Protocol

TD-P Revision 2.0

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Goof-Proof™ qPCR Protocol

Procedure for using Goof-Proof Master Mix in qPCR

Introduction

Goof-Proof™ qPCR Master Mix is an EvaGreen® dye-based hot start master mix for use in DNA melt curve analysis and in Real Time PCR (RT-PCR) applications. Goof-Proof™ is a mistake-prevention master mix which contains a low concentration of blue dye. Additionally, Goof-Proof™ includes a DNA template buffer which has a higher concentration of blue dye. With the addition of the 2X Goof-Proof™ Master Mix to the reaction, it will appear light blue. And when you add the template, containing DNA template buffer, the color will turn darker blue (see Figure 2). Goof-Proof™ allows you to see quickly and easily whether you forgot to add the master mix or the template to any of your reactions, preventing pipetting mistakes and wasted time, reagents and money. The 2X Goof-Proof™ Master Mix can also be used without the Template Buffer, if desired.

An added benefit of an EvaGreen®-based master mix is that you can analyze your PCR product by gel electrophoresis without the need to add another DNA binding dye to either your loading buffer or gel. The EvaGreen® dye in the master mix can act as a DNA prestain, permitting direct visualization of DNA bands following electrophoresis.

GoldBio's HotStart Taq DNA Polymerase is a proprietary chemically modified hot-start DNA polymerase. Our HotStart Taq is fully activated in 2 minutes with high activity recovery, making it particularly suitable for fast PCR and is completely inactive at room temperature.

Kit Components

Component	G-700-1	G-700-5	G-705-1	G-705-5
Goof-Proof™ qPCR Master Mix	1 ml	5 x 1 ml	1 ml	5 x 1 ml
40X Template Buffer	1 ml	2 x 1 ml	1 ml	2 x 1 ml
ROX Reference Dye	n/a	n/a	0.2 ml	1 ml

Storage/Handling

Store the kit at -20°C. The Goof-Proof™ qPCR Master Mix is shipped on blue ice and should be stored immediately upon arrival at -20°C. When stored under the recommended condition and handled correctly, the kit should be stable for at least 1 year from the date of receipt. Before

use, thaw at room temperature and mix well by gentle vortexing. After thawing, the master mix should be kept on ice before use. It can be refrozen for storage.

Spectral Properties

λ_{abs} = 471 nm (without DNA)
λ_{abs} / λ_{em} = 500/530 nm (with DNA)

Performance Properties

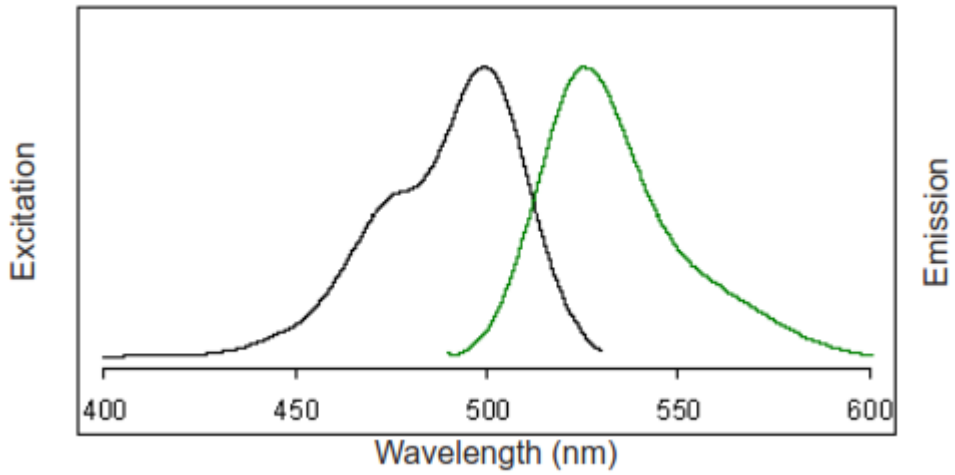

Figure 1. Excitation (left) and emission (right) spectra of EvaGreen® dye bound to dsDNA in PBS Buffer, pH 7.3

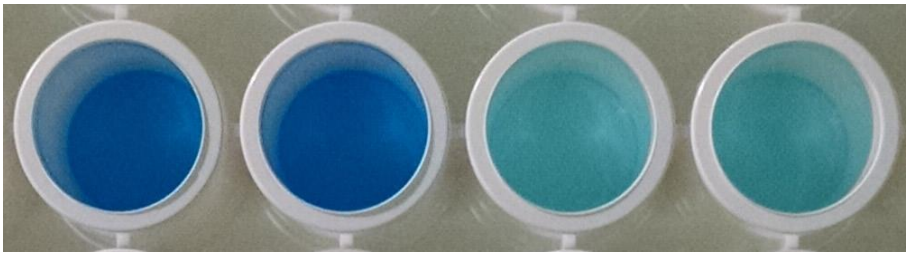

Figure 2. Left two wells contain qPCR reaction mix including Goof-Proof™ Master Mix and have had the DNA template or NTC control added in Template Buffer, hence the darker blue color. The right two wells have not yet had the DNA template or NTC control in Template Buffer added, and therefore are still the lighter blue of the 1X Goof-Proof™ Master Mix.

Method

Table 2. PCR Reaction Mix

Reaction Component	Amount required per 20 µl reaction	Final Concentration
2X Goof-Proof™ Master Mix	10 µl	1X
Primers	x µl each	0.1-0.5µM each
Template	x µl (See Note 1)	See Note 2
ROX	Optional	See Note 3
H2O	Add to 20 µl	

Note: Template may be added directly, or diluted first in template buffer. Template buffer is provided at 40X and can be diluted to 20X in PCR grade water prior to use. Template buffer should be at 1X in the final reaction. For example, if 1 µl of DNA template is to be added, a 1:2 dilution of DNA with the 20X Template Buffer should be made and 2 µl added to the final reaction. If 5 µl of DNA template is to be added, 5 µl of DNA would be added to 1 µl 20X Template Buffer, then 6 µl total would be added to the final reaction. The use of template buffer is optional, but all reactions in a given experiment should contain the same amount for accurate comparisons. Important Note: Template buffer should be well thawed and vortexed prior to use, and care should be used during pipetting to ensure no dye sticks to the outside of the pipette tip or is left remaining inside the tip.

Note: Template concentration: The optimal amount of template DNA varies by application. Recommended amounts of genomic DNA template per reaction typically range from 50 pg to 50 ng per reaction. Recommended amounts of cDNA typically range from 50 fg to 50 pg, based on the amount of input RNA in the RT reaction.

Note: ROX reference dye: For certain instruments, ROX is necessary for accurate Ct determination from well to well. Refer to Table 6 for the recommended ROX concentration for your instrument (minor adjustments may be needed). ROX may add noise to melt curve analysis, which could be mistaken for real peaks. Thus, in case of unexpected peaks, un-check "ROX" in the "Passive Reference Dye" box in the software so that data is not collected from the ROX fluorescence channel, then re-analyze the data.

PCR Cycling Protocols

Choose one of the following three protocols, depending on the nature of your amplicon and instrument capability.

A. Two-step fast cycling protocol

This cycling protocol should be applicable to most amplifications where the primer T_m are designed to be 60°C. Melt curves may be performed by following instructions provided for your instrument.

Cycling Step	Temperature	Holding Time	Number of Cycles
Enzyme activation	95°C	2 minutes	1
Denaturation	95°C	5 seconds	45
Annealing & Extension	60°C	30 seconds	

B. Three-step fast cycling protocol

This cycling protocol can be used if you would like to have the extension step to be performed at a higher temperature than the annealing step. For example, if you have relatively long primers that tend to anneal non-specifically, carrying out the extension step at a higher temperature can reduce nonspecific amplification. Melt curves may be performed by following instructions provided for your instrument.

Cycling Step	Temperature	Holding Time	Number of Cycles
Enzyme activation	95°C	2 minutes	1
Denaturation	95°C	5 seconds	45
Annealing	50-60°C	5 seconds	
Extension	72°C	25 seconds	

C. Universal cycling protocol

This cycling protocol can be used on nearly all qPCR instruments. The protocol also may be useful for targets that are relatively difficult to amplify under fast cycling conditions.

Cycling Step	Temperature	Holding Time	Number of Cycles
Enzyme activation	95°C	2 minutes	1
Denaturation	95°C	15 seconds	45
Annealing & Extension	60°C	30 seconds	

Tips

- qPCR instruments. For iCycler users, you do not need to add FAM to your PCR mix as EvaGreen® dye has a slight background fluorescence that provides adequate and stable baseline level fluorescence. For Roche LightCycler users using glass capillaries for reactions, you need to add BSA to your PCR reactions (~0.5 mg/ml final concentration). BSA is not necessary if transparent plastic capillary tubes are used.

- Instruments for melt curve analysis. Suitable instruments include RotorGene 6000, ABI 7500 FAST and HR1™, 384-well LightScanner™ and Roche LightCycler 480. Rotor-Gene 6000, ABI 7500 FAST and Roche LightCycler 480 are capable of performing both qPCR

and melt curve analysis. Follow the manufacturer's instruction for data collection and analysis.

- Amplicon length. To maximize amplification efficiency with Fast EvaGreen® master mix, the optimal amplicon length is 50-200 bp. For longer amplicons you may need to extend the elongation time.

- Gel electrophoresis analysis of PCR product. To analyze your PCR product by gel electrophoresis using the EvaGreen® dye in the master mix as a prestain, simply add DNA loading buffer to your PCR reaction solution, load on a gel, and conduct electrophoresis as usual. No additional DNA-binding dye needs to be added to either the loading buffer or the gel. Gel visualization can be carried out using a 254 nm UV box, or a gel imager or Dark Reader using a SYBR® Green filter. Alternatively, the gel may be imaged using a 488 nm laser-based gel scanner.

Table 6. Recommended ROX Concentration for PCR Instruments

PCR Instrument	Recommended ROX Concentration	Amount of ROX per 20 µl reaction
BioRad: iCycler, MyiQ, MiQ 2, iQ 5, CFX-96, CFX-384, MJ Opticon, Option2, Chromo4, MiniOpticon Qiagen: Rotor-Gene Q, Rotor-Gene 3000, Rotor-Gene 6000 Eppendorf: Mastercycler realplex Illumina: Eco RealTime PCR System Cepheid: SmartCyler Roche: LightCycler 480, LightCycler 2.0	No ROX	None
ABI: 7500, 7500 Fast, ViiA 7 Stratagene: MX4000P, MX3000P, MX3005P	Low ROX	If using Template Buffer, dilute ROX 1:10 with dH_2O and add 1.8 µl diluted ROX per 20 µl reaction.
		If not using Template Buffer, dilute ROX 1:100 with dH_2O and add 3 µl diluted ROX per 20 µl reaction.
ABI: 5700, 7000, 7300, 7700, 7900, 7900HT, 7900HT Fast, StepOne, StepOne plus	High ROX	If using Template Buffer add 2uL ROX Reference Dye per 20 µl reaction.
		If not using Template Buffer, dilute ROX 1:10 with dH_2O and add 3 µl diluted ROX per 20 µl reaction.

Associated Products

GoldBio Catalog #	Product Name
A-201	Agarose LE (Molecular Biology Grade)
D010	1 kb DNA Ladder
D011	1 kb PLUS™ DNA Ladder
D001	100 bp DNA Ladder
E-670	EvaGreen® Dye, 20x (25µM) in Water
G-725	GelRed™ Nucleic Acid Stain Gel Stain, 10,000X in Water
G-745	GelGreen™ Nucleic Acid Stain Gel Stain, 10,000X in Water

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