Summary of Mutagenic Toxicity Test Results for EvaGreen™

Compiled by Biotium, Inc. from the results of an independent testing service:

Litron Laboratories, Inc., Rochester, NY

Biotium

Overview

When our scientists designed EvaGreen™ for quantitative real-time PCR (qPCR) use, sensitivity and stability were not their only objectives—safety of the dye was also their top priority. Because qPCR is now so widely practiced both in laboratory settings and in the fields, we thought that having a low-toxicity qPCR dye is important.

SYBR® Green I is a DNA-binding dye that has been widely used for a variety of applications including nucleic acid gel staining, DNA quantitation in solutions, staining of cellular DNA in live cells and qPCR. SYBR® Green I is an asymmetric cyanine dye having a delocalized positive charge and a relatively small molecular weight (Zipper, et al. *Nucleic Acids Res*. **32**(12), e103). Organic dyes with such structure features are generally known for their ability to be rapidly taken up by live cells. For example, numerous fluorescent mitochondrial dyes, membrane dyes and nucleic acid dyes designed for live cell staining all have a delocalized positive charge and a small molecular weight. The rapid cellular uptake of these dyes is probably due to the fact that the resting membrane potential for most of the cell types has a negative value, which should facilitate the cell membrane penetration of a positively-charged organic molecule with a small molecular size. In fact, the excellent membrane permeability of SYBR® Green I has made the dye a valuable tool for staining mitochondrial and nuclear DNA in a variety of live cells. On the other hand, this unique property of SYBR® Green I may also make the dye an unnecessary hazard for applications such as qPCR and nucleic acid gel staining, where membrane permeability of the dye is not required. Indeed, cytotoxicity and a small mutagenic effect of the dye have previously been reported (Singer, et al. *Mutat. Res*. **439**, 37(1999). Perhaps, even more importantly, Ohta, et al. showed that SYBR® Green I can strongly enhance the genotoxicity of UV-irradiation and chemical mutagens (Ohta, et al. *Mutat. Res*. **492**, 91(2001). It was proposed that SYBR® Green I might inhibit the nucleotide excision repair of DNA damaged by UV or a chemical treatment. The finding suggests that serious caution should be taken when handling SYBR® Green I under UV light, as in the case of gel staining, or when another potential mutagen is present.

So, how did we make EvaGreen™ a safer DNA dye without sacrificing other desirable properties? Before we began the project, we recognized that all DNA dyes are, by definition, potentially hazardous and are particularly so if they can easily enter cells. This is because intracellular DNA binding of the dyes may result in any number of harmful effects such as DNA mutation, interference of DNA transcription and inhibition of the DNA repair mechanism in cells. We reasoned that, since cell membrane-permeability is not required for a qPCR dye, we might be able to improve the dye safety by making the dye less likely to enter a cell. Thus, as a first line of defense, we built an innovative structure feature into EvaGreen™ so that the dye not only has improved PCR performance but also becomes extremely difficult to cross cell membranes. Additionally, we also recognized that once a DNA dye enters a cell it may be subject to metabolism, which may convert the dye into a chemical that could be either more mutagenic (as in the case of ethidium bromide) or less mutagenic than the unmetabolized dye. Thus, as a second line of defense, our chemists incorporated into the structure of EvaGreen™ chemical bonds at strategic positions so that on enzymatic cleavage the products will become very weak DNA-binding molecules. We believe that these unique structure features of EvaGreen™ are at least partially responsible for the observed low mutagenicity and low cytotoxicity of the dye.

Using standard Ames test, as measured in two bacterial strains, EvaGreen™ is confirmed to be essentially nonmutagenic. EvaGreen™ was not mutagenic at all 10 different doses ranging from 0.1 μg/plate (or 0.037 μg/mL) to 50 μg/plate (or 18.5 μg/mL) in the presence or absence of a mammalian S9 fraction. On the other hand, SYBR® Green I showed a weak dose-dependent mutagenic response in strain TA98 with the absence of S9 fraction, and consistently displayed cytotoxicity at higher dye concentrations in both bacterial strains with or without S9 metabolic activation. We believe that these data, coupled with its low membrane permeability, should make EvaGreen™ a safer alternative to SYBR® Green I for PCR application.

This document is intended to provide a brief summary of the safety data on EvaGreen™ obtained by Litron Laboratories, an independent toxicity test service in Rochester, New York. For more detailed information, please download the original test reports at our website: www.biotium.com.

Test System Description

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 Description

EvaGreen™ and SYBR® Green I along with ethidium bromide (EB) as a reference were tested under the same conditions. 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 each dye concentration for each dye or control chemical, and either 0.5 mL of S9/Cofactor mix or 0.5 mL of phosphate buffered saline. By using the above 10 stock solutions for each dye plus the control, the following per plate doses for each dye were used: 0, 0.1, 0.25, 0.5, 1, 2.5, 5, 7.5, 10, 25, and 50 μg/plate. These doses 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.

Mutagenicity Tests in Salmonella Strain TA98 without S9 Metabolic Activation

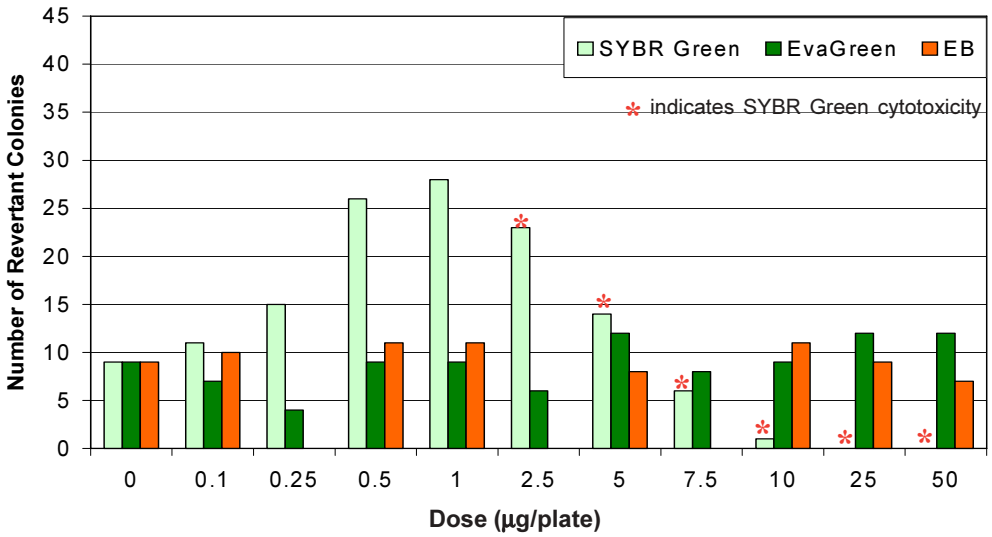

Figure 1. Comparison of mutagenicity of EvaGreen™, SYBR® Green I and EB in +1 frameshift indicator strain TA98 without the presence of S9 fraction. Tests were performed by Litron Laboratories Inc., Rochester, NY.

Conclusion

- EvaGreen™ is not mutagenic over the dose range in +1 frameshift indicator strain TA98 without S9 metabolic activation.
- SYBR® Green I shows weak dose-dependent mutagenic response at up to 1 µg/plate (or 0.37 µg/mL) and becomes cytotoxic thereafter, consistent with earlier reports (Singer, et al. *Mutat. Res.* **439**, 37(1999)).
- Ethidium bromide (EB) is not mutagenic without S9 metabolic activation, consistent with earlier reports (McCann, et al. *Proc. Natl. Acad. Sci. USA* **72**, 5135)1975)).

Mutagenicity Tests in Salmonella Strain TA98 with S9 Metabolic Activation

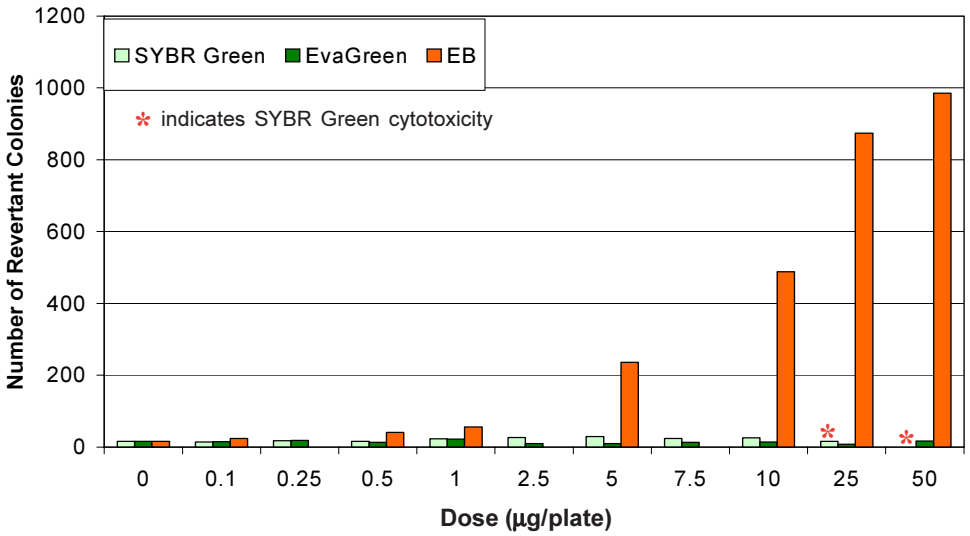

Figure 2. Comparison of mutagenicity of EvaGreen™, SYBR® Green I and EB in +1 frameshift indicator strain TA98 with the presence of S9 fraction. Tests were performed by Litron Laboratories Inc., Rochester, NY.

Conclusion

- EvaGreen™ is not mutagenic over the dose range in +1 frameshift indicator stain TA98 with S9 metabolic activation.
- SYBR® Green I is not mutagenic, but becomes cytotoxic at higher doses (≥ 25 µg/plate or 9.3 µg/mL) when S9 fraction was present.
- EB is highly mutagenic with S9 metabolic activation, consistent with the known toxicity of the dye.

Mutagenicity Tests in Salmonella Strain TA1537 without S9 Metabolic Activation

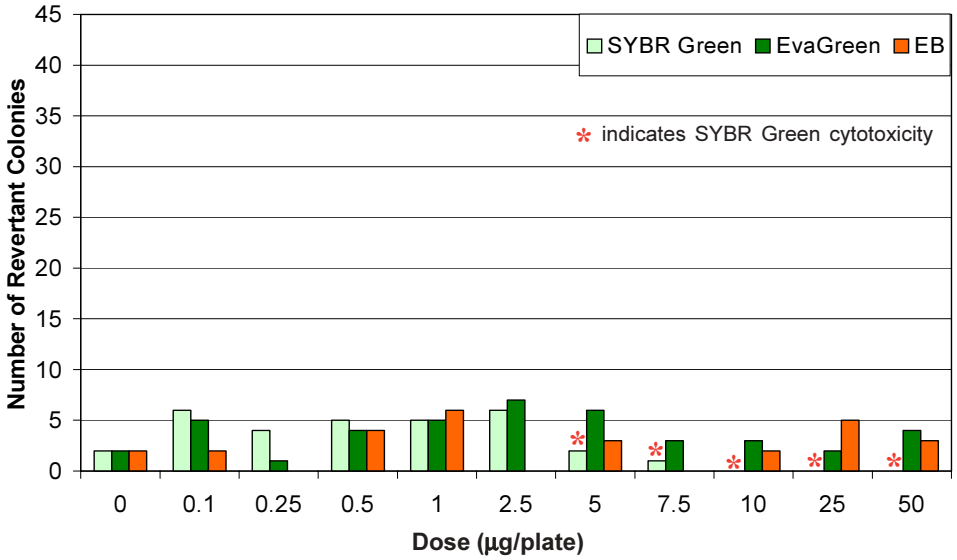

Figure 3. Comparison of mutagenicity of EvaGreen™, SYBR® Green I and EB in -1 frameshift indicator strain TA1537 without the presence of S9 fraction. Tests were performed by Litron Laboratories Inc., Rochester, NY.

Conclusion

- EvaGreen™ is not mutagenic over the dose range in -1 frameshift indicator strain TA1537 without S9 metabolic activation.
- SYBR® Green I is not mutagenic, but becomes cytotoxic at higher doses (≥ 2.5 µg/plate or 0.93 µg/mL) without S9 metabolic activation.
- EB is not mutagenic over the dose range in -1 frameshift indicator strain TA1537 without S9 metabolic activation.

Mutagenicity Tests in Salmonella Strain TA1537 with S9 Metabolic Activation

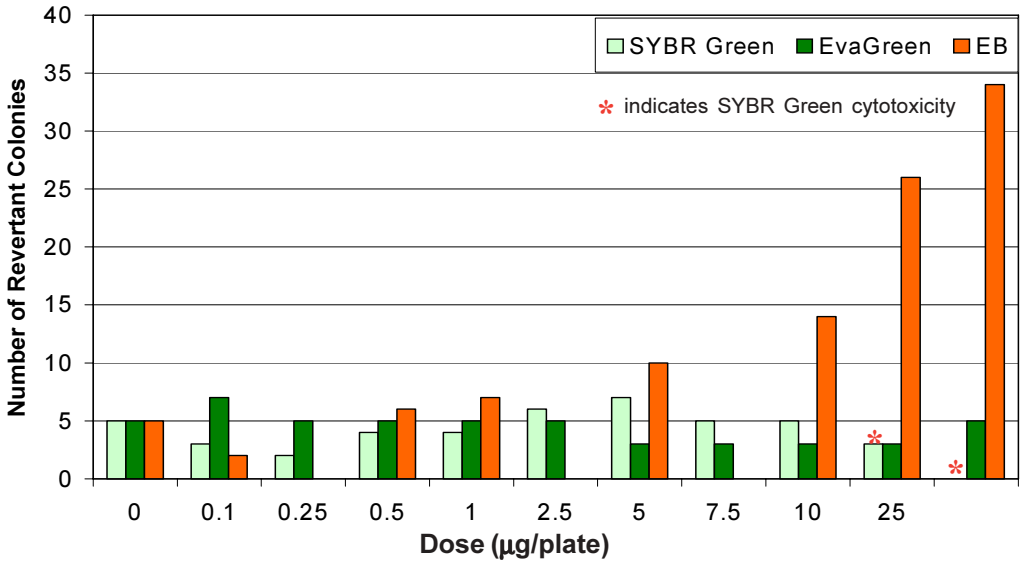

Figure 4. Comparison of mutagenicity of EvaGreen™, SYBR® Green I and EB in -1 frameshift indicator strain TA1537 with the presence of S9 fraction. Tests were performed by Litron Laboratories Inc., Rochester, NY.

Conclusion

- EvaGreen™ is not mutagenic over the dose range in -1 frameshift indicator strain TA1537 with S9 metabolic activation.
- SYBR® Green I is not mutagenic, but becomes cytotoxic at higher doses (≥ 25 µg/plate or 9.3 µg/mL) when S9 fraction was present.
- EB is mutagenic with S9 metabolic activation, consistent with the known toxicity of the dye.