Cat. # RR091A

For Research Use

TaKaRa

TB Green® Premix DimerEraser™ (Perfect Real Time)

Product Manual

v202202Da

Table of Contents

I. Description

TB Green Premix DimerEraser (Perfect Real Time) is a reagent specifically designed for intercalator-based real-time PCR using TB Green for detection.This is a 2X concentration premix type reagent, which contains TB Green; its concentration is adjusted for real-time monitoring. This makes preparation of the reaction mixture easier.
In comparison to TB Green *Premix Ex Taq*™ II (Tli RNaseH Plus) (Cat. #RR820A/B), this product has an improved buffer system and is added with the original accessory protein, which results in improved reaction specificity.
This product has the effect of depression of primer dimers, which is an especially important matter in intercalator method. It makes it possible to accurately perform quantitative analysis for a wide range of template concentrations by suppression of non-specific amplification and high detection of a lower amount of template.
We recommend a 3-step PCR method as the standard protocol of this product.

Applicable real-time PCR instruments:

- 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)
- Applied Biosystems 7500/7500 Fast Real-Time PCR System, StepOnePlus Real-TimePCR System (Thermo Fisher Scientific)
- LightCycler/LightCycler 480 System (Roche Diagnostics)
- Smart Cycler System/Smart Cycler II System (Cepheid) etc.

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

II. Principle

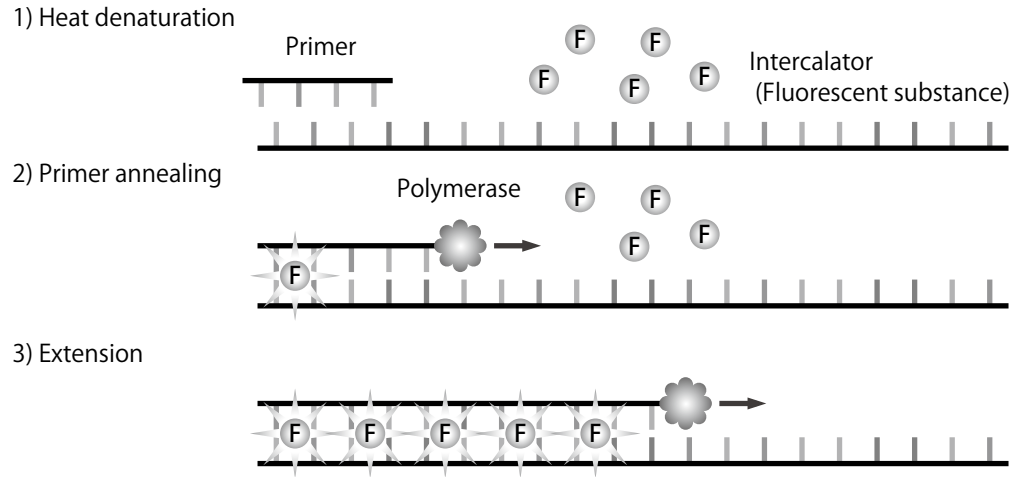
This product employs *TaKaRa Ex Taq*® HS for PCR reactions. PCR amplification products are monitored in real time using TB Green as an intercalator.

1) PCR

PCR is a simple and powerful method to amplify a tiny amount of target DNA by cycling through three incubation steps at different temperatures: double-stranded target DNA is heat denatured (denaturation step), the two primers complementary to the target segment are annealed at low temperature (annealing step), and the annealed primers are then extended at an intermediate temperature (extension step) with a DNA polymerase. Since the target copy number doubles upon each cycle, PCR can therefore amplify DNA fragments up to 10^6-fold in a short period. As this product utilizes an enzyme for Hot Start PCR, *TaKaRa Ex Taq* HS, non-specific amplification due to mispriming prior to cycling or due to primer dimers can be minimized. Accordingly, highly specific and sensitive detection is achieved.

2) Fluorescence detection

[Intercalator method]
It is a detection method utilizing a DNA-intercalating dye (e.g., TB Green) that fluoresces once bound to double-stranded DNA. The dye is added in the reaction system and its fluorescence is detected during amplification.
When an intercalator binds to double-stranded DNA synthesized in PCR amplification, fluorescence is emitted. By measuring the fluorescence intensity, the melting temperature of the amplified DNA is also available, as well as quantification of PCR products.



III. Components (200 reactions, 50 μl PCR)

TB Green Premix DimerEraser (Perfect Real Time) (2X conc.)*1	1 ml x 5
ROX Reference Dye (50X conc.)*2	200 μl
ROX Reference Dye II (50X conc.)*2	200 μl

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

*2 ROX Reference Dyes are used for analyses with instruments that correct for crosstalk between wells, such as the real-time PCR instruments by Applied Biosystems.

◆ Add ROX Reference Dye (50X) when using the following instruments:
- StepOnePlus Real-Time PCR System (Thermo Fisher Scientific)

◆ Add ROX Reference Dye II (50X) when using the following instruments:
- Applied Biosystems 7500 Real-Time PCR System
- Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific)

◆ No ROX Reference Dye is required when using the following instruments:
- Thermal Cycler Dice Real Time System III (Cat. #TP950/TP970/TP980/TP990)*3
- Thermal Cycler Dice Real Time System *II/Lite* (Cat. #TP900/TP960/TP700/TP760: discontinued)
- Smart Cycler System/Smart Cycler II System (Cepheid)
- LightCycler/LightCycler 480 System (Roche Diagnostics)

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

Materials required but not provided

1. Thermal cycler for real-time PCR
2. Reaction tube or plate for real-time PCR
3. PCR primers*4
4. Sterile purified water
5. Micropipettes and micropipette tips (autoclaved prior to use)

*4 Please refer to VIII. Guidelines for Primer Design for instructions on the design PCR primers.

IV. Storage

Stable at 4℃ for 6 months.
It should be protected from light, and careful attention should be made not to cause contamination.

Note: Storage at -80℃ is most recommended for long-term storage. Storage at -20℃ is not recommended. Once thawed, store at 4℃ and use up within 6 months.

V. Features

1) Quick and accurate detection and quantification of target gene through real-time PCR.
2) Easy-to-use 2X premix reagent including TB Green:
   Ready to perform real-time PCR in the presence of a fluorescent intercalator. Just add PCR primers, template, and sterile purified water to start the reaction.
3) High amplification efficiency and highly sensitive detection:
   This product utilizes an enzyme for hot start PCR, *TaKaRa Ex Taq* HS. Since this enzyme-buffer system is optimized for real-time PCR, this product offers highly efficient amplification and highly sensitive detection. Moreover, the addition of the accessory protein strongly suppresses mispriming of primers during PCR reactions and non-specific amplifications, such as primer dimer.

VI. Precautions before Use

Please read carefully before use.

1) A white or yellowish precipitate may form in this product when stored frozen. This precipitate can be completely dissolved by briefly warming the tube with your hands or placing the tube at room temperature while protected 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, lowered signal intensity and reactivity may result due to an inadequate concentration of TB Green and other components. USE THE REAGENT ONLY AFTER COMPLETELY REDISSOLVING 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 the preparation of the reaction mixture, all the reagents should be placed on ice.
3) Avoid direct light in preparation of the PCR reaction mixture because TB Green is included.
4) For preparing and dispensing the reagents, a new disposable tip should be used to minimize contamination among samples.

VII. Protocol

Note: We recommend a 3-step PCR method as the standard protocol of TB Green Premix DimerEraser (Perfect Real Time).

【 Protocol using the Thermal Cycler Dice Real Time System series 】

1) Prepare PCR reaction mixture.

<Per reaction>

Reagent	Volume	Final conc.
TB Green Premix DimerEraser (2X)	12.5 μl	1X
PCR forward primer (10 μM)	0.75 μl	0.3 μM*1
PCR reverse primer(10 μM)	0.75 μl	0.3 μM*1
template (<100 ng)*2	2 μl	
sterile purified water	9 μl	
Total	25 μl	

*1 The final concentration of primers can be 0.3 μM in most reactions. When it does not work, determine the optimal concentrations within the range of 0.2 - 1.0 μM.

*2 Final template concentration varies depending on the copy number of target present in the template solution. Optimal amount should be determined by preparing a dilution series. It is recommended to add DNA template at less than 100 ng. When the RT reactant (cDNA) is used as a template, it should be added in at less than 10% volume of the PCR reaction mixture.

2) Start the reaction.

Perform a 3-step PCR method using the standard protocol of TB Green Premix DimerEraser (Perfect Real Time). Set the 'Normal Mode' program (default) on the Thermal Cycler Dice Real Time System III or the 'Fast Mode' program (default) on the Thermal Cycler Dice Real Time System *II* and *Lite* (discontinued).

<u>3-step PCR Standard Protocol</u>

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

3-step PCR
Cycles: 40
95℃ 5 sec
55℃ 30 sec
72℃ 30 sec

Dissociation

Note:
This product contains *TaKaRa Ex Taq* HS, which includes a *Taq* antibody. There is no need to heat at 95℃ for 5 - 15 min for initial denaturation (as is required for chemically modified *Taq* polymerases). If longer heat treatment is used with this kit, the enzyme activity decreases and amplification efficiency and the accuracy of quantification may also be affected.
Perform heat inactivation of RTase prior to PCR at 95℃ for 30 sec.

3) After the reaction is completed, verify the amplification curve and melting curve. Establish the standard curve when quantification is done.

Refer to the operation manual of each apparatus.

【 Protocol using the Applied Biosystems 7500/7500 Fast Real-Time PCR System and StepOnePlus Real-Time PCR System 】

1) Prepare PCR reaction mixture.

<Per reaction>

Reagent	Volume	Volume	Final conc.
TB Green Premix DimerEraser (2X)	10 μl	25 μl	1X
PCR forward primer (10 μM)	0.6 μl	1.5 μl	0.3 μM*1
PCR reverse primer(10 μM)	0.6 μl	1.5 μl	0.3 μM*1
ROX Reference Dye or Dye II*3 (50X)	0.4 μl	1 μl	1X
template*2	2 μl	4 μl	
sterile purified water	6.4 μl	17 μl	
Total	20 μl*4	50 μl*4	

*1 The final concentration of primers can be 0.3 μM in most reactions. When it does not work, determine the optimal concentrations within the range of 0.2 - 1.0 μM.

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

*3 The ROX Reference Dye/Dye II is supplied for performing normalization of fluorescent signal intensities among wells when used with real-time PCR instruments that have option.
For StepOnePlus, the use of ROX Reference Dye (50X) is recommended. For Applied Biosystems 7500/7500 Fast Real-Time PCR System, the use of ROX Reference Dye II (50X) is recommended.

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

2) Start the reaction.
3-step PCR standard protocol is recommended. Try this protocol first, and optimize the reaction condition if needed.

< Applied Biosystems, 7500 Real-Time PCR System, and StepOnePlus >

3-step PCR Standard Protocol

Stage 1: Initial denaturation
Reps: 1
95℃ 30 sec

Stage 2: PCR
Reps: 40
95℃ 5 sec
55℃ 30 sec
72℃ 30 or 34 sec*5

Stage 3: Melt Curve

*5 This step should be 30 sec with the StepOnePlus Real-TimePCR System and 34 sec with the 7500 Real-Time PCR System.

< Applied Biosystems 7500 Fast Real-Time PCR System >

3-step PCR Standard Protocol

Holding Stage
Number of Cycles: 1
95℃ 30 sec

Cycling Stage
Number of Cycles: 40
95℃ 3 sec
55℃ 30 sec
72℃ 30 sec

Melt Curve Stage

Note:
This product contains *TaKaRa Ex Taq* HS, which includes a *Taq* antibody. There is no need to heat at 95℃ for 5 - 15 min for initial denaturation (as is required for chemically modified *Taq* polymerases). If longer heat treatment is used with this kit, the enzyme activity decreases and amplification efficiency and the accuracy of quantification may also be affected.
Perform heat inactivation of RTase prior to PCR at 95℃ for 30 sec.

3) After the reaction is completed, verify the amplification curve and dissociation curve. Establish the standard curve when quantification is done.
Refer to the operation manual of an used real-time PCR instrument.

【 Protocol using the LightCycler/LightCycler 480 System 】

1) Prepare PCR reaction mixture.

<Per reaction>

Reagent	Volume	Final conc.
TB Green Premix DimerEraser (2X)	10 μl	1X
PCR forward primer (10 μM)	0.6 μl	0.3 μM*1
PCR reverse primer(10 μM)	0.6 μl	0.3 μM*1
template (<100 ng)*2	2 μl	
sterile purified water	6.8 μl	
Total	20 μl	

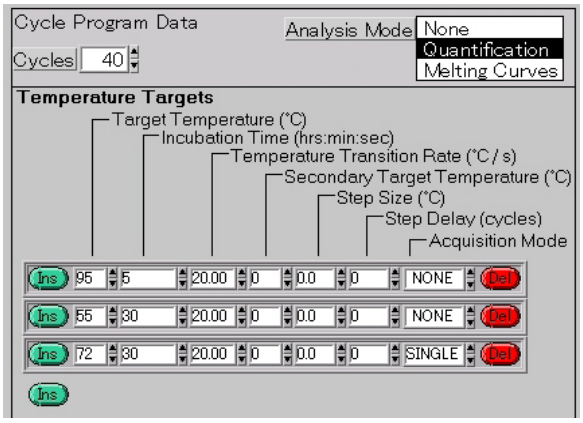
*1 The final concentration of primers can be 0.3 μM in most reactions. When it does not work, determine the optimal concentrations within the range of 0.2 - 1.0 μM.

*2 Final template concentration varies depending on the copy number of target present in the template solution. Optimal amount should be determined by preparing the dilution series. It is recommended to apply DNA template in less than 100 ng. When the RT reactant (cDNA) is used as a template, it should be added in at less than 10% volume of the PCR reaction mixture.

2) Start the reaction.

3-step PCR standard protocol is recommended. Try this protocol first, and optimize the reaction condition if needed. Try shuttle PCR protocol when 3-step protocol is difficult.

< LightCycler >



3-step PCR Standard Protocol

Stage 1: Initial denaturation
95℃ 30 sec 20℃/sec
1 Cycle

Stage 2: PCR
95℃ 5 sec 20℃/sec
55℃ 30 sec 20℃/sec
72℃ 30 sec 20℃/sec
40 Cycles

Stage 3: Melting Curve analysis
95℃ 0 sec 20℃/sec
65℃ 15 sec 20℃/sec
95℃ 0 sec 0.1℃/sec

< LightCycler 480 System >

3-step PCR Standard Protocol

Denature
95℃ 30 sec (Ramp Rate 4.4℃/sec)
1 Cycle

PCR
Analysis Mode: Quantification
95℃ 5 sec (Ramp Rate 4.4℃/sec)
55℃ 30 sec (Ramp Rate 2.2℃/sec)
72℃ 30 sec (Ramp Rate 4.4℃/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:
This product contains *TaKaRa Ex Taq* HS, which includes a *Taq* antibody. There is no need to heat at 95℃ for 5 - 15 min for initial denaturation (as is required for chemically modified *Taq* polymerases). If longer heat treatment is used with this kit, the enzyme activity decreases and amplification efficiency and the accuracy of quantification may also be affected.
Perform heat inactivation of RTase prior to PCR at 95℃ for 30 sec.

3) After the reaction is completed, verify the amplification curve and melting curve. Establish the standard curve when quantification is done.
Refer to the operation manual of LightCycler.

【 Protocol using the Smart Cycler II System 】

1) Prepare PCR reaction mixture.

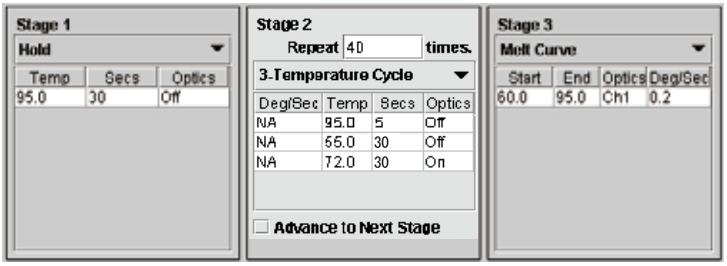
<Per reaction>

Reagent	Volume	Final conc.
TB Green Premix DimerEraser (2X)	12.5 μl	1X
PCR forward primer (10 μM)	0.75 μl	0.3 μM*1
PCR reverse primer(10 μM)	0.75 μl	0.3 μM*1
template (<100 ng)*2	2 μl	
sterile purified water	9 μl	
Total	25 μl	

*1 The final concentration of primers can be 0.3 μM in most reactions. When it does not work, determine the optimal concentrations within the range of 0.2 - 1.0 μM.

*2 Final template concentration varies depending on the copy number of target present in the template solution. Optimal amount should be determined by preparing the dilution series. It is recommended to apply DNA template in less than 100 ng. When the RT reactant (cDNA) is used as a template, it should be added in at less than 10% volume of the PCR reaction mixture.

2) Gently centrifuge the reaction tubes using the centrifuge exclusive for use with the Smart Cycler. Load the reaction tubes on Smart Cycler II System and start the reaction.
3-step PCR standard protocol is recommended. Try this protocol first, then optimize the reaction condition if needed. Try shuttle PCR protocol when 3-step protocol is difficult.



3-step PCR Standard Protocol

Stage 1: Initial denaturation
Hold
95℃ 30 sec

Stage 2: PCR
Repeats: 40 times
95℃ 5 sec
55℃ 30 sec
72℃ 30 sec

Stage 3: Melting Curve analysis

Note:
This product contains *TaKaRa Ex Taq* HS, which includes a *Taq* antibody. There is no need to heat at 95℃ for 5 - 15 min for initial denaturation (as is required for chemically modified *Taq* polymerases). If longer heat treatment is used with this kit, the enzyme activity decreases and amplification efficiency and the accuracy of quantification may also be affected.
Perform heat inactivation of RTase prior to PCR at 95℃ for 30 sec.

3) After the reaction is completed, verify the amplification curve and melting curve. Establish the standard curve when quantification is done.
Refer to the operation manual of Smart Cycler System.

Optimization

If the recommended conditions (3-step 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 TB Green Premix series (Cat. #RR820A/B, #RR420A/B, RR430A/B) may greatly improve the results.

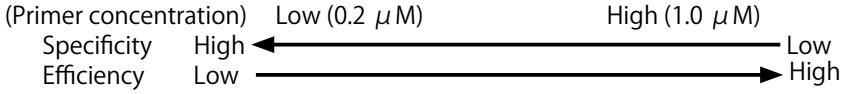
Select PCR conditions based on comprehensive analysis of reaction specificity and amplification efficiency. Using conditions that balance these two aspects will allow accurate measurement over a wide range of concentrations.

○ 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

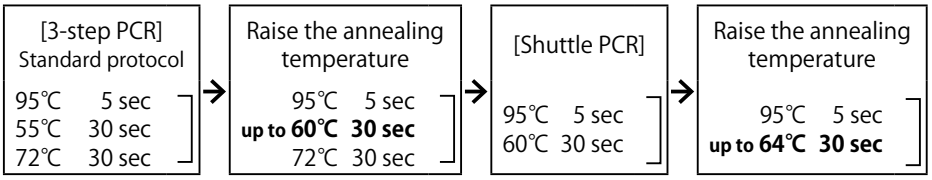
The relationship between primer concentration, reaction specificity, and amplification efficiency is illustrated below. Reducing primer concentration raises reaction specificity whereas increasing the primer concentration raises amplification efficiency.



2. Evaluation of PCR conditions

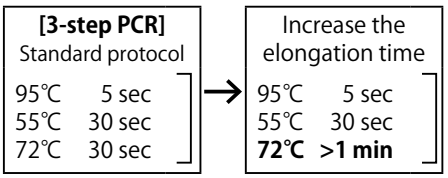
○ To improve reaction specificity

Raising the annealing temperature or shuttle PCR protocol may improve reaction specificity. Perform optimization while checking amplification efficiency.



○ To improve amplification efficiency

Increasing the elongation time may improve amplification efficiency. Perform optimization using the step below.



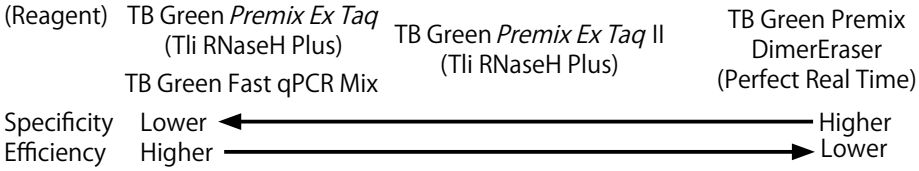
○ Initial denaturation

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.



VIII. Guidelines for Primer Design

It is essential to design primers which allow good reactivity for a successful real-time PCR reaction. Please follow the guideline stated as below to design primers which offer high amplification efficiency and minimizes non-specific reaction.

Amplification product

Amplified size	80 - 150 bp is most recommended. (Possible to amplify a target up of 300 bp.)

Primer

Length	17 - 25 mer
GC content	40 - 60% (45 - 55% is recommended.)
Tm	Tm values of forward primer and reverse primer must not largely differ. Tm value is calculated with the software. OLIGO*1: 63 - 68℃ Primer 3: 60 - 65℃
Sequence	The sequence should not be partially rich in any base throughout the whole sequence. Avoid including parts which have high GC or AT content, (especially 3'-end). Not include polypyrimidine (serial T/C sequence). Not include polypurine (serial A/G sequence).
Sequence of 3' end	The termini part of 3' end should not have high content of GC or AT. It is recommended to have G or C at 3' end. It is not recommended to have T at 3' end.
Complementarity	Complementary sequences of more than 3 bases should not exist within a primer or even between primer pairs. Primer pair should not have a complementary sequence of more than 2 bases at the 3' end each.
Specificity	Specificity of primers should be confirmed through BLAST search.*2

*1 OLIGO Primer Analysis Software (Moleculor Biology Insights, Inc.)
*2 https://blast.ncbi.nlm.nih.gov/Blast.cgi

IX. Related Products

TB Green® *Premix Ex Taq*™ (Tli RNaseH Plus) (Cat. #RR420A/B)
TB Green® Fast qPCR Mix (Cat. #RR430A/B)
TB Green® *Premix Ex Taq*™ II (Tli RNaseH Plus) (Cat. #RR820A/B)
Probe qPCR Mix (Cat. #RR391A/B)
One Step TB Green® PrimeScript™ PLUS RT-PCR Kit (Perfect Real Time) (Cat. #RR096A/B)*

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