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Product Information

This document contains information for three EvaGreen® Master Mix product groups:

1. Fast-Plus EvaGreen® Master Mix (no ROX) (Cat # 31020, 31020-1, 31020-2 and 31020-T)
2. Fast-Plus EvaGreen® Master Mix with Low ROX (Cat# 31014, 31014-1, 31014-2 and 31014-T)
3. Fast-Plus EvaGreen® Master Mix with High ROX (Cat# 31015, 31015-1, 31015-2 and 31015-T)

See table below for product information including instrument compatibility related to the product you have purchased.

Table 1. EvaGreen® Master Mix product information and instrument compatibility

Product Group	Cat #	Packaging Size	Component	PCR Instrument
Fast-Plus EvaGreen Master Mix (no ROX)	31020	200 rxn (2 X 1 mL)	EvaGreen® dye, dNTP, buffer composition (including Tris and $MgCl_2$) and Cheetah™ hot-start Taq polymerase. Optimized for most of the non-ABI instruments which do NOT require ROX reference dye.	BioRad: iCycler, MyiQ, MiQ 2, iQ 5, CFX-96, CFX-384, MJ Opticon, Option2, Chromo4, MiniOpticon Qiagen: Rotor-Gene Q, Rotor-Gene3000, Rotor-Gene 6000 Eppendorf: Mastercycler realplex Illumina: Eco RealTime PCR System Cepheid: SmartCyler Roche: LightCycler 480, LightCycler 2.0
	31020-1	500 rxn (5 X 1 mL)		
	31020-2	5,000 rxn (50 X 1 mL)		
	31020-T	100 rxn (1 X 1 mL) (trial size)		
Fast-Plus EvaGreen Master Mix with Low ROX	31014	200 rxn (2 X 1 mL)	EvaGreen® dye, dNTP, buffer composition (including Tris and $MgCl_2$), Cheetah™ hot-start Taq polymerase and low concentration of ROX reference dye.	ABI: 7500, 7500 Fast, ViiA 7 Stratagene: MX4000P, MX3000P, MX3005P
	31014-1	500 rxn (5 X 1 mL)		
	31014-2	5,000 rxn (50 X 1 mL)		
	31014-T	100 rxn (1 X 1 mL) (trial size)		
Fast-Plus EvaGreen Master Mix with High ROX	31015	200 rxn (2 X 1 mL)	EvaGreen® dye, dNTP, buffer composition (including Tris and $MgCl_2$), Cheetah™ hot-start Taq polymerase and high concentration of ROX reference dye.	ABI: 5700, 7000, 7300, 7700, 7900, 7900HT, 7900HT Fast, StepOne, StepOne plus
	31015-1	500 rxn (5 X 1 mL)		
	31015-2	5,000 rxn (50 X 1 mL)		
	31015-T	100 rxn (1 X 1 mL)		

Storage and Handling

Fast-Plus EvaGreen® Master Mix is shipped on blue ice and should be stored immediately upon arrival at -20 °C. When stored in a constant temperature freezer at -20 °C, the kit is stable for at least 12 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.

EvaGreen® dye is the first and only qPCR dye that is environmentally safe to dispose down drain due to its inability to cross cell membranes. See Appendix for more on EvaGreen® dye features. A safety report can be downloaded at www.biotium.com.

EvaGreen® Dye Properties

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 Page 4, Figure 1); λ_{abs} = 471 nm (without DNA).

Product Description

Fast-Plus EvaGreen® Master Mix is a ready-to-use hot-start mix for nucleic acid quantitation and melt analysis of PCR amplicons. It delivers clean PCR product even with the most challenging samples tested. Although the master mix is formulated for qPCR using a fast cycling protocol, it is also compatible with qPCR using a regular cycling protocol (see below for recommended cycling protocols of 2-step fast, 3-step fast and universal). ROX reference dye is included in the mix (Cat #31014 and Cat #31015 series) for well-to-well fluorescence normalization in ABI and some other instrument platforms. Please refer to the master mix selection guide (Table 1) to decide which master mix is best suited for real time instruments.

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-Plus EvaGreen® 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 recovered in 2 minutes with high activity, 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 binding genomic DNA in live cells. All other commercial PCR dyes enter live 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 (Ohta et al. 2001). Independent labs have confirmed that EvaGreen dye is nonmutagenic, noncytotoxic and safe to aquatic life for direct disposal in the drain. Visit www.biotium.com to view the EvaGreen® dye safety report.

An added benefit of the Fast-Plus 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.

Selected references

1. Khan, et al. Detection of aacA-aphD, qacEδ1, marA, floR, and tetA genes from multidrug-resistant bacteria: comparative analysis of real-time multiplex PCR assays using EvaGreen® and SYBR® Green I dyes, Molecular and Cellular Probes (2011), doi: 10.1016/j.mcp.2011.01.004

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

3. Cheng, et al. Detection of hemi/homozygotes through heteroduplex formation in high-resolution melting analysis, Anal. Biochem. 410, 158 (2011).

4. 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, 1960 (2007).

5. 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 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) 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-Plus 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 peak wavelength 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.

3) Expected kinetic curve: Based on our comparative studies, amplification curves of Fast-Plus EvaGreen® 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-Plus EvaGreen® Master Mix continues to amplify for as many as 50 cycles.

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

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

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-Plus 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
H_2O	Add to 20 uL	

Notes:

1) cDNA templates: Fast-Plus EvaGreen® master mix can be used for mRNA quantitation using a two-step RT-PCR reaction. In the first step cDNA is synthesized using mRNA by reverse transcription (components not provided; we recommend cDNA synthesis kits from Quanta or Invitrogen). A portion of the cDNA is used for qPCR with Fast-Plus EvaGreen master mix. 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. 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 well-to-well normalization. ROX reference dye is included in the mix (Cat #31014 and Cat #31015). See Table 1 on page 1 to determine which master mix is recommended for your instrument.

2. 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. DNA melt curve analysis 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)	40
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 second in the program, it merely means that the temperature will ramp to 96 °C and then will immediately ramp down with no delay. 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. DNA melt curve analysis 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	40
Annealing	50-60 °C (See Note 6)	5 s	
Extension	72 °C (See Note 7)	25 s	

Notes:

6) Annealing temperature: 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.

7) 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 may give better results.

C. Universal cycling protocol

This traditional 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 condition.

Cycling Step	Temperature	Holding Time	Number of Cycles
Enzyme activation	95 °C	5 min	1
Denaturation	95 °C	15 s	40
Annealing & Extension	60 °C	60 s	

Gel electrophoresis analysis of PCR product

To analyze your PCR product by gel electrophoresis using the EvaGreen® dye in the master mix as 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.

Related Products

EvaGreen dye, 20X in H_2O, Cat # 31000

Cheetah Hotstart Taq DNA polymerase, Cat # 29050

Fast Probe Master Mix, Cat # 31005

Fast Probe Master Mix with ROX, Cat # 31016

dNTP Set, 100mM, Cat # 40052

dNTP Mix, 25mM, Cat # 40053

dNTP Mix, 10mM, Cat # 40054

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

AccuBlue Broad Range dsDNA Quantitation Kit, Cat # 31007

AccuBlue High Sensitivity dsDNA Quantitation Kit, Cat # 31006

Appendix. EvaGreen Dye® Characteristics

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

Spectral Characteristics

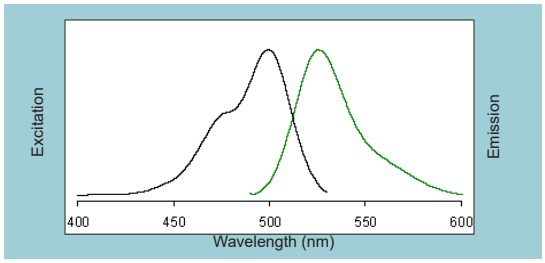


Figure 1. Excitation (left) and emission (right) spectra of EvaGreen® dye bound to dsDNA in pH 7.3 PBS buffer. Also see ref. 1.

Stability Comparison of EvaGreen® Dye and SYBR® Green I

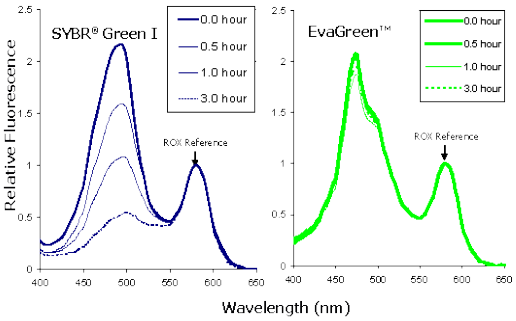
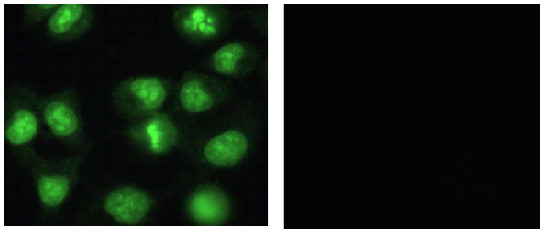


Figure 2. A solution of EvaGreen® dye or SYBR® Green I each at 1.2 μM in pH 9 Tris buffer was incubated at 99 °C. The absorption spectrum of each solution was followed over a period of 3 hours. ROX was added as a stable reference. SYBR Green I nearly completely disappeared while no decomposition was noticeable for EvaGreen dye.

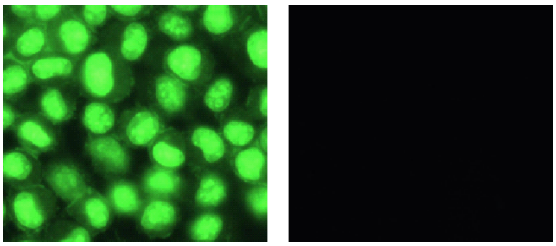
Comparison of Cell Membrane Permeability between EvaGreen® Dye and SYBR® Green I

A) 5 min. incubation



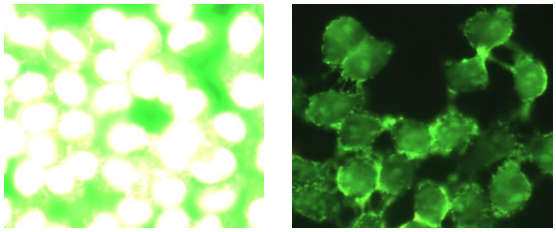
SYBR Green I, 1.2 μM EvaGreen dye, 1.2 μM

B) 30 min. incubation



SYBR Green I, 1.2 μM EvaGreen dye, 1.2 μM

C) 30 min. incubation, long photographing exposure time



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 (panels B and C). SYBR Green I entered cells rapidly while EvaGreen appeared membrane-impermeable as evident from the absence of cell nuclear staining (panels A and B). Image taking with long exposure time revealed that EvaGreen dye only associated with cell membranes (panel C). SYBR Green I has been suggested to interfere with the DNA repair mechanism in living cells, a rationale used to explain the observation that the dye is even more environmentally toxic than the widely known mutagen ethidium bromide (Ohta, et al. *Mutation Research*, **492**, 91-97(2001)). In contrast, EvaGreen dye has been confirmed to be nonmutagenic and noncytotoxic. See EvaGreen safety report at Biotium website.