Biotium

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Product Information

Fast EvaGreen® Master Mix for qPCR and HRM

Catalog Number: 31003, 31003-1, 31003-2 or 31003-T

Unit Size: 200 reactions (cat # 31003), 500 reactions (cat # 31003-1), 5,000 reactions (cat# 31003-2), or 100 reactions (cat # 31003-T, trial size)

Components:

The product has two components: component A and component B. Component A is 2X master mix containing EvaGreen® dye, dNTP, buffer composition and Cheetah™ hot-start *Taq* polymerase. Component B is 10X Rox reference, which may be required on certain ABI instruments (See protocol below).

Components Supplied

Cat #	Component	
	31003A (2X master mix)	31003B (10X ROX reference)
31003	2 X 1 mL	1 X 1 mL
31003-1	5 X 1 mL	1 X 1 mL
31003-2	50 X 1 mL	10 X 1 mL
31003-T	1 X 1 mL	1 X 1 mL

Spectral Property of EvaGreen® Dye:

The absorption and fluorescence emission spectra of DNA-bound EvaGreen® dye are very similar to those of SYBR® Green I or FAM: $\lambda_{abs}/\lambda_{em}$ = 500/530 nm (DNA bound) (See Figure 1); λ_{abs} = 471 nm (without DNA)

Storage and Handling

Fast EvaGreen® 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 6 months 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.

Product Description

Fast EvaGreen® Master Mix is suitable for both quantitative real-time PCR (qPCR) and high resolution DNA melt curve analysis (HRM). It is formulated for qPCR using a fast cycling protocol. However, the master mix is also compatible with qPCR using a regular cycling protocol.

The dual use of Fast EvaGreen® master mix is unique among the commercially available PCR master mixes as master mixes based on SYBR® Green I or LC Green dye are usually only optimal for either qPCR or HRM, not for both applications, particularly not for both fast qPCR and HRM.

An important component of the master mix is EvaGreen® dye, a DNA-binding dye with features ideal for both qPCR and HRM. EvaGreen® dye binds to dsDNA via a novel "release-on-demand" mechanism, which permits relatively high dye concentration in qPCR without causing PCR inhibition.[1] Furthermore, EvaGreen® shows equal binding affinity for both GC-rich and AT-rich regions, an essential requirement for HRM analysis. Unlike other HRM dyes, such as SYTO 9 and LC Green dyes, EvaGreen® dye is a non-saturation dye. This means that EvaGreen® dye can be used for HRM at a concentration below its saturation concentration, lowering the chance for the dye to inhibit PCR.[2]

Another critical component of the master mix is Cheetah™ Taq, our proprietary chemically-modified hot-start DNA Polymerase. Unlike AmpliTaq Gold®, which is also a chemically modified Taq but takes 10 minutes or longer to activate, Cheetah™ Taq is fully activated in 2 minutes with high activity recovery, making it particularly suitable for fast PCR. Cheetah Taq is completely inactive at room temperature and largely free of DNA contamination. This makes Cheetah Taq superior to any antibody-based hotstart Taq, which is typically not completely inactive at room temperature and is prone to DNA contamination due to the nature of antibody production.

This kit is suitable for mRNA quantitation if a two-step procedure is followed. The first step involves converting the mRNA to cDNA by reverse transcription (components not provided). A portion of the synthesized cDNA can then be quantitated by using Fast EvaGreen kit in the second step. To ensure optimal amplification efficiency in real-time PCR, the aliquot of the cDNA sample to be amplified should not exceed 10% of the volume of the PCR reaction. We recommend cDNA synthesis kits from Quanta or Invitrogen. For accurate quantitation of mRNA level, a none-RT control is recommended to eliminate the possibility of genomic DNA contamination.

Reference

1. Mao, et al. Characterization of EvaGreen Dye and the implication of its physicochemical properties for qPCR applications. *BMC Biotechnology* 7, 76 (2007).
2. White, et al. Methylation-sensitive high-resolution melt-curve analysis of the SNRPN gene as a diagnostic screen for Prader-Willi and Angelman Syndromes. *Clin. Chem.* 53(11), 1 (2007):1960-2.

Additional Notes:

1) 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 an adequate and stable baseline level fluorescence; For Roche LightCycler users using glass capillaries for reactions, you need to add BSA (~0.5 mg/mL final concentration). BSA is not necessary if transparent plastic capillary tubes are used.

2) HRM instruments: Suitable instruments include Rotor-Gene 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 HRM. Follow the manufacturer's instruction to perform experiment and data analysis.

3) Expected ΔR and ΔR_N: When comparing signal strength among various commercial qPCR master mixes, one needs to be mindful of the method used in the comparison. Conventionally, ΔR is the fluorescence gain above the baseline. In general, in our hands, 10 µL of 1X Fast EvaGreen® qPCR reaction generates higher ΔR than 50 µL 1X PowerSYBR from ABI or 1X SYBR GreenER from Invitrogen. ΔRn is defined as ΔR divided by the signal in the ROX channel. Therefore, a higher concentration of ROX will generate smaller ΔRn. ΔRn will also become smaller when ROX is excited at its maximal as in the case of ABI 7500, iCycler IQ, MJ opticon, MJ Chromo4, MX3000, and MX4000. Accordingly, a lower ROX concentration in a SYBR Green master mix will produce a higher ΔRn, a technique sometimes used in some of the commercial SYBR Green Kits.

4) Expected kinetic curve: Based on our comparative studies, amplification curves of Fast EvaGreen® qPCR Master Mix are generally more robust than other commercial master mixes formulated using SYBR Green I. Because of SYBR's inhibitory effect, SYBR-based master mixes may tend to stall amplification in 5-7 cycles after the signal reaches the Ct line. In contrast, Fast EvaGreen® qPCR Master Mix continues to amplify for as many as 50 cycles.

5) Expected Ct value: Under similar conditions, Ct values generated by EvaGreen and SYBR Green I may differ from each other by +1 or –1.

PCR Protocol

1. Reaction Setup

Pipet reaction components into each well according to the table below:

Reaction component	Amount required for 20-uL reaction	Final concentration
2X Fast EvaGreen Master Mix	10 uL	1X
Primers (See Helpful Tip #1)	x uL each	0.1-0.5 uM each
Template (See Helpful Tip #1)	x uL	
ROX	Optional	See Helpful Tip # 2 below
H_2O	Add to 20 uL	

Helpful Tips:

1) Amplicon length: To maximize amplification efficiency with Fast EvaGreen master mix, the optimal amplicon length is 50-200 bp. If longer amplicon is intended, you may need to extend elongation time.

2) ROX reference dye: If you are using an ABI 7500, 7700 or 7900, ROX is necessary for accurate Ct determination from well to well. The optimal ROX for ABI 7700 or 7900 is 1X; this can be achieved by dispensing 2 µL of 10X ROX into each 20 µL reaction well. The optimal ROX for ABI 7500 is 0.05 to 0.1X, which would require adding 0.1 to 0.2 µL of 10X ROX to each 20 µL reaction; this is more conveniently achieved by first diluting the 10X ROX to 1X ROX and then dispensing 1 to 2 µL of the 1X ROX into each reaction well. 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, and then re-analyze the data.

ROX is optional for qPCR on iCycler IQ, MJ Opticon, MJ Chromo4, MX3000, MX4000, RotorGene 3000, RotorGene 6000 or LightCycler 480. In general, adding ROX to the reaction does not interfere with the operation.

2. Cycling Protocol

You may 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's are designed to be 60 °C. HRM may be performed by following instruction provided for your instrument.

Cycling Step	Temperature	Holding Time	Number of Cyles
Enzyme activation	96 °C	2 min	1
Denaturation	96 °C	5 s (See Helpful Tip #3)	45
Annealing & Extension	60 °C	30 s	

Helpful Tip:

3) Denaturation time: The holding time for denaturation can be lower than 5 seconds, including as low as 0 second, if you have a relatively short amplicon. When the denaturation time is set to "0" in the program, it merely means that the temperature is ramped up to 96 °C and then immediately ramped down with no stay. Setting the time to 5 s will ensures a more robust denaturation for relatively long or high GC amplicons. Instruments with fast ramping capability further add reliability to amplicon denaturation.

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 nonspecifically, carrying out the extension step at a higher temperature can reduce nonspecific amplification. HRM may be performed by following instruction provided for your instrument.

Cycling Step	Temperature	Holding Time	Number of Cyles
Enzyme activation	96 °C	2 min	1
Denaturation	96 °C	5 s	45
Annealing	50-60 °C (See Helpful Tip #4)	5 s	
Extension	72 °C (See Helpful Tip #5)	25 s	

Helpful Tips:

4) Annealing temperature: The annealing temperature should be set at your primer T_m, which should generally be 50-60 °C for optimal result. However, whenever possible, primer T_m (and thus extension temperature) should be designed closer to 60 °C (but still within 50-60 °C range) to minimize the gap between annealing and denaturation temperatures. This way, the temperature ramping will take less time, which in turn facilitates amplification.

5) Extension temperature: Extension at 72 °C is usually more efficient for most amplicons. However, for AT-rich amplicons (>70% AT) or amplicons that have an AT-rich patch, extension at 60 °C usually gives better results.

C. Universal cycling protocol

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

Cycling Step	Temperature	Holding Time	Number of Cyles
Enzyme activation	96 °C	2 min	1
Denaturation	96 °C	15 s	45
Annealing & Extension	60 °C	60 s	

Safety of EvaGreen® Dye

Ames test performed by an independent lab, Litron Laboratories (Rochester, NY), showed that EvaGreen® dye is nonmutagenic as well as noncytotoxic. EvaGreen® dye appears to be completely cell membrane-impermeable (Figure 3), which may be a key factor responsible for the observed low toxicity. On the other hand, SYBR® Green I is known to be a powerful mutation enhancer, possibly by inhibiting the natural DNA repairing mechanism in cells (Ohta, et el. *Mutat. Res.* 492, 91(2001)). The toxicity of SYBR® Green I may be associated with its ability to enter cells rapidly (Figure 3).

For more information on the Ames test result, you may download a complete report at Biotium website.

Spectral Characteristics

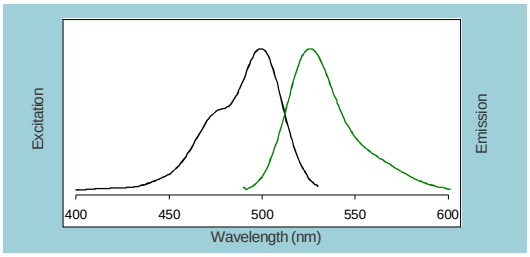


Figure 1. Excitation (left) and emission (right) spectra of EvaGreen® dye bound to dsDNA in pH 7.3 PBS buffer. Also see ref. 1.

Stability Comparison of EvaGreen™ Dye and SYBR® Green I

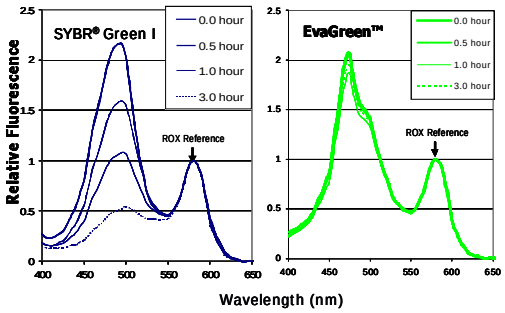
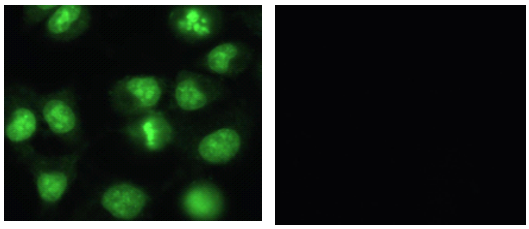


Figure 2. A solution of EvaGreen® dye or SYBR® Green I each at 1.2 µM in pH 9 Tris buffer was incubated at 99 °C. The absorption spectrum of each solution was followed over a period of 3 hours. ROX was added as a stable reference. Also see ref. 1.

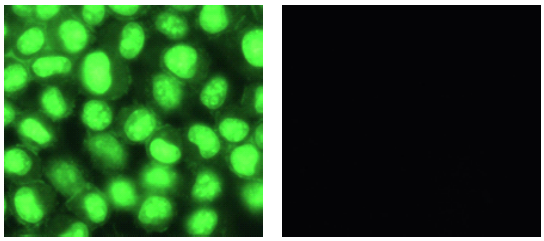
Comparison of Cell Membrane Permeability between EvaGreen® Dye and SYBR® Green I

A) 5 min. incubation



SYBR Green I, 1.2 µM — EvaGreen dye, 1.2 µM

B) 30 min. incubation



SYBR Green I, 1.2 µM — EvaGreen dye, 1.2 µM

Figure 3. HeLa cells were incubated with SYBR Green I (1.2 µM) or EvaGreen dye (1.2 µM) at 37 °C. Photographs were taken following incubation for 5 min (panel A) and 30 min (panel B). SYBR Green I entered cells rapidly while EvaGreen appeared membrane-impermeable.

Related Products:

EvaGreen dye, 20X in H_2O, cat# 31000
Cheetah hotstart Taq DNA polymerase, cat# 29050
PMA for selective detection of live pathogens by PCR, cat# 40013
GelRed nucleic acid gel stain, 10,000X in H_2O, cat# 41003
GelGreen nucleic acid gel stain, 10,000X in H_2O, cat# 41005