# Safety Report of EvaGreen® Dye

A Summary of Mutagenicity and Environmental Safety Test Results
from Three Independent Laboratories

Biotium
Glowing Products for Science™

Last updated: March 7, 2011

# Overview

Real-time PCR (qPCR) is a sequence-specific DNA quantitation technique widely practiced in research and diagnostic fields by laboratory technicians and scientists of all levels. A large number of qPCR experiments are carried out using a DNA-binding dye as a reporter molecule. However, despite the fact that DNA-binding dyes are inherently dangerous due to their potential to cause mutation, very few PCR dyes have been thoroughly examined for their safety. Thus, handling and disposal of PCR master mixes can be a health and environmental issue. Indeed, SYBR® Green I is found to be even more environmentally toxic than ethidium bromide, a well-known mutagen.[1] It has been suggested that SYBR® Green I interferes with the natural DNA-repair mechanism in cells and as a result it potentiates genotoxicity of chemicals as well as DNA damage caused by UV light. Although no safety data are available on other PCR and HRM dyes (*e.g.*, SYTO9, LC Green, BRYT Green and ResoLight), these dyes are known to enter cells in a matter of minutes, thus posing potential genotoxicity risk. Bearing this information in mind, Biotium's scientists engineered the EvaGreen® dye structure to maximize the dye's safety without compromising PCR performance.

## Dye Design Principle

At the outset of the EvaGreen® dye project, we recognized that one way to make a PCR dye safe is to eliminate or minimize the chance for the dye to interact with genomic DNA in living cells. Armed with this insight, we sought to improve the dye on three fronts: 1) to make the dye impenetrable to latex gloves; 2) to make the dye impenetrable to cell membranes; and 3) to design the dye so that its metabolites have little or no interaction with DNA. By achieving these objectives, successive lines of safety defense are built to offer maximal protection. Based on this design principle, chemists at Biotium incorporated structural features into the dye to achieve the desired dye properties.

## Safety Tests

EvaGreen® dye was subjected to a series of tests both by Biotium and by three independent testing services to assess the dye's safety for routine handling and disposal. These tests include: 1) glove penetration test; 2) cell permeability test; 3) Ames test; and 4) environmental safety tests. Results of the tests are summarized in the table below. The data show that EvaGreen® dye passed all of the tests, thus validating the dye design principle. Detailed test results are described in pages 3-10.

## Conclusion

EvaGreen® dye possesses novel chemical features designed to minimize dye-DNA interaction in living cells. Test results confirm that the dye is impenetrable to both latex gloves and cell membranes. The dye is noncytotoxic and nonmutagenic at concentrations well above the working concentrations used in qPCR. Furthermore, EvaGreen® dye has successfully passed environmental safety tests in compliance with CCR Title 22 Hazardous Waste Characterization, an environmental regulation in the state of California . As a result, EvaGreen® dye is not only safe to handle but also can be conveniently disposed of down the drain.

References:
1. Ohta et al. Ethidium bromide and SYBR Green I enhances the genotoxicity of UV-irradiation and chemical mutagens in E. coli, *Mut. Res.* **492**, 91(2001).

Table 1. Summary of EvaGreen Dye Safety Test Results

| Test | Latex and Nitrile Glove Penetration | Cell Membrane Permeability | Ames Test | Hazardous Waste Screening (aquatic toxicity test) |
|---|---|---|---|---|
| Result | Impenetrable | Impermeable | Nonmutagenic | Non-toxic to aquatic life |

*This document is intended to provide a brief summary of the safety data on EvaGreen® dye obtained from several laboratories. If you wish to see the original test reports, you may contact Biotium Technical Support.*

# Glove Penetration Test

## Purpose

Latex and nitrile gloves are commonly worn by researchers in laboratories as protective gear. The purpose of this test is to see if these glove materials can act as effective barriers to EvaGreen® dye.

## Method

A finger of either a latex glove or nitrile glove filled with deionized water was submerged in a beaker containing 20X EvaGreen dye solution. After 24 hours, the water in the glove finger and the EvaGreen dye solution in the bath were analyzed by absorption spectrum.

## Results

No trace of EvaGreen® dye was detected in the finger of either the latex glove or the nitrile glove (Figure 1).

## Conclusion

Latex or nitrile gloves can serve as an adequate barrier to EvaGreen® dye at a concentration at least as high as 20X (*i.e.*, a concentration 20 times higher than the typical concentration used in qPCR).

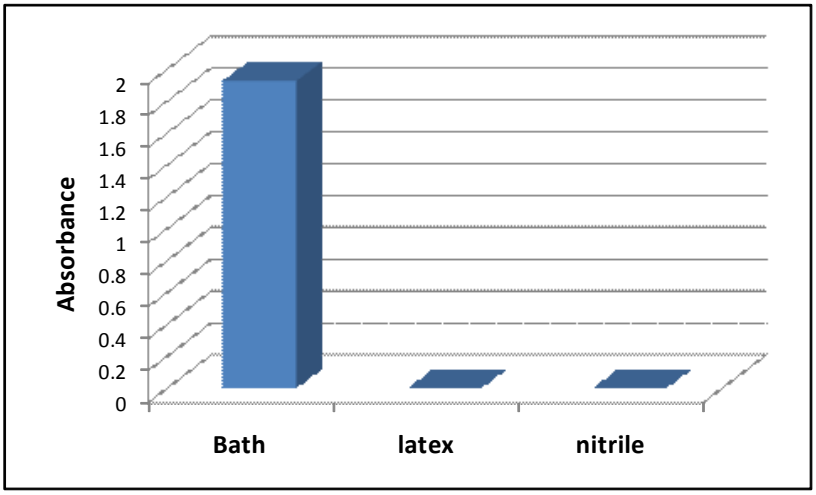


**Figure 1.** Absorbance values of the bath, water from the latex glove finger and water from the nitrile glove finger taken at EvaGreen® dye absorption maximum (471 nm) following 24 hours of dialysis.

# Cell Permeability Test

## Purpose

The purpose of this test is to see if EvaGreen® dye can cross cell membranes to stain nuclear DNA.

## Method

HeLa cells were incubated at 37 °C with EvaGreen® dye or SYBR® Green I at 1.2 μM dye concentration (*i.e.*, 1X concentration used for qPCR). Fluorescence images of the cells were taken following incubation for 5 and 30 minutes, respectively.

## Results

SYBR® Green I entered cells in as little as 5 minutes whereas no nuclear staining was observed for EvaGreen® dye even after 30 minutes of incubation. Image taken with prolonged photoexposure revealed that EvaGreen® dye mainly associated with the cell membranes.

## Conclusion

EvaGreen® dye is cell membrane impermeable whereas SYBR® Green I enters cell rapidly.

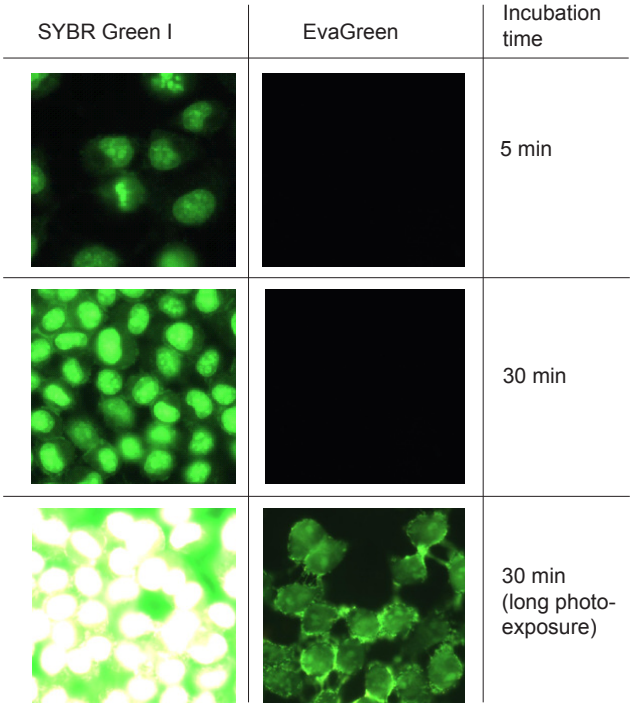


**Figure 2**. Comparison of cell membrane permeability between EvaGreen® dye and SYBR® Green I. HeLa cells were incubated with SYBR® Green I (1.2 μM) or EvaGreen® dye (1.2 μM) at 37 °C. Photographs were taken following incubation for 5 and 30 minutes. SYBR® Green I entered cells rapidly while EvaGreen® dye appeared membrane-impermeable as evident from the absence of cell nuclear staining. Image taken with long photo-exposure time revealed that EvaGreen® dye only associated with cell membranes. 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 ethidium bromide (Ohta et al. *Mutation Research*, **492**, 91-97(2001)).

# Ames Test

## Purpose

Ames test is a standard assay to assess the mutagenic potential of chemicals. As cancer is often associated with DNA damage, the test can be used to estimate the carcinogenic potential of a chemical compound.

## Test System

The test employed two Salmonella strains, TA98 and TA1537, both of which carry mutation(s) in the operon encoding for histidine biosynthesis. When these bacteria are exposed to mutagenic agents, under certain conditions reverse mutation from amino acid (histidine) auxotrophy to prototrophy occurs, giving colonies of revertants. Both strains of bacteria used in the assays are among those recommended by OECD 471 for use in the Ames test. These two strains of S. typhimurium have been shown to be reliably and reproducibly responsive between laboratories. In order to test the mutagenic toxicity of metabolized products, S9 fraction, a rat liver extract, was used in the assays. The S9 fraction contains a mixture of several enzymes and is known to be able to convert some chemicals into mutagens.

## Test Articles and Vehicle

EvaGreen® dye along with ethidium bromide (EB) as a reference were tested under the same condition. DMSO was used for dissolving each dye to give the following stock concentrations: 0 (control), 1, 2.5, 5, 10, 25, 50, 75, 100, 250 and 500 μg/mL.

## Test Procedure

The following was added to each sterile culture tube containing 2.0 mL top agar: 0.1 mL of overnight cell culture (TA98 or TA1537), 0.1 mL of a dye stock solution, and either 0.5 mL of S9/Cofactor mix or 0.5 mL of phosphate buffered saline. Thus, the control and the ten dye stock solutions result in the following per plate dosages: 0, 0.1, 0.25, 0.5, 1, 2.5, 5, 7.5, 10, 25, and 50 μg/plate. These dosages corresponded to a final dye concentration of: 0, 0.04, 0.09, 0.19, 0.37, 0.93, 1.85, 2.78, 3.7, 9.3, and 18.5 μg/mL, respectively.

The contents of each tube were vortexed, poured onto Vogel-Bonner media plates, and evenly distributed. The agar on the test plates was allowed to harden. The plates were inverted and incubated at 37 °C for 2 days.

Revertant colonies were counted using a New Brunswick Biotran III automatic colony counter.

## Ames Test Using *Salmonella* Strain TA98 without S9 Metabolic Activation

(Tests performed by Litron Laboratories Inc., Rochester, NY)

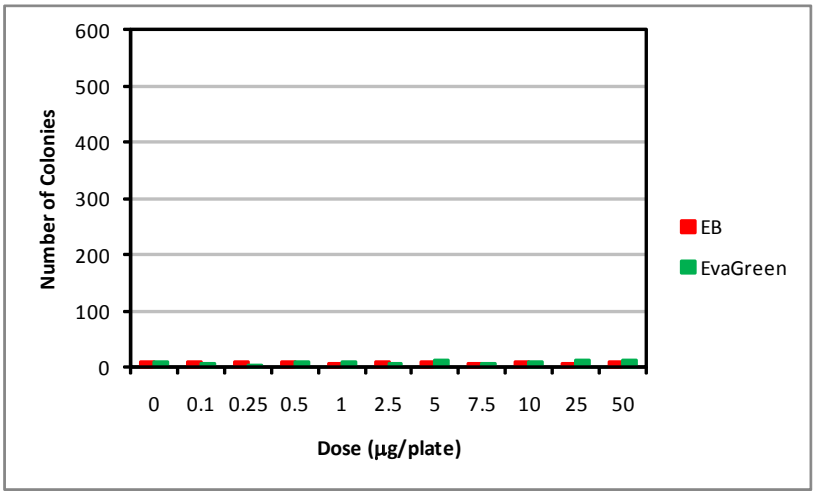


**Figure 3.** Comparison of mutagenicity between EvaGreen® dye and EB in +1 frameshift Salmonella indicator strain TA98 without the presence of S9 fraction.

## Conclusion

- In Salmonella strain TA98 bacteria and without S9 metabolic activation, EvaGreen® dye is nonmutagenic over the entire dose range from 0.1 µg/plate (or 40 ng/mL) to 50 µg/plate (or 18.5 µg/mL). The 1X concentration of EvaGreen® dye used in qPCR is about 1 µg/mL, which is well within this range.

- Under the same condition, EB is nonmutagenic at dosage up to 25 µg/plate but becomes weakly mutagenic at 50 µg/plate. The low genotoxicity of EB in the absence of S9 activity is consistent with an earlier report (McCann, et al. *Proc. Natl. Acad. Sci. USA* **72**, 5135)1975)).

## Ames Test Using *Salmonella* Strain TA98 with S9 Metabolic Activation

(Tests performed by Litron Laboratories Inc., Rochester, NY)

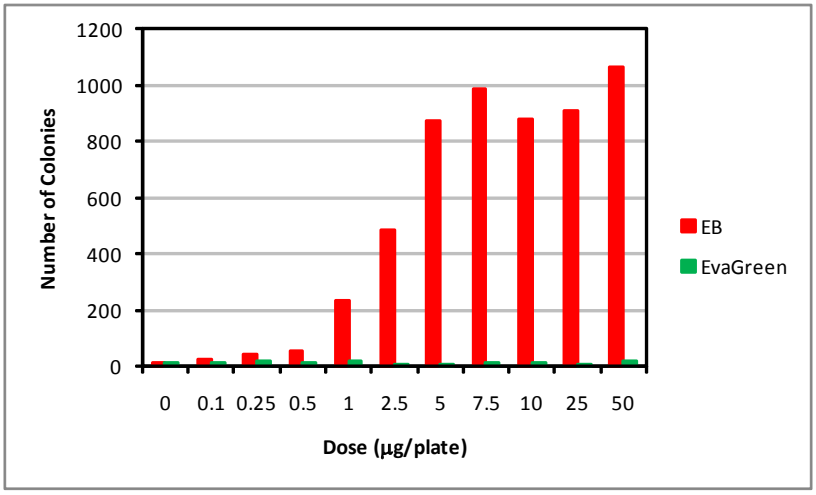


**Figure 4**. Comparison of mutagenicity between EvaGreen® dye and EB in +1 frameshift Salmonella indicator strain TA98 with the presence of S9 fraction.

## Conclusion

- In Salmonella strain TA98 bacteria and in the presence of S9 fraction, EvaGreen® dye is nonmutagenic over the entire dose range from 0.1 µg/plate (or 40 ng/mL) to 50 µg/plate (or 18.5 µg/mL). The 1X concentration of EvaGreen® dye used in qPCR is about 1 µg/mL, which is well within this range.

- Under the same condition, EB is highly mutagenic, consistent with the known genotoxicity of the dye.

## Ames Test Using *Salmonella* Strain TA1537 without S9 Metabolic Activation

(Tests performed by Litron Laboratories Inc., Rochester, NY)

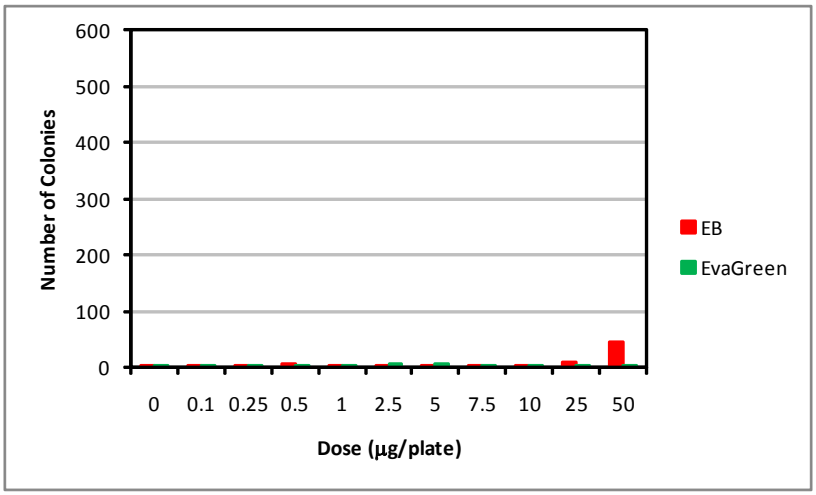


**Figure 5.** Comparison of mutagenicity between EvaGreen® and EB in -1 frameshift Salmonella indicator strain TA1537 without the presence of S9 fraction.

## Conclusion

- In Salmonella strain TA1537 bacteria and without S9 metabolic activation, EvaGreen® dye is nonmutagenic over the entire dose range from 0.1 µg/plate (or 40 ng/mL) to 50 µg/plate (or 18.5 µg/mL). The 1X concentration of EvaGreen® dye used in qPCR is about 1 µg/mL, which is well within this range.

- Under the same condition, EB is nonmutagenic at dosage up to 10 µg/plate but becomes weakly mutagenic at above 25 µg/plate.

## Ames Test Using *Salmonella* Strain TA1537 with S9 Metabolic Activation

(Tests performed by Litron Laboratories Inc., Rochester, NY)

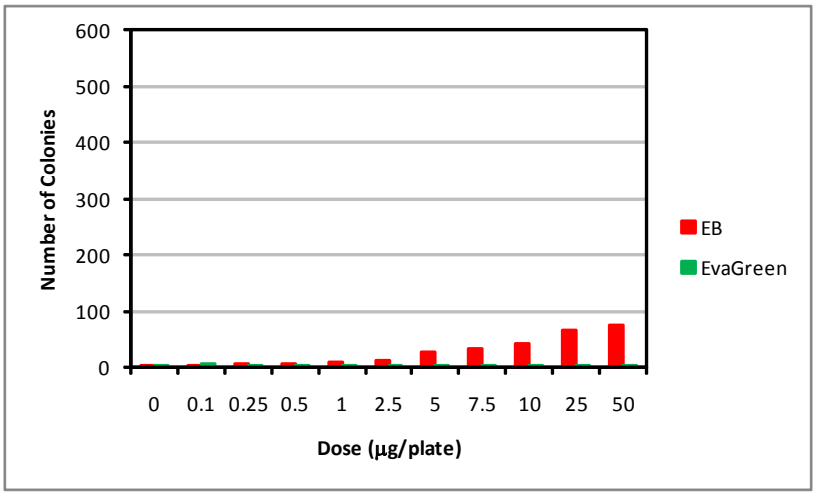


**Figure 6.** Comparison of mutagenicity between EvaGreen® and EB in Salmonella -1 frameshift indicator strain TA1537 with the presence of S9 fraction.

## Conclusion

- In Salmonella strain TA1537 bacteria and with S9 metabolic activation, EvaGreen® dye is nonmutagenic over the entire dose range from 0.1 µg/plate (or 40 ng/mL) to 50 µg/plate (or 18.5 µg/mL). The 1X concentration of EvaGreen® dye used in qPCR is about 1 µg/mL, which is well within this range.

- Under the same condition, EB becomes significantly mutagenic at above 2.5 µg/plate, consistent with the known genotoxicity of the dye.

# Aquatic Toxicity Test

(Performed by Nautilus Environmental, San Diego, CA)

## Purpose

This test assesses the acute toxicity of EvaGreen® dye to aquatic life. The results of the test are used to determine if the dye can be directly released into the environment for disposal as required by regulatory guideline CCR Title 22 in the state of California.

## Test Specifications

Test start date and time: 02/06/2009, 15:45

Test end date and time: 02/10/2009, 14:00

Test organism: *Pimephales promelas* (Fathead minnow)

Organism mean length/weight: 33.2 mm/0.20 g

Test concentration: 750, 500, and 250 mg/L sample (EvaGreen® dye at 2X); plus Lab Control

Number of replicates and fish: 2 replicates with 10 fish each (20 fish total per concentration)

Method used: California Department of Fish & Game, 1988 Acute Procedures; EPA/600/4-85/013, 1985 Acute Manual

Regulatory guideline: CCR Title 22 Hazardous Waste Characterization

Passing requirements: Sample must result in greater than 50% survival at a concentration of 500 mg/L ($LC_{50}$ > 500 mg/L) to be "not hazardous" to aquatic life.

## Results

The results are summarized in Table 2 below. The sample resulted in an $LC_{50}$ > 750 mg/L.

## Conclusion

EvaGreen® dye at 2X is classified as nonhazardous to aquatic life under CCR Title 22 regulation. Thus, EvaGreen® dye at 2X or lower concentrations can be safely released into the environment.

Table 2. Summary of EvaGreen® Dye Aquatic Toxicity Data

| Sample | Dose (mg/L) | % Survival |
|---|---|---|
| Lab Control | | 100 |
| EvaGreen® Dye | 250 | 100 |
| | 500 | 100 |
| | 750 | 100 |