Protocol

TD-P Revision 2.0

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EvaGreen® Dye qPCR Protocol

Procedure for qPCR with non-Hot Start Taq

Introduction

EvaGreen® dye is a green fluorescent nucleic acid dye with features that make the dye useful for several applications including qPCR and DNA melt curve analysis, real-time monitoring of thermophilic helicase-dependent amplification (tHDA), and capillary gel electrophoresis. The DNA-bound dye has excitation and emission spectra very close to those of fluorescein (FAM) or SYBR® Green I, making the dye readily compatible with instruments equipped with the 488 nm argon laser or any visible light excitation with wavelength in the region. EvaGreen® dye is extremely stable both thermally and hydrolytically, providing convenience during routine handling. The dye is essentially nonfluorescent by itself, but becomes highly fluorescent upon binding to dsDNA. EvaGreen® dye is nonmutagenic and noncytotoxic by being completely impermeable to cell membranes, making it a great alternative to SYBR® Green I, which enters cell rapidly and is known to be a powerful mutation-enhancer.

The unique properties of EvaGreen® dye have made it particularly useful in quantitative real-time PCR (qPCR) applications. Compared with the widely used SYBR® Green I, EvaGreen® dye is generally less inhibitory toward PCR and less likely to cause nonspecific amplification. As a result, EvaGreen® dye can be used at a much higher dye concentration than SYBR® Green I, resulting in a high resolution signal for droplet digital PCR, real-time PCR and melt curve analysis.

EvaGreen® Dye is specifically formulated for qPCR use. The PCR reaction can be monitored using your existing optical setting for SYBR Green I or FAM on any commercial real-time PCR cycler. The qPCR protocol provided below is for PCR using regular non-hot start Taq. Use of a hot start Taq may require some adjustment of PCR buffer composition in terms of ionic strength and pH to best take the advantage of EvaGreen® dye. For example, chemically-modified Taq, such as AmpliTaq Gold, may prefer a lower concentration of KCl or no KCl and higher Tris concentration (up to 50mM). In addition, a water soluble solvent such as DMSO or glycerol are frequently added to stabilize master mixes. These components and pH may need to be optimized depending on the enzyme used.

Note: For convenience, the EvaGreen® 2000x in DMSO concentrated solution (2.5mM) may be diluted 10 times to a 0.25mM solution in either dH_2O or Tris (10mM, pH 7-9), which may be stored at 4°C.

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Performance Properties

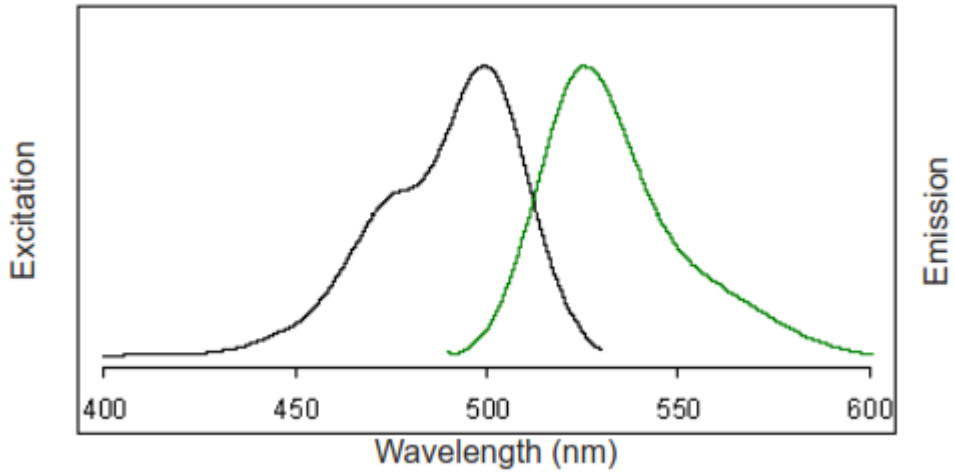

Figure 1. Excitation (left) and emission (right) spectra of EvaGreen® dye bound to dsDNA in PBS Buffer, pH 7.3

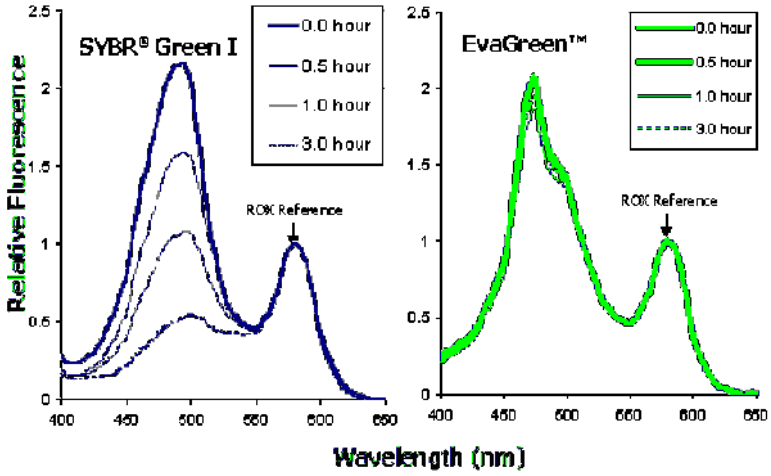

Figure 2. Stability comparison of EvaGreen® and SYBR Green dyes. Solutions of EvaGreen® dye or SYBR Green I at 1.2µM in pH 9 Tris buffer were incubated at 99°C. The absorption spectrum of each solution was measured over a period of 3 hours. ROX was added as a stable reference.

Spectral Properties

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

Materials

- EvaGreen® Dye, 20x (25µM) in Water (Catalog # E-670)
- EvaGreen® Dye, 2000x (2.5mM) in DMSO (Catalog # E-675)

Method

Using non-hot start Taq and the protocol provided below, EvaGreen® dye shows higher fluorescent signals than SYBR Green I for both qPCR and melt curve analysis. Because the optical settings vary slightly from instrument to instrument and the wavelengths of EvaGreen® dye are slightly longer than those of SYBR Green I, Ct value may differ slightly by +1 or –1 when compared with SYBR Green I side-by-side.

The following protocol is recommended for use with non-hot start Taq.

1. Set up the PCR reaction as follows[1]:
   5 µL of 10x polymerase buffer without magnesium[2]
   2.5 µL of 50mM $MgCl_2$[3]
   5 µL each of 2 mM dNTP
   2.5 µL of 20X EvaGreen[4]
   1-5 units of Taq DNA polymerase[5]
   0.1-1µM each of primers (final concentrations)
   dH_2O to a final volume of 50 µl.

2. Perform real-time PCR on a thermocycling fluorometer and record the fluorescence signal at the annealing or extension step.

Note: When using ABI Sequence Detection Systems, make sure to select NONE for the passive reference under the tab WELL INSPECTOR.

Note: BSA may be required if the reaction is run on a Roche LightCycler. A final BSA concentration of 0.5 mg/mL may be sufficient. With SYBR Green, addition of a protein such as BSA results in a fluorescence increase, which provides a background signal that triggers the start of a LightCycler. Because EvaGreen® dye is less sensitive to proteins, you may need to adjust the instrument setting (for background fluorescence) so that the instrument will start.

[1] – For iCycler users, you do not need to add FAM to your PCR mix because EvaGreen® has a slight background fluorescence that provides an adequate and stable baseline level fluorescence for well calibration.

[2] – For chemically-modified Taq, it may be necessary to reduce the KCl concentration and increase the Tris concentration.

[3] – The optimal Mg^{2+} concentration for PCR with EvaGreen® dye is 2.5mM.

[4] – Before pipetting, warm up the 20X solution to room temperature and thoroughly mix the solution by vortexing. EvaGreen® is highly stable. However, dye may adsorb to the vial during storage. Vortex the vial for a few seconds to make sure the dye is fully dissolved.

[5] – For best results, a hot-start enzyme should be used. However, buffer formulation may need to be adjusted.

Safety

Ames testing performed by an independent lab, Litron Laboratories (Rochester, NY), showed that EvaGreen® dye is nonmutagenic as well as non-cytotoxic. EvaGreen® dye appears to be completely cell membrane-impermeable, which may be a key factor responsible for the observed low toxicity. Although EvaGreen® has undergone extensive safety testing, we advise researchers to exercise universal laboratory safety precautions when handling EvaGreen® dye or any other DNA-binding agents.

Disposal

EvaGreen® dye at 2X is classified as nonhazardous for drain disposal under CCR Title 22 regulation. If required by your local regulations, EvaGreen® can be adsorbed onto activated charcoal for disposal. Pour 10 liters of EvaGreen® waste solution through ~1 g of activated charcoal. The filtrate may directly go to the drain while the charcoal may be treated as solid waste.

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
G-725	GelRed™ Nucleic Acid Stain Gel Stain, 10,000X in Water
G-745	GelGreen™ Nucleic Acid Stain Gel Stain, 10,000X in Water

References

Ohta, T., Tokishita, S., & Yamagata, H. (2001). Ethidium bromide and SYBR Green I enhance the genotoxicity of UV-irradiation and chemical mutagens in E. coli. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 492*(1-2), 91-97. Doi:10.1016/s1383-5718(01)00155-3.