da

Table of Contents

I. Description

TB Green *Premix Ex Taq* (Tli RNaseH Plus), ROX plus is a kit specifically designed for intercalator-based real-time PCR using TB Green for detection. The Premix is supplied at a 2X concentration and contains TB Green and ROX Reference Dye at a concentration appropriate for real-time monitoring, making it easy to prepare reaction mixtures.

A combination of *TaKaRa Ex Taq*® HS (a hot-start PCR enzyme that uses an anti-*Taq* antibody) and a buffer optimized for real-time PCR allows high amplification efficiency and high detection sensitivity in real-time PCR. The 2X premixed reagent also contains Tli RNaseH, a heat-resistant RNase H, which minimizes PCR inhibition due to residual mRNA when using cDNA as template. This product is suitable for high-speed PCR and allows accurate assay and detection of targets, making real-time PCR analyses with good reproducibility and high reliability possible.

Compatible instrument systems include:

- ♦ Devices that require signal correction using ROX Reference Dye
  - StepOnePlus Real-Time PCR System (Thermo Fisher Scientific)＊1
- ♦ Devices that do not require signal correction using ROX Reference Dye
  - Thermal Cycler Dice™ Real Time System III (Cat. #TP950/TP970/TP980/TP990)＊2, 3
  - Thermal Cycler Dice Real Time System *Lite* (Cat. #TP700/TP760: discontinued)
  - Smart Cycler II System (Cepheid)

＊1 The Applied Biosystems 7500/7500 Fast Real-Time PCR System (Thermo Fisher Scientific) requires correction with ROX Reference Dye II; TB Green *Premix Ex Taq* (Tli RNaseH plus), Bulk (Cat. #RR420L/W) is recommended.

＊2 When using Thermal Cycler Dice Real Time System *II* (Cat. #TP900/TP960: discontinued), the use of TB Green *Premix Ex Taq* II (Tli RNaseH Plus), Bulk (Cat. #RR820L/W) is recommended.

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

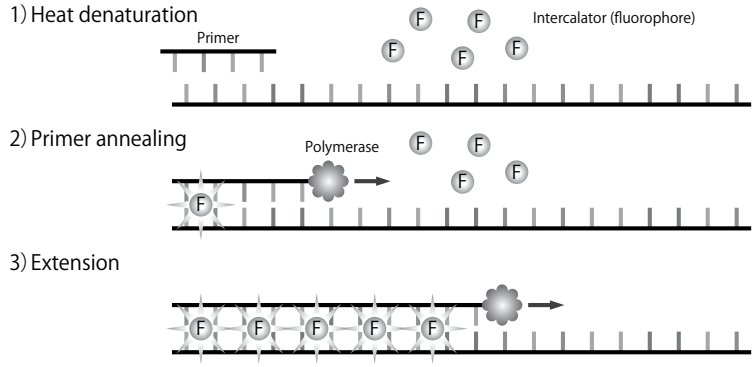
II. Principle

TB Green *Premix Ex Taq* (Tli RNaseH Plus) is used for PCR amplification and monitoring of amplification products in real time by TB Green as an intercalator.

TaKaRa Ex Taq HS, a hot-start PCR enzyme, prevents non-specific amplification from mispriming or primer dimer formation during reaction mixture preparation or other pre-cycling steps, thereby allowing high-sensitivity detection.

Fluorescence detection - intercalator method

This method involves the addition of an intercalating reagent (e.g., TB Green) that emits fluorescence when bound to double-strand DNA in the reaction mixtures. This enables the detection of amplified DNA by monitoring fluorescence. Fluorescence measurement not only allows for quantitative determination of target DNA but also for DNA composition by melting curve analysis.



III. Components (200 reactions, 50 μl volume)

TB Green *Premix Ex Taq* (2X) (Tli RNaseH Plus), ROX plus＊1	5 ml

＊1 Contains *TaKaRa Ex Taq* HS, dNTP Mixture, Mg^{2+}, Tli RNaseH, TB Green, and ROX Reference Dye.

IV. Materials Required but not Provided

- Gene amplification system for real-time PCR (authorized instruments)
- Reaction tubes and plates designed specifically for the qPCR instrument used
- PCR primers＊2
- Sterile purified water
- Micropipettes and tips (sterile, with filter)

＊2 For designing real-time PCR primers, please see Section VIII.-(2) Primer design.

V. 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.

VI. Precautions before Use

Read these precautions before use and follow them when using this product.

1. Prior to use, make sure the reagent is evenly mixed by inverting the bottle several times without creating bubbles. Uneven reagent mixing will result in inadequate reactivity.
   - Do not mix by vortexing.
   - When stored at -20℃, TB Green *Premix Ex Taq* (2X) (Tli RNaseH Plus), ROX plus may develop a white to pale yellow precipitate. To dissolve the precipitate completely, let stand briefly protected from light at room temperature (below approximately 30℃), then invert the bottle several times.
   - 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 they have thawed.
3. This product contains TB Green and ROX Reference Dye. Avoid exposing to strong light when preparing the reaction mixture.
4. Use fresh disposable tips to avoid contamination between samples when preparing or dispensing reaction mixtures.
5. *TaKaRa Ex Taq* HS is a hot-start PCR enzyme with an anti-*Taq* antibody that inhibits polymerase activity. Do not perform the pre-PCR incubation (5 to 15 min at 95℃) that is required with other companies' chemically modified hot-start PCR enzymes. The activity of *TaKaRa Ex Taq* HS decreases with longer heat treatment and the amplification efficiency and quantification accuracy can be affected.

   Even for the initial denaturation step, 95℃ for 30 sec is generally sufficient.

VII. Protocol

【 For the StepOnePlus Real-Time PCR System 】

Note: Please follow the procedures provided in the manual of the instrument.

1. Prepare the PCR mixture shown below.

< Per reaction >

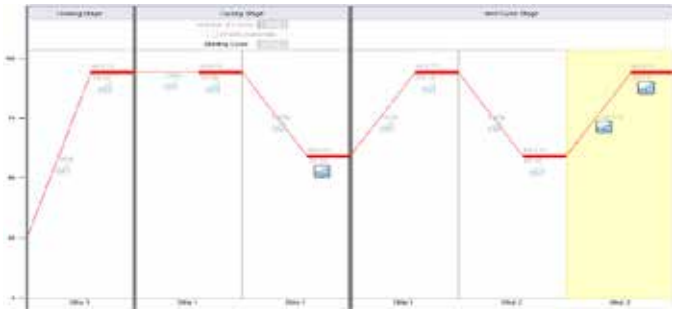
Reagent	Volume	Final conc.
TB Green *Premix Ex Taq* (2X) (Tli RNaseH Plus), ROX plus	10 μl	1X
PCR Forward Primer (10 μM)	0.4 μl	0.2 μM*1
PCR Reverse Primer (10 μM)	0.4 μl	0.2 μM*1
Template (< 100 ng)*2	2 μl	
Sterile purified water	7.2 μl	
Total	20 μl	

*1 A final primer concentration of 0.2 μM is likely to yield good results. However, if there is an issue with reactivity, use a primer concentration between 0.1 and 1.0 μM.

*2 The optimal quantity varies depending on the number of target copies in the template solution. Make serial dilutions to determine the appropriate amount. It is preferable to use no more than 100 ng of DNA template. Furthermore, if cDNA (RT reaction mixture) is used as template, the template volume should be no more than 10% of the PCR mixture.

2. 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 further, please see VIII.-(1) Optimization.)



Shuttle PCR standard protocol

Holding Stage
Step 1: 95℃ 30 sec

Cycling Stage
Number of cycles: 40
Step 1: 95℃ 5 sec
Step 2: 60℃ 30 sec

Melt Curve Stage

3. After the reaction is complete, check the amplification and melting curves and plot a standard curve if quantification will be performed. For analytical methods, refer to the manual for the StepOnePlus Real-Time PCR System.

【 For the Thermal Cycler Dice Real Time System III (*Lite*: discontinued) 】

1. Prepare the PCR mixture shown below.

< Per reaction >

Reagent	Volume	Final conc.
TB Green *Premix Ex Taq* (2X) (Tli RNaseH Plus), ROX plus	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	2 μl	
Sterile purified water	9.5 μl	
Total	25 μl*3	

*1 A final primer concentration of 0.2 μM is most likely to yield good results. However, if there is an issue with reactivity, use a primer concentration between 0.1 and 1.0 μM.

*2 The optimal quantity varies depending on the number of target copies in the template solution. Make serial dilutions to determine the appropriate amount. It is preferable to use no more than 100 ng of DNA template. Furthermore, if cDNA (RT reaction mixture) is used as template, the template volume should be no more than 10% of the PCR mixture.

*3 The recommended reaction volume is 25 μl.

2. 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 further, please see VIII.-(1) Optimization.

Shuttle PCR standard protocol

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

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

Dissociation

3. After the reaction is complete, check the amplification and melting curves and plot a standard curve if quantification will be performed. For analytical methods, refer to the manual for the Thermal Cycler Dice Real Time System.

【 For the Smart Cycler II System 】

1. Prepare the PCR mixture shown below.

< Per reaction >

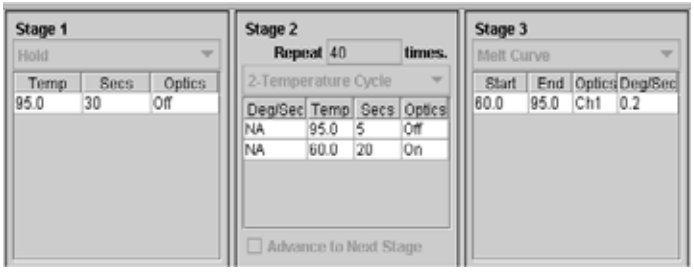
Reagent	Volume	Final conc.
TB Green *Premix Ex Taq* (2X) (Tli RNaseH Plus), ROX plus	12.5 μl	1X
PCR Forward Primer (10 μM)	0.5 μl	0.2 μM＊1
PCR Reverse Primer (10 μM)	0.5 μl	0.2 μM＊1
Template (< 100 ng)＊2	2 μl	
Sterile purified water	9.5 μl	
Total	25 μl	

＊1 A final primer concentration of 0.2 μM is likely to yield good results. However, if there is an issue with reactivity, use a primer concentration between 0.1 and 1.0 μM.

＊2 The optimal quantity varies depending on the number of target copies in the template solution. Make serial dilutions to determine the appropriate amount. It is preferable to use no more than 100 ng of DNA template. Furthermore, if cDNA (RT reaction mixture) is used as template, the template volume should be no more than 10% of the PCR mixture.

2. Briefly centrifuge reaction tubes with Smart Cycler centrifuge and then set them in Smart Cycler to 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 a shuttle PCR is not feasible. To optimize PCR conditions further, please refer to Section VIII.-(1) Optimization.



Shuttle PCR standard protocol

Stage 1: Initial denaturation
Hold
95℃ 30 sec

Stage 2: PCR
Repeat: 40
95℃ 5 sec
60℃ 20 sec

Stage 3: Melt Curve

3. After the reaction is complete, check the amplification and melting curves and plot a standard curve if quantification will be performed. For analytical methods, refer to the manual for the Smart Cycler II System.

VIII. Appendix

(1) 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. In addition, other real-time TB Green Premix PCR reagents in the Perfect Real Time series (Cat. #RR820A/B/L/W, RR82LR/WR, RR091A/B*) may greatly improve the results.

Select PCR conditions based on comprehensive analysis of both 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
- Non-specific amplification (e.g., primer-dimer) does not occur in the No Template Control reactions.
- 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 C_t value).
- PCR amplification efficiency is high (near the theoretical maximum of 100%).

[Evaluation of primer concentration]

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

(Primer concentration)	Low (0.1 μM)		High (1.0 μM)
Reaction specificity	High	←	Low
Amplification efficiency	Low	→	High

[Evaluation of PCR conditions]

○ To improve reaction specificity

Raising the annealing temperature may improve reaction specificity. Optimize the annealing temperature while balancing specificity with amplification efficiency.

[Shuttle PCR] Standard protocol	→	Raise the annealing temperature
95℃ 5 sec 60℃ 30 sec]		95℃ 5 sec **up to 64℃ 30 sec**]

○ To improve amplification efficiency

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

[Shuttle PCR] Standard protocol	→	Increase the elongation time	→	**[3-step PCR]**	→	Increase the elongation time
95℃ 5 sec 60℃ 30 sec]		95℃ 5 sec **60℃ > 1 min**]		95℃ 5 sec 55℃ 30 sec 72℃ 30 sec]		95℃ 5 sec 55℃ 30 sec **72℃ > 1 min**]

○ 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. Denaturation may be extended to 1 to 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 longer than 2 min.

[Relationship between reagent and reactivity]

Takara Bio supplies several different real-time PCR 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/L/W, RR42LR/WR) provides a high amplification efficiency and is suitable for high-speed reactions. TB Green *Premix Ex Taq* II (Tli RNaseH Plus) (Cat. #RR820A/B/L/W, RR82LR/WR) and TB Green Premix DimerEraser™ (Perfect Real Time) (Cat. #RR091A/B)＊ are effective in raising the reaction specificity.

(Reagent)	TB Green *Premix Ex Taq* (Cat.#RR420A, RR42LR)	TB Green *Premix Ex Taq* II (Cat. #RR820A, RR82LR)	TB Green Premix DimerEraser* (Cat. #RR091A)
Specificity:	Lower	⟶	High
Efficiency:	High	⟵	Lower

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

(2) Primer design

Designing a primer set with good reactivity is critical to efficient real-time PCR. Please follow the guidelines below to design primers that yield high amplification efficiency without nonspecific amplification.

■ Amplification product

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

■ Primer

Length	17 - 25mer
GC content	40 - 60% (preferably 45 - 55%)
Tm	Make sure that the Tm values for the forward primer and the reverse primer do not differ greatly. Use software specifically designed to determine Tm values. OLIGO＊1 : 63 - 68℃ Primer3 : 60 - 65℃
Sequence	Make sure that there are no sequence biases overall. Avoid having GC-rich or AT-rich regions in the sequence (particularly 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. It is better to avoid a primer design with T as the terminal base at the 3′ end.
Complementation	Avoid having any complementary sequences of 3 bases or more within a primer and between primers. Avoid having any complementary sequences of 2 bases or more at primer 3' ends.
Specificity	Verify primer specificity by a BLAST search.＊2

＊1 OLIGO Primer Analysis Software (Molecular Biology Insights)

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

(3) When performing real-time RT-PCR

To perform reverse transcription reactions 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 highly reliable results.

The following is a protocol when using PrimeScript RT Master Mix (Perfect Real Time) (Cat. #RR036A/B).

1. Prepare the reverse transcription mixture shown below and keep on ice.

   < Per reaction >

Reagent	Amount	Final conc.
5X PrimeScript RT Master Mix (Perfect Real Time)	2 μl	1X
Total RNA*	x μl	
RNase Free dH_2O	to 10 μl	

   * Scale up the reverse transcription reaction as necessary. Approximately 500 ng total RNA can be reverse transcribed in a 10 μl reaction volume.

2. Perform a reverse transcription reaction.

   37℃ 15 min (reverse transcription reaction)
   85℃ 5 sec (heat inactivation of reverse transcriptase)
   4℃

3. Perform PCR according to the method described in VII. Protocol.

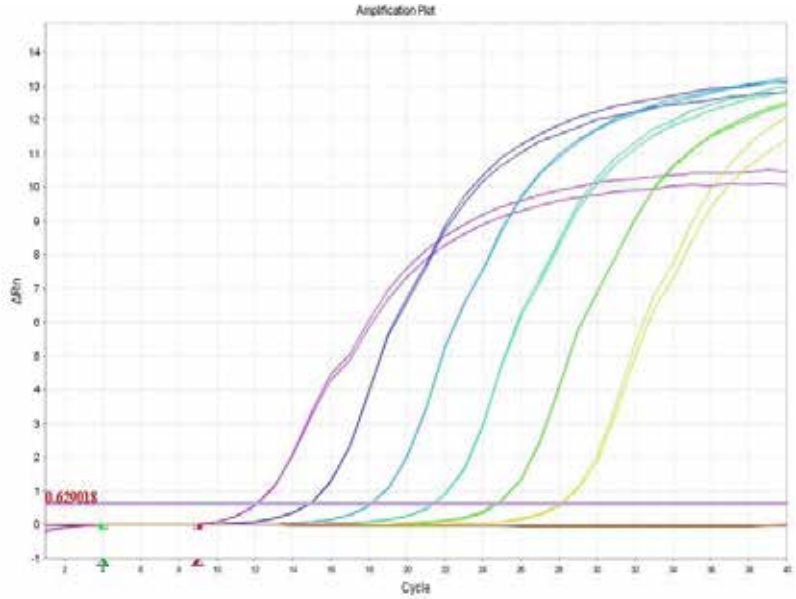
■ Experimental Example (StepOnePlus Real-Time PCR System)

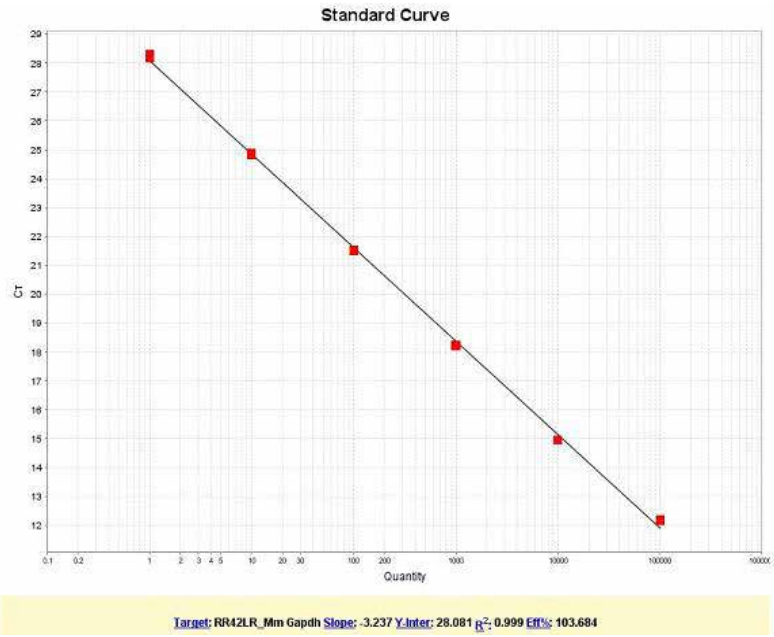
Reverse Transcription Reaction

Template: Mouse heart total RNA (1 pg to 100 ng)

Real-time PCR

Target: Mouse GAPDH





IX. Related Products

TB Green® *Premix Ex Taq*™ (Tli RNaseH Plus) (Cat. #RR420A/B)
TB Green® *Premix Ex Taq*™ (Tli RNaseH Plus), Bulk (Cat. #RR420L/W)
TB Green® Fast qPCR Mix (Cat. #RR430A/B)
TB Green® *Premix Ex Taq*™ II (Tli RNaseH Plus) (Cat. #RR820A/B)
TB Green® *Premix Ex Taq*™ II (Tli RNaseH Plus), Bulk (Cat. #RR820L/W)
TB Green® *Premix Ex Taq*™ II (Tli RNaseH Plus), ROX plus (Cat. #RR82LR/WR)
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.