GENTAUR

DATA SHEET

Product Information

Fast EvaGreen® qPCR Master Mix

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, PCR buffer (including Tris and $MgCl_2$) and Cheetah™ hot-start Taq polymerase. Component B is 10X Rox reference, which may be required on certain ABI instruments (See protocol below).

Cat #	Component	
	31003A (2X master mix)	99939 (10X ROX reference dye)
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 Properties 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 (see Page 4, Figure 1).

$\lambda_{abs}/\lambda_{em}$ = 500/530 nm (DNA bound) 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 a ready-to-use hot-start mix for qPCR and DNA melt curve analysis of PCR amplicons. It is formulated for qPCR using a fast cycling protocol, but also can be used for qPCR using regular cycling protocols.

EvaGreen® dye is a unique DNA-binding dye with features ideal for both qPCR and melt curve analysis. EvaGreen® dye binds to dsDNA via a novel "release-on-demand" mechanism, which permits the use of a relatively high dye concentration in qPCR without PCR inhibition.

Fast EvaGreen® qPCR Master Mix contains 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.

A unique feature of EvaGreen® dye is its safety. DNA-binding dyes are inherently dangerous due to their potential to cause mutation. With this in mind, Biotium's scientists designed EvaGreen® dye such that it cannot cross cell membranes, thus preventing the dye from being in contact with genomic DNA in live cells. All other commercial PCR dyes enter into cells in a matter of minutes. SYBR® Green I, for example, has been shown to be environmentally more toxic than ethidium bromide, a well-known mutagen.[3] Independent labs have confirmed that EvaGreen dye is nonmutagenic, noncytotoxic and safe to aquatic life for direct disposal in the drain. Visit Biotium website for a full EvaGreen® dye safety report.

An added benefit of EvaGreen® 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.

References

1. Mao, et al. Characterization of EvaGreen Dye and the implication of its physico-chemical properties for qPCR applications. BMC Biotechnology 7, 76-91 (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), 1960-1962 (2007).

3. Ohta, et al. Ethidium bromide and SYBR Green I enhance the genotoxicity of UV-irradiation and chemical mutagens in E. coli. Mutation Res. 492, 91-97 (2001).

PCR Protocols

General Considerations

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 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.

2) Instruments for melt curve analysis: 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 melt curve analysis. Follow the manufacturer's instruction for data collection and 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, 10 μL of 1X Fast EvaGreen® 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, the lower ROX concentration used in some commercial SYBR Green master mixes will produce a higher ΔRn.

4) Expected kinetic curve: Based on our comparative studies, amplification curves of Fast EvaGreen® Master Mix generally are 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 5-7 cycles after the signal reaches the Ct threshold. In contrast, reactions in Fast EvaGreen® Master Mix can continue 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.

6) 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.

7) 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 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.

PCR Reaction Setup

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

Reaction component	Amount required per 20 uL reaction	Final concentration
2X Fast EvaGreen Master Mix	10 uL	1X
Primers	x uL each	0.1-0.5 uM each
Template	x uL See Notes #1 & #2	See Note #3
ROX	Optional	See Note #4 and Table 1
H_2O	Add to 20 uL	

Notes:

1) cDNA templates: Fast EvaGreen® master mix 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, 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 transcript levels, a no-RT control is recommended to check for possible genomic DNA contamination.

2) One-step RT-qPCR can also be applied for mRNA quantitation. Primer sets must be well characterized to ensure no primer-dimer formation. We recommend that you titrate the amount of reverse transcriptase and the duration of the RT step. Heat-resistant reverse transcriptases that have been tested to be compatible include those from Agilent, Fermentas, Lucigen and Life Technologies. If possible, design primers to have T_m at 55 °C, run both RT step and extension step at 55 °C. For accurate quantitation of transcript levels, a no-RT control is recommended to check for possible genomic DNA contamination.

3) 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.

4) ROX reference dye: For certain instruments, ROX is necessary for accurate Ct determination from well to well. Refer to Table 1 for the recommended ROX concentration for your instrument. 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.

Table 1. Recommended ROX Concentration for PCR Instruments

PCR Instrument	Recommended Rox Concentration	Amount of 10X ROX per 20 uL reaction
BioRad: iCycler, MyiQ, MiQ 2, iQ 5, CFX-96, CFX-384, MJ Opticon, Option2, Chromo4, MiniOpticon Qiagen: Roto-Gene Q, Roto-Gene3000, Roto-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 Stratagene: MX4000P, MX3000P, MX3005P	Low ROX 0.05-0.1X final	Dilute 10X ROX 1:10 with dH2O to obtain 1X ROX; add 1 to 2 uL of 1X ROX per 20 uL reaction
ABI: 5700, 7000, 7300, 7700, 7900, 7900HT, 7900HT Fast, StepOne, StepOne plus	High ROX 1X final	2 uL of 10X ROX per 20 uL reaction

Cycling Protocols

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. 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 min	1
Denaturation	95 °C	5 s (See Note #5)	45
Annealing & Extension	60 °C	30 s	

Note:

5) 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 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 min	1
Denaturation	95 °C	5 s	45
Annealing	50-60 °C (See Note #6)	5 s	
Extension	72 °C (See Note #7)	25 s	

Notes:

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

7) Extension temperature: Extension at 72 oC 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 oC usually gives better results.

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 min	
Denaturation	95 °C	15 s	45
Annealing & Extension	60 °C	60 s	

Appendix. EvaGreen Dye® Characteristics

The following figures provide additional information on EvaGreen® dye in regard to its fluorescence spectra, stability and cell membrane permeability.

Spectral Characteristics

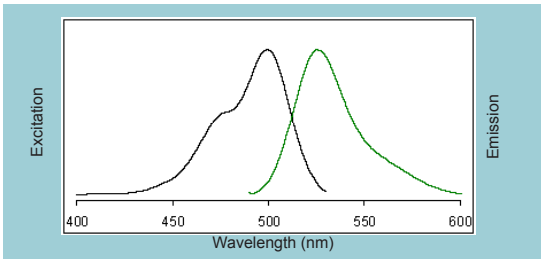

Figure 1. Excitation (left) and emission (right) spectra of EvaGreen® dye bound to dsDNA in pH 7.3 PBS buffer. Also see ref. 1.

Stability Comparison of EvaGreen® Dye and SYBR® Green I

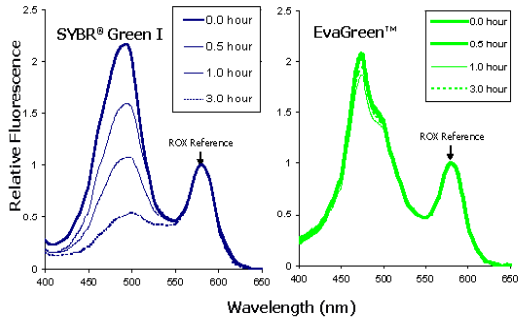

Figure 2. A solution of EvaGreen™ dye or SYBR® Green I each at 1.2 μM in pH 9 Tris buffer was incubated at 99 °C. The absorption spectrum of each solution was followed over a period of 3 hours. ROX was added as a stable reference.

Safety of EvaGreen® Dye

Ames testing 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).

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

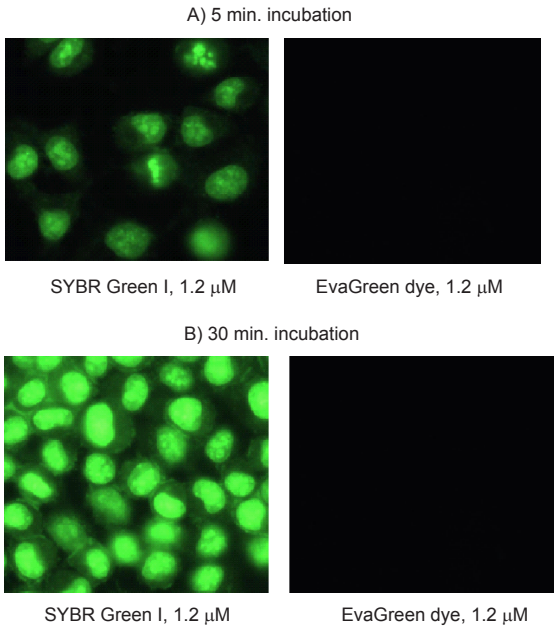

Figure 3. HeLa cells were incubated with SYBR Green I (1.2 μM) or EvaGreen dye (1.2 μM) at 37 °C. Photographs were taken following incubation for 5 min (panel A) and 30 min (panel B). SYBR Green I stained cells rapidly while EvaGreen appeared to be membrane impermeable.

Related Products:

EvaGreen dye, 20X in H_2O, cat# 31000
Cheetah hotstart Taq DNA polymerase, cat# 29050
Fast Plus EvaGreen Master Mix, no ROX, cat# 31020
Fast Plus EvaGreen Master Mix, low ROX, cat# 31014
Fast Plus EvaGreen Master Mix, high ROX, cat# 31015
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