Takara Bio USA, Inc.

Terra™ qPCR Direct TB Green® Premix User Manual

Cat. No. 638319
(011222)

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I. Introduction

Terra qPCR Direct TB Green Premix is a 2X master mix designed specifically for real-time PCR with TB Green chemistry. In addition to TB Green I dye, this mix contains Terra qPCR Direct Polymerase, a novel enzyme developed for optimal amplification from crude or dirty templates; it's perfect for amplifying short DNA targets (up to 2 kb), regardless of GC content or template purity. The premix also contains a monoclonal antibody that suppresses polymerase activity up to 98°C, allowing automatic hot-start PCR.

IMPORTANT: Blood and whole tissues exhibit strong quenching effects on TB Green dye fluorescence. Therefore, Terra qPCR Direct TB Green Premix should not be used to perform direct qPCR on blood samples or whole tissues.

II. List of Components

Store all components at –80°C. After thawing, store the kit, protected from light, at 4°C and use within 6 months. **Do not refreeze!**

Terra qPCR Direct TB Green Premix (200 rxns; Cat. No. 638319)

- 5 tubes 2X Terra qPCR Direct TB Green Premix (1 ml per tube)
- 200 µl ROX Reference Dye LSR (50X)
- 200 µl ROX Reference Dye LMP (50X)

III. Additional Materials Required

The following materials are required but not supplied:

- Gene-specific PCR primers
- PCR-grade water

  NOTE: Avoid using autoclaved H_2O; the recycled steam in some autoclaves can introduce contaminants that may interfere with PCR.
- PCR reaction tubes or plates (reaction-specific)
- Pipettors dedicated for pre-PCR
- Aerosol-resistant pipette tips suitable for use with the above pipettors and preferably equipped with hydrophobic filters.
- Real-time thermal cycler

IV. Protocol

NOTE: Please read the entire protocol before starting.

A. Preparing for Terra qPCR Direct TB Green Premix Assays

- Before use, mix the 2X Terra qPCR Direct TB Green Premix well by inverting the tube several times—be careful not to create air bubbles. **Do not vortex!**

  NOTE: The premix may form a white precipitate when stored at –80°C. To dissolve the precipitate, gently warm the tube with your hands; allow the liquid to equilibrate to room temperature while protecting it from light. Invert the tube several times until the precipitate is completely dissolved.

  Do not use the reagent until the precipitate has dissolved completely.

- During reaction setup, keep all reagents on ice.
- Terra qPCR Direct TB Green Premix contains TB Green I dye, which is sensitive to light; avoid exposure to direct light during reaction setup.
- Use 0.1–1.0 µM of each primer per reaction. For most targets, 0.2 µM of each primer is adequate.
- The quantity of template required depends on the number of target copies present in the sample. Make serial dilutions to select an appropriate quantity to use. Use no more than 100 ng of template per 20–25 µl reaction. If using single-stranded cDNA as a template, the volume of the cDNA added should be no more than 1/10th the volume of the PCR reaction.
- If you are using ROX as a reference dye on a real-time PCR thermal cycler not mentioned in this user manual, use the amount recommended by the manufacturer of your thermal cycler.

B. Protocol: Setting up Terra qPCR Direct TB Green Premix Assays

1. In a pre-PCR area or a template-free PCR workstation, prepare a Master Mix on ice by adding each of the components indicated in Table 1. Add all reagents in the order shown.

NOTE: Make sure you use enough of each reagent for all of your reactions, plus an additional reaction to accommodate pipetting errors. Ideally, each reaction should be performed in triplicate; however, duplicate reactions will suffice.

Table 1. Recommended Master Reaction Mixes for Different Real-Time PCR Instruments.

	qPCR instrument			
	Roche LightCycler	Takara Thermal Cycler Dice™ Real Time System	ABI PRISM 7000 & ABI 7500/7500 Fast Real-Time PCR Systems	
Reagent	**Reagent volume (µl per well) for each instrument**			
Sterile PCR-Grade Water	7.2	9.5	6.8	18.0
Terra PCR Direct TB Green Premix (2X)	10.0	12.5	10.0	25.0
Forward Primer (10 µM)*	0.4	0.5	0.4	1.0
Reverse Primer (10 µM)*	0.4	0.5	0.4	1.0
ROX Reference Dye LSR or LMP (50X)†	–	–	0.4	1.0
Template‡	2.0	2.0	2.0	4.0
Total volume per well	**20.0**	**25.0**	**20.0§**	**50.0****

* Use 0.1–1.0 µM of each primer per reaction. For most targets, 0.2 µM of each primer is adequate.

† The kit is supplied with two different ROX formulations that allow you to normalize fluorescence signals on instruments that are equipped with this option. ROX Reference Dye LSR is for instruments whose excitation source is a 488-nm laser, while ROX Reference Dye LMP is for instruments whose excitation source is either a lamp or an LED. **Be certain to use the formulation that is appropriate for your real-time instrument!**

‡ See Section IV. A. 5. (above) for information on template quantity.

§ This 20 µl reaction is for ABI instruments using 384-well plates and 96-well Fast Thermal Cycling plates.

** This 50 µl reaction is for ABI instruments using 96-well plates, single tubes, and 8-tube strips.

2. Mix the Master Mix well by tapping the tube at least 5 times, then centrifuge briefly. Transfer the amount of Mix indicated for your real-time instrument (see the last row of Table 1) into each well of a PCR plate or 8-well strip, or individual PCR capillary tubes. Centrifuge briefly.
3. Seal the wells according to the procedure recommended for the real time instrument being used.
4. Program your thermal cycler using the cycling conditions recommended in Table 2.

Table 2. Recommended Thermal Cycling Conditions for Different Real-Time PCR Instruments.

	qPCR instrument							
	Roche LightCycler		Takara Thermal Cycler Dice RealTime System		ABI PRISM 7000 & ABI 7500 Real-Time PCR System		ABI 7500 Fast Real-Time PCR System	
Reaction cycles	**Thermal cycling conditions for each instrument**							
Initial Denaturation* (1 cycle):	98°C	2 min*†	98°C	2 min*	98°C	2 min*	98°C	2 min*
qPCR (40 cycles):	98°C 60°C 68°C	10 sec† 15 sec† 1 min/kb†‡	98°C 60°C 68°C	10 sec 15 sec 1 min/kb‡	98°C 60°C 68°C	10 sec 15 sec 1 min/kb‡	98°C 60°C 68°C	10 sec 15 sec 1 min/kb‡
Melting/Dissociation Curve (1 cycle):	95°C 65°C 95°C	0 sec† 15 sec† 0 sec†	98°C 60°C 98°C	15 sec 30 sec 15 sec	Dissociation Curve		Melt Curve	

*** The initial denaturation step must be performed at 98°C for 2 min in order to denature the hot-start antibody.**

† 20°/sec.

‡ 30 sec for 500 bp or less.

IMPORTANT:

- The initial denaturation step must be performed at 98°C for 2 min in order to denature the hot-start antibody.
- Although Table 2 shows the optimized cycling conditions for a selection of commonly used real-time PCR instruments, the Terra qPCR Direct TB Green Premix can be used with a variety of real-time instruments and is not limited to those listed in the table. If your instrument is not listed in the table, please refer to the user manual supplied with the instrument to determine optimal cycling conditions. (For any real-time instrument used, the initial denaturation step must be performed at 98°C for 2 min in order to denature the hot-start antibody.)

5. Spin the reactions briefly, then place them into the real-time PCR instrument and begin thermal cycling.
6. After the reaction is complete, verify the amplification and melting curves. Establish a standard curve when quantitative analysis is necessary.
7. If this protocol does not produce the desired results, the reaction conditions will need to be optimized for your particular system. Template and primer concentrations can be adjusted within the recommended parameters. In addition, the PCR cycling conditions may need to be optimized—see Section IV.C.

C. Optimizing Your qPCR Cycling Conditions

- As noted above, the initial denaturation step must be performed at 98°C for 2 min in order to denature the hot-start antibody; however, the remaining cycling conditions can be modified as necessary.
- Reaction specificity can be improved by raising the annealing temperature. We recommend raising the annealing temperature in 2°C increments until you find an optimal balance between reaction specificity and amplification efficiency. It might also help to switch from a three-step protocol to a two-step protocol by combining the annealing and extension steps. Table 3 describes an example procedure to help you optimize your reaction specificity.

Table 3. Recommended Thermal Cycling Conditions to Improve Reaction Specificity.

	Standard Protocol		→	Raise Annealing Temperature		OR	Two-Step PCR	
(1 cycle):	98°C	2 min		98°C	2 min		98°C	2 min
(40 cycles):	98°C 60°C 68°C	10 sec 15 sec 1 min/kb		98°C ~64°C 68°C	10 sec 15 sec 1 min/kb		98°C 68°C	10 sec 1 min/kb

- Amplification efficiency can be improved by increasing the time allowed for the extension step or by lowering the annealing temperature. Table 4 describes an example procedure to help you optimize your amplification efficiency.

Table 4. Recommended Thermal Cycling Conditions to Improve Amplification Efficiency.

	Standard Protocol		→	Increase Extension Time		OR	Decrease Annealing Temperature		→	Increase Extension Time	
(1 cycle):	98°C	2 min		98°C	2 min		98°C	2 min		98°C	2 min
(40 cycles):	98°C 60°C 68°C	10 sec 15 sec 1 min/kb		98°C 60°C 68°C	10 sec 15 sec ~2 min/kb		98°C 55°C 68°C	10 sec 15 sec 1 min/kb		98°C 55°C 68°C	10 sec 15 sec ~2 min/kb

V. Examples

A. Example 1: qPCR with Crude Extracts

Crude alkaline heat extracts of cow muscle (beef foodstuff) and mouse spleen were obtained by adding 180 µl of 50 mM NaOH to 13.4 mg of beef and 10.5 mg of mouse spleen and incubating for ten minutes at 95°C. Each extract was neutralized with 20 µl of 1 M Tris-HCl (pH 8.0). qPCR was then performed using 1.5 µl of undiluted, 4x diluted, and 16x diluted extract along with either Terra qPCR Direct TB Green Premix or a conventional qPCR premix to amplify a 289-bp portion of the cytochrome oxidase gene (COXI) from the beef extract and a 165-bp portion of beta globin gene Hbb-b1 from the mouse spleen extract. The amplification plots of each are compared in Figure 1.

The amplification plots (and the resulting Ct values) generated by Terra qPCR Direct TB Green Premix correspond to the theoretical quantities of each gene assayed (Figure 1, Panel A). The conventional product, on the other hand, was markedly affected by inhibitors present in the crude samples (Figure 1, Panel B), as can be seen by the right-shifted amplification plots (and resulting Ct values) generated with the beef extract, and the complete lack of data generated with the mouse spleen extract. These results demonstrate the superior ability of the Terra premix to provide sensitive real-time detection with samples containing high levels of PCR inhibitors.

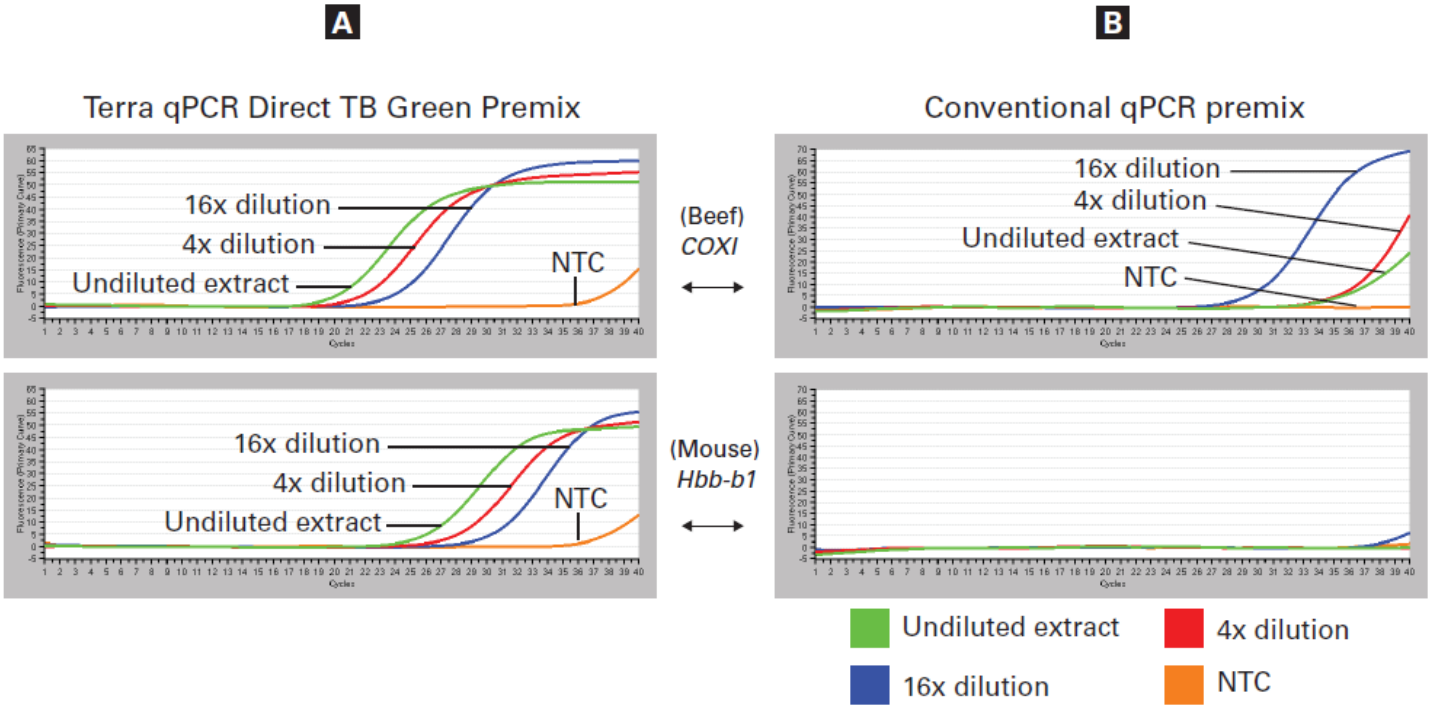

Figure 1. Real-time PCR with crude extracts—Terra qPCR Direct TB Green Premix versus a conventional qPCR premix. qPCR was performed using undiluted, 4x diluted, and 16x diluted crude alkaline heat extracts of cow muscle (beef foodstuff) or mouse spleen, and either Terra qPCR Direct TB Green Premix (**Panel A**) or a conventional qPCR premix (**Panel B**). Using conditions recommended for each enzyme, a 289-bp region of the cytochrome oxidase gene (COXI) was amplified from the beef extract and a 165-bp region of beta globin gene Hbb-b1 was amplified from the mouse spleen extract. Data generated by Terra qPCR Direct TB Green Premix corresponded to the theoretical quantities of each gene, while the conventional product was clearly affected by inhibitors present in the crude samples.

B. Example 2: qPCR of Targets with GC Content Greater than 70%

Targets with GC content greater than 70% were amplified by real time qPCR using either human genomic DNA, or cDNA generated from human testis total RNA as template, and either Terra qPCR Direct TB Green Premix or one of two conventional SYBR premixes (one specifically for GC-rich targets). Prior to the assay, the template DNA was serially diluted (10-fold), to obtain 100 ng–10 pg of genomic DNA, or the equivalent in cDNA of 50 ng–5 pg of total RNA. In the assay, gene-specific primers were used to amplify portions of the cyclin I gene (CCNI), the BTB domain-containing protein 6 gene (BTBD6), the jun D proto-oncogene (JUND) from the diluted cDNA (Figure 2, Panel A), and a portion of the b-actin CpG island (ACTB_CpG) from the diluted genomic DNA (Figure 2, Panel B). The resulting Ct values were plotted against the initial quantity of DNA used in each assay.

Terra qPCR Direct TB Green Premix consistently demonstrated sensitive and specific amplification of all of the targets assayed (Figure 2; triangles). The conventional premixes, on the other hand, were unable to consistently or specifically amplify any of the targets having GC content greater than 71.3% (Figure 2; diamonds and squares).

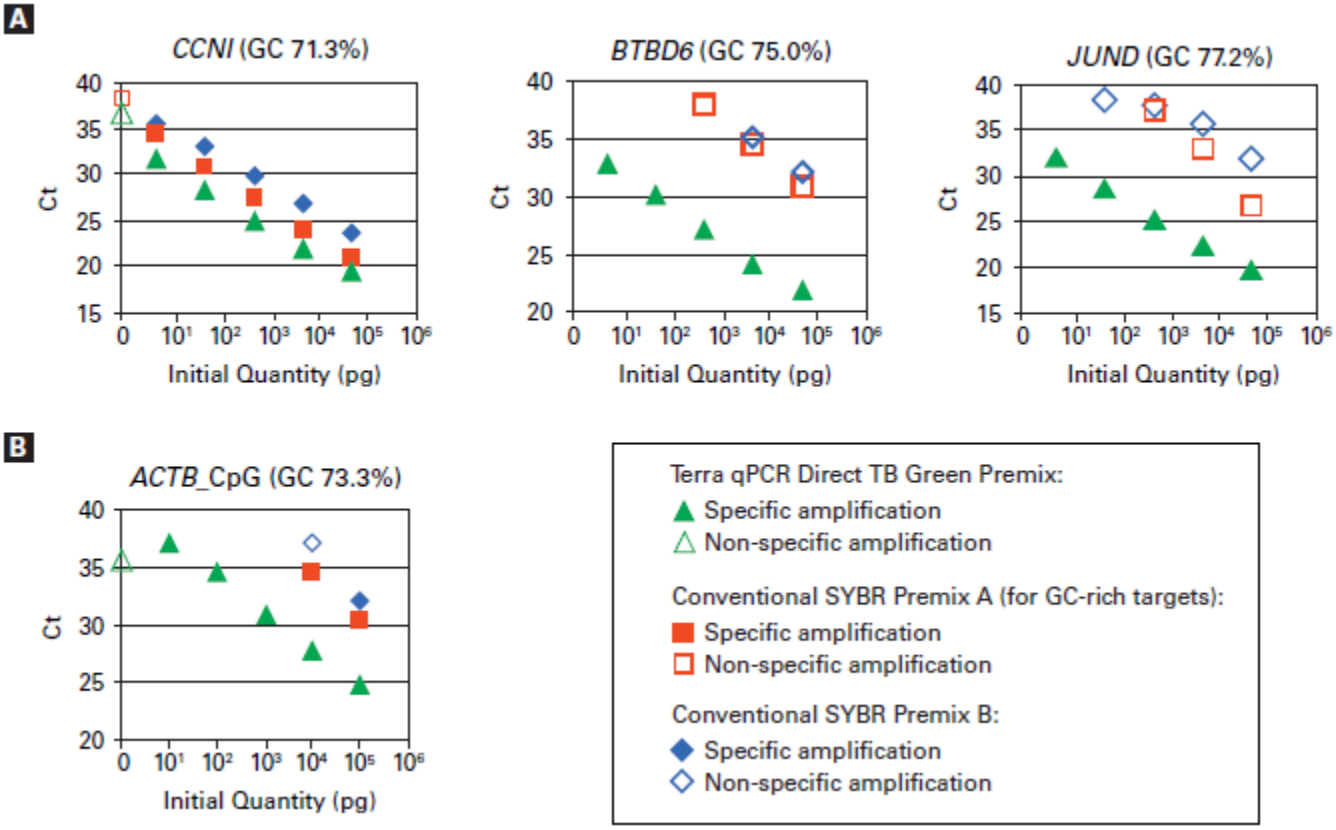

Figure 2. Real-time PCR of GC-rich targets—Terra qPCR Direct TB Green Premix versus conventional 2X qPCR premixes. Targets with GC content greater than 70% were amplified by real-time qPCR using either human testis cDNA (**Panel A**) or human genomic DNA (**Panel B**) as template, and either Terra qPCR Direct TB Green Premix (triangles) or one of two conventional SYBR premixes (one specifically for GC-rich targets). Prior to the assay, template DNA was serially diluted (10-fold), to obtain 100 ng–10 pg of genomic DNA, or the equivalent in cDNA of 50 ng–5 pg of total RNA. Under the conditions recommended for each enzyme mix, gene-specific primers were used to amplify portions of the cyclin I gene (CCNI), the BTB domain-containing protein 6 gene (BTBD6), and the jun D protooncogene (JUND) from the diluted cDNA (**Panel A**); and a portion of the b-actin CpG island (ACTB_CpG) from the diluted genomic DNA (**Panel B**). The resulting Ct values were plotted against the initial quantity of DNA used in each assay. Terra qPCR Direct TB Green Premix was the only premix able to consistently amplify all of the targets assayed (triangles).

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