TB Green® Advantage®/TB Green Advantage GC qPCR Premix Protocol-At-A-Glance

I. Introduction

TB Green Advantage qPCR Premix (Cat. No. 639676) and **TB Green Advantage GC qPCR Premix** (Cat. No. 638320) are 2X master mixes designed specifically for real-time PCR with green intercalating dye chemistry. Both premixes are formulated to provide superior specificity and increased amplification efficiency in high-speed real-time PCR—resulting in high sensitivity, a broad dynamic range, and accurate quantification. The TB Green Advantage GC qPCR Premix is specially formulated to allow highly accurate quantification of GC-rich targets containing up to 70% GC content, but also works well on standard, non-GC-rich targets.

II. Recommended Precautions

1. Mix the 2X TB Green Advantage (or TB Green Advantage GC) qPCR Premix by inverting the tube several times before use—be careful not to create air bubbles. **Do not vortex!** The premix may form a white or yellowish 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.**
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, use a new disposable tip each time to minimize cross-contamination.

III. Protocol

A. Reaction Mix Preparation

1. Prepare a Master Mix on ice by adding each of the components indicated in Table 1 (see next page).

   NOTE: Table 1 provides 3 master mix options–choose the option that suits the requirements of your thermal cycler. Mix A produces 20 µl reactions that lack ROX reference dye. Mixes B and C both contain ROX, but Mix B produces a 20 µl reaction, while Mix C produces a 50 µl reaction.

2. Make sure you use enough of each reagent for all of your reactions, plus an additional reaction to accommodate pipetting errors.

Table 1. Master Mix Recommendations for Different Reaction Volumes

Reagent	Reagent Volume (µl per sample)			Final Conc.
	Mix A	Mix B	Mix C	
PCR-Grade H_2O	~7.2	~6.8	~18	—
TB Green Advantage (or TB Green Advantage GC) qPCR Premix	10	10	25	1X
Forward Primer (10 µM)[a]	0.4	0.4	1.0	0.2 µM [a]
Reverse Primer (10 µM)[a]	0.4	0.4	1.0	0.2 µM [a]
ROX Reference Dye LSR or LMP (50X)[b]		0.4	1.0	1X [b]
Template (<100 ng per 25 µl rxn) [c]	~2.0	~2.0	~4.0	<100 ng [c]
Total volume per rxn	20.0	20.0	50.0	

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

[b]The Premix 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!**

[c]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.

3. 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.
4. Seal the wells according to the procedure recommended for the real time instrument being used.

B. Recommended Thermal Cycling Conditions

1. Program your thermal cycler using the cycling conditions recommended below as a guide:

Initial Denturation	95°C	30 sec	1 Cycle
Denaturation	95°C	5–10 sec	40 Cycles
Annealing/extension[a]	60°C	30–34 sec	

[a] For standard, non-GC-rich targets, if optimization of cycling conditions is required, optimize the extension/annealing temperature 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 (see User Manual, PT3883-1).

NOTE: For the initial denaturation cycle, heating the reactions to 95ºC for 30 sec is sufficient to denature both the hot start antibody and the template DNA. In addition, because real-time PCR targets are generally <300 bp, denaturation at 95°C for 5–10 seconds is sufficient during subsequent qPCR cycles.

2. Spin the reactions briefly, place them into the real-time PCR instrument, and begin thermal cycling.
3. After the reaction is complete, verify the amplification and melting curves. Establish a standard curve when quantitative analysis is necessary.

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