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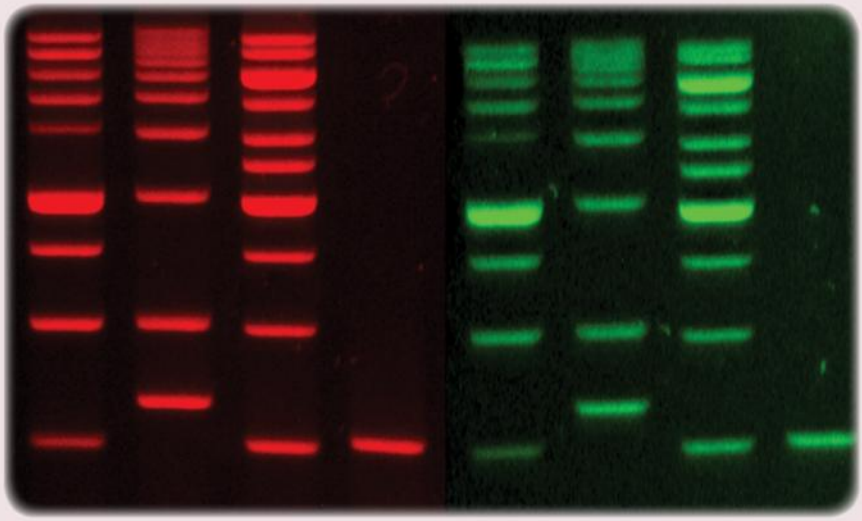
Safety Report of GelRed™ and GelGreen™



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

GelRed™/GelGreen™ Safety Report

Procedure for use with Gold Biotechnology GelRed™ and GelGreen™ Nucleic Acid Gel Stain
Catalog #: G-720; G-725; G-740; G-745

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GelRed™/GelGreen™ Safety Report

Overview

Ethidium bromide (EtBr) has been the stain of choice for nucleic acid gel staining for decades. The dye is inexpensive, sufficiently sensitive and very stable. However, EtBr is also a known powerful mutagen. It poses a major health hazard to the user, and efforts in decontamination and waste disposal ultimately make the dye expensive to use. To overcome the toxicity problem of EtBr, scientists developed GelRed™ and GelGreen™ nucleic acid gel stains as superior alternatives. Extensive tests demonstrate that both dyes have significantly improved safety profiles over EtBr.

Dye Design Principle

At the very beginning of GelRed™ and GelGreen™ development, we made a fundamental recognition that an important way to make a gel stain safe is to eliminate or minimize the chance for the dye to interact with genomic DNA in living cells. Based on this design principle, chemists incorporated structural features into the dyes to achieve maximal protection on three fronts: 1) to make the dyes impenetrable to latex gloves; 2) to make the dyes impenetrable to cell membranes; and 3) to make the dyes metabolizable to form compounds that have no or minimal interaction with DNA.

Safety Tests

GelRed™ and GelGreen™ were subjected to a series of tests both by the manufacturer and by three independent testing services to assess the dyes' safety for routine handling and disposal. These tests include:
1) glove penetration test; 2) cell membrane permeability and cytotoxicity test; 3) Ames test; and 4) environmental safety tests. Results of the tests are summarized in Table 1 below. The data show that GelRed™ and GelGreen™ have passed all of the tests, thus validating the dye design principle. Detailed test results are described on pages 5-14.

Conclusion

GelRed™ and GelGreen™ are a new generation of nucleic acid gel stains. They possess novel chemical features designed to minimize the chance for the dyes to interact with nucleic acids in living cells. Test results confirm that the dyes do not penetrate latex gloves or cell membranes.

In the AMES test, GelRed™ and GelGreen™ are noncytotoxic and nonmutagenic at concentrations well above the working concentrations used in gel staining. The highest dye concentrations shown to be non-toxic and non-mutagenic in the Ames test for GelRed™ and GelGreen™ dyes are 18.5-times higher than the 1X working concentration used for gel casting, and 6-times higher than the 3X working concentration used for gel staining. In manufacturer publications, SYBR® Safe has been reported to show mutagenicity in several strains in the presence of S9 mix[1]. SYBR® Safe was reported to be non-mutagenic in Syrian hamster embryo (SHE) cells and L5178YTK +/- mouse lymphoma cells[2]. However, in these assays mutagenicity was only tested for concentrations of SYBR Safe below its 1X working concentration of 0.66 µg/ml. This is because excessive toxicity was observed at concentrations above 0.333 µg/ml in SHE cells and above 0.25 µg/ml in L5178YTK +/- mouse lymphoma cells[1, 3]. These results are consistent with the observation that SYBR® Safe rapidly penetrates cell membranes and stains the cytoplasm and nucleus of live cells (see p. 6).

Furthermore, GelRed™ and GelGreen™ have successfully passed environmental safety tests in compliance with CCR Title 22 Hazardous Waste Characterization. As a result, GelRed™ and GelGreen™ are not classified as hazardous waste, thus can be safely disposed of down the drain or as regular trash, providing convenience and reducing cost in waste disposal.

References

1. Report: SYBR Safe DNA Gel Stain, Assessment of Mutagenicity and Environmental Toxicity. http://probes.invitrogen.com/media/publications/494.pdf

2. Beaudet, MP, Hendrickson, JE, Ruth, JL. Safety testing of SYBR Safe, a non-hazardous alternative to ethidium bromide. http://probes.invitrogen.com/media/publications/519.pdf

3. The working concentration of SYBR Safe was calculated using the absorbance of the 1X solution, the extinction coefficient for SYBR dyes (70,000) and the molecular weight of SYBR Safe reported in reference 4 (MW 505).

4. Evenson, WE, Boden, LM, Muzikar, KA, and O'Leary, DJ. 1H and 13C NMR Assignments for the Cyanine Dyes SYBR Safe and Thiazole Orange. The Journal of Organic Chemistry 2012 77: 10967-10971.

Table 1. Summary of GelRed™ and GelGreen™ Safety Test Results

	Latex Glove Penetration	Cell Staining		Ames Test	Hazardous Waste Screening (aquatic toxicity test)	Reactivity test	Corrosivity test	Ignitability test
		Cell Membrane Permeability	Cytotoxity					
GelRed™	Impermeable	Impermeable	Nontoxic	Nonmutagenic	Nontoxic to aquatic life	Unreactive	Noncorrosive	Nonflammable
GelGreen™	Impermeable	Impermeable	Nontoxic	Nonmutagenic	Nontoxic to aquatic life	Unreactive	Noncorrosive	Nonflammable

This document is intended to provide a brief summary of the safety data on GelRed™ and GelGreen™ obtained from several laboratories.

Glove Penetration Test

Purpose

Latex gloves are commonly worn by researchers in laboratories as protective gear. Thus, it is important to show GelRed™ and GelGreen™ do not diffuse through the latex material.

Method

A finger of a latex glove containing TAE buffer was dialyzed against TAE buffer containing 5X GelRed™ or GelGreen™ for 48 hours. The solution in the finger was then analyzed for presence of the dye by fluorescence. As a reference, the fluorescence of the dye at 1X was also measured. To increase the sensitivity of the detection, all fluorescence measurements were made in the presence of 100 µg/ml salmon sperm dsDNA.

Results

The results of the test show that both GelRed™ and GelGreen™ do not penetrate latex gloves (Figure 1).

Conclusion

Latex gloves provide an effective barrier to GelRed™ and GelGreen™.

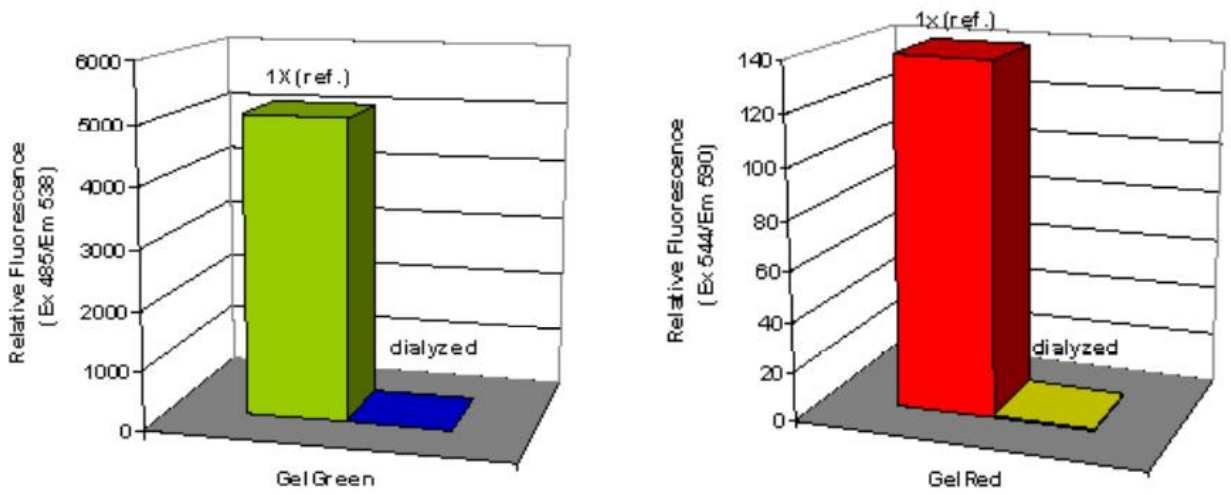

Figure 1. Relative fluorescence of solutions dialyzed in latex glove fingers against 5X GelGreen™ (blue) or 5X GelRed™ (yellow) and the relative fluorescence of the corresponding 1X dye solution as a reference. The data show that the amount of the fluorescence for the dialyzed solutions is negligible, suggesting that neither dye penetrates latex gloves at 5X concentration.

Cell Staining Test

Purpose
The purpose of this test is to see if GelRed™ and GelGreen™ can cross cell membranes to stain nuclear DNA.

Method
Hela cells were incubated at 37°C with GelRed™, GelGreen™, SYBR Safe, and SYBR Green I, respectively. The dye concentrations were all 1X based on the respective dye concentrations used for gel staining for each dye. The SYBR dyes were used as controls as they are known to be able to stain DNA in live cells. Cell staining was followed by fluorescence microscopy using optical filter sets appropriate for each dye.

Results
Microscopic images obtained following 5 and 30 minutes of incubation are shown in Figure 2. SYBR Safe and SYBR Green stained cell cytoplasm and nuclear DNA with bright green fluorescence in only a few minutes. GelRed™ or GelGreen™ did not stain live cells even after 30 minutes of incubation.

Conclusion
GelRed™ and GelGreen™ do not penetrate cell membranes.

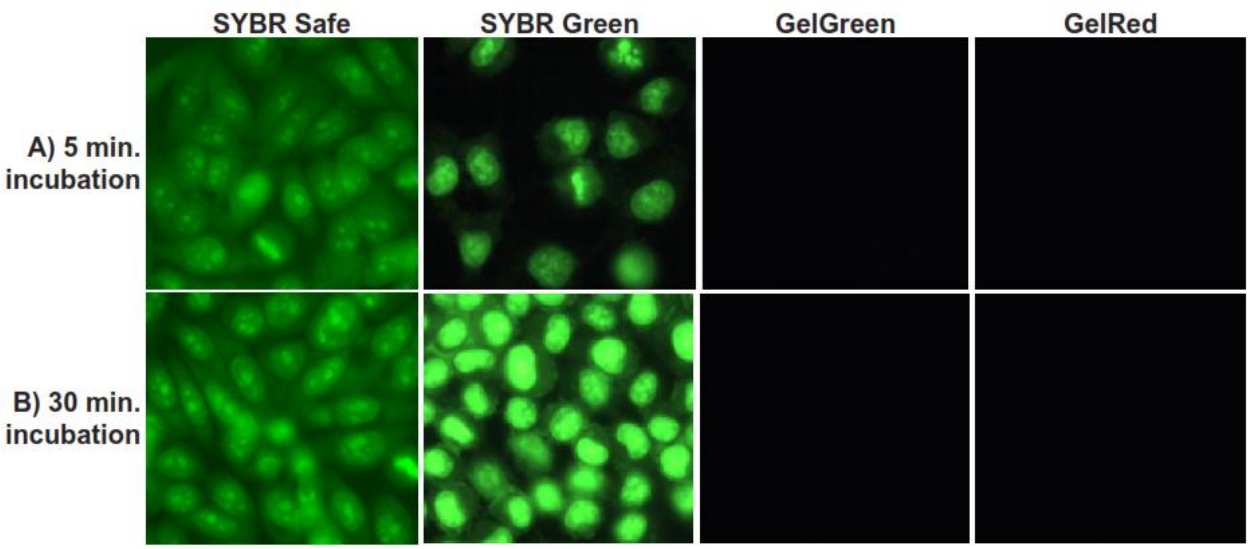

Figure 2. HeLa cells were incubated at 37°C with 1X of SYBR Safe, SYBR Green I, GelGreen™ and GelRed™, respectively. Images were taken following incubation for 5 min (panel A) and 30 min (panel B), respectively. SYBR Safe and SYBR Green entered into cells rapidly as evident from the bright green staining. However, GelRed™ and GelGreen™ were unable to cross cell membranes as shown by the absence of fluorescence staining.

Ames Test

Purpose

The 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 coding 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

GelRed™ and GelGreen™ along with ethidium bromide (EtBr) 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 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 dosages 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 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 VogelBonner 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.

Results from Ames Test Using Salmonella Strain TA98 without S9 Metabolic Activation

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

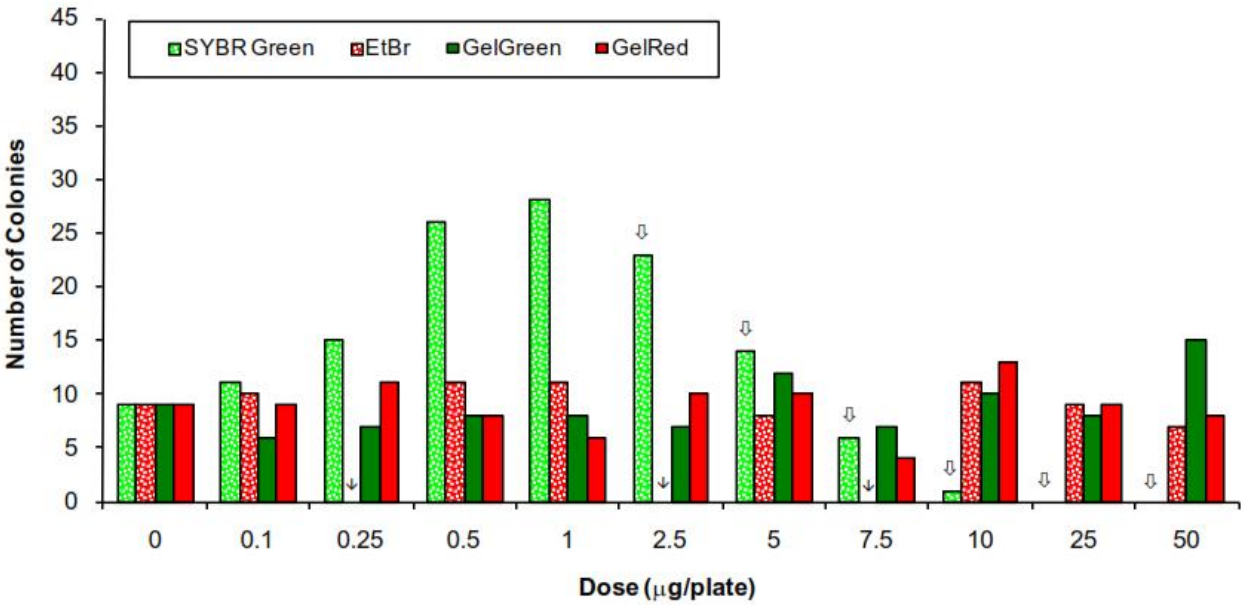

Figure 3. Comparison of mutagenicity among GelGreen™, GelRed™, SYBR Green I and EtBr in +1 frameshift Salmonella indicator strain TA98 without the presence of S9 fraction. "↓" indicates EtBr was not tested at this concentration. "⇩" indicates SYBR Green I became cytotoxic at this concentration.

Conclusion

GelGreen™ and GelRed™ are nonmutagenic over the dose range from 0.1 µg/plate (or 40 ng/ml) to 50 µg/plate (or 18.5 µg/ml) in +1 frameshift Salmonella indicator strain TA98 without S9 metabolic activation. The working concentration used in gel staining for both GelRed™ and GelGreen™ is 1-3 µg/ml (1X-3X), which is well within the safety range.

EtBr is nonmutagenic without S9 metabolic activation, consistent with an earlier report McCann, et al. Proc. Natl. Acad. Sci. USA 72, 5135) (1975)).

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 an earlier report (Singer, et al. Mutat. Res. 439, 37(1999)).

Results from Ames Test Using Salmonella Strain TA98 with S9 Metabolic Activation

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

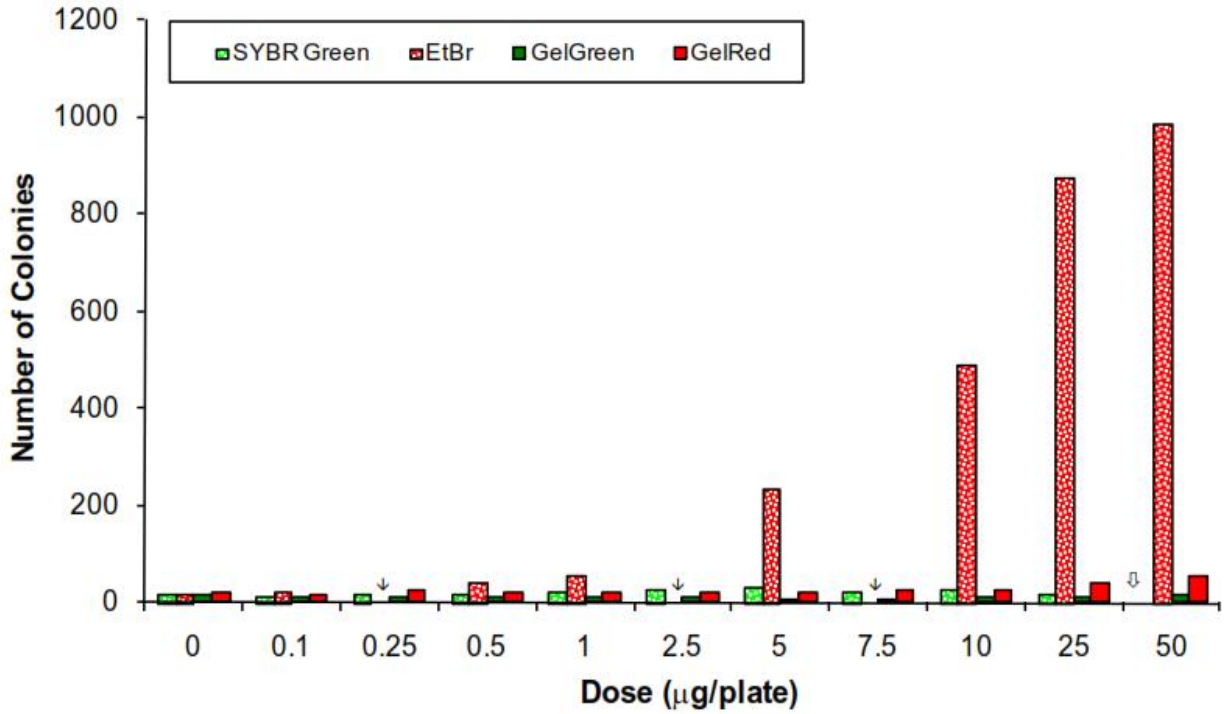

Figure 4. Comparison of mutagenicity among GelGreen™, GelRed™, SYBR Green I and EtBr in +1 frameshift Salmonella indicator strain TA98 with the presence of S9 fraction. "↓" indicates EtBr was not tested at this concentration. "⇩" indicates SYBR Green I became cytotoxic at this concentration.

Conclusion

GelGreen™ is nonmutagenic over the dose range from 0.1 µg/plate (or 40 ng/ml) to 50 µg/plate (or 18.5 µg/ml) in +1 frameshift Salmonella indicator strain TA98 with S9 metabolic activation. GelGreen™ working concentration used in gel staining is 1-3 µg/ml (1X-3X), which is well within the safety range.

GelRed™ is only weakly mutagenic at very high dose (50 µg/plate or 18.5 µg/ml) with S9 metabolic activation. GelRed™ working concentration used in gel staining is 1-3 µg/ml (1X-3X), which is well within the safety range.

SYBR Green I is nonmutagenic at lower concentrations (0.1-25 µg/plate or 0.04-9.3 µg/ml), but becomes cytotoxic at higher concentrations (≥25 µg/plate or 9.3 µg/ml), consistent with an earlier report (Singer, et al. Mutat. Res. 439, 37(1999)).

EtBr is highly mutagenic with S9 metabolic activation, consistent with the known toxicity of the dye.

Results from Ames Test Using Salmonella Strain TA1537 without S9 Metabolic Activation

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

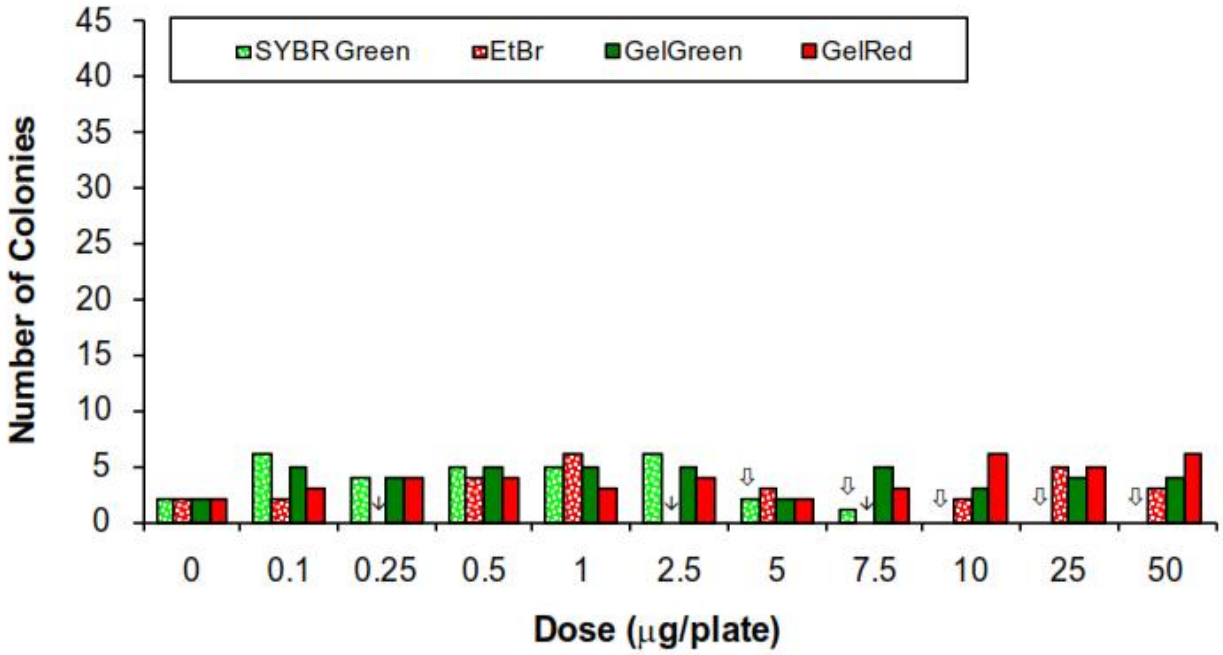

Figure 5. Comparison of mutagenicity among GelGreen™, GelRed™, SYBR Green I and EtBr in -1 frameshift Salmonella indicator strain TA1537 without the presence of S9 fraction. "↓" indicates EtBr was not tested at this concentration. "⇩" indicates SYBR Green I became cytotoxic at this concentration.

Conclusion

GelGreen™ and GelRed™ are nonmutagenic over the dose range from 0.1 µg/plate (or 40 ng/ml) to 50 µg/plate (or 18.5 µg/ml) in -1 frameshift Salmonella indicator strain TA1537 without S9 metabolic activation. The working concentration used in gel staining for both GelRed™ and GelGreen™ is 1-3 µg/ml (1X-3X), which is well within the safety range.

SYBR Green is nonmutagenic at lower concentrations (0.1-2.5 µg/plate or 0.04-0.93 µg/ml), but becomes cytotoxic at higher concentrations (≥2.5 µg/plate or 0.93 µg/ml), consistent with an earlier report (Singer, et al. Mutat. Res. 439, 37(1999)).

EtBr is non mutagenic without S9 metabolic activation, consistent with an earlier report (McCann, et al. Proc. Natl. Acad. Sci. USA 72, 5135) (1975)).

Results from Ames Test Using Salmonella Strain TA1537 with S9 Metabolic Activation

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

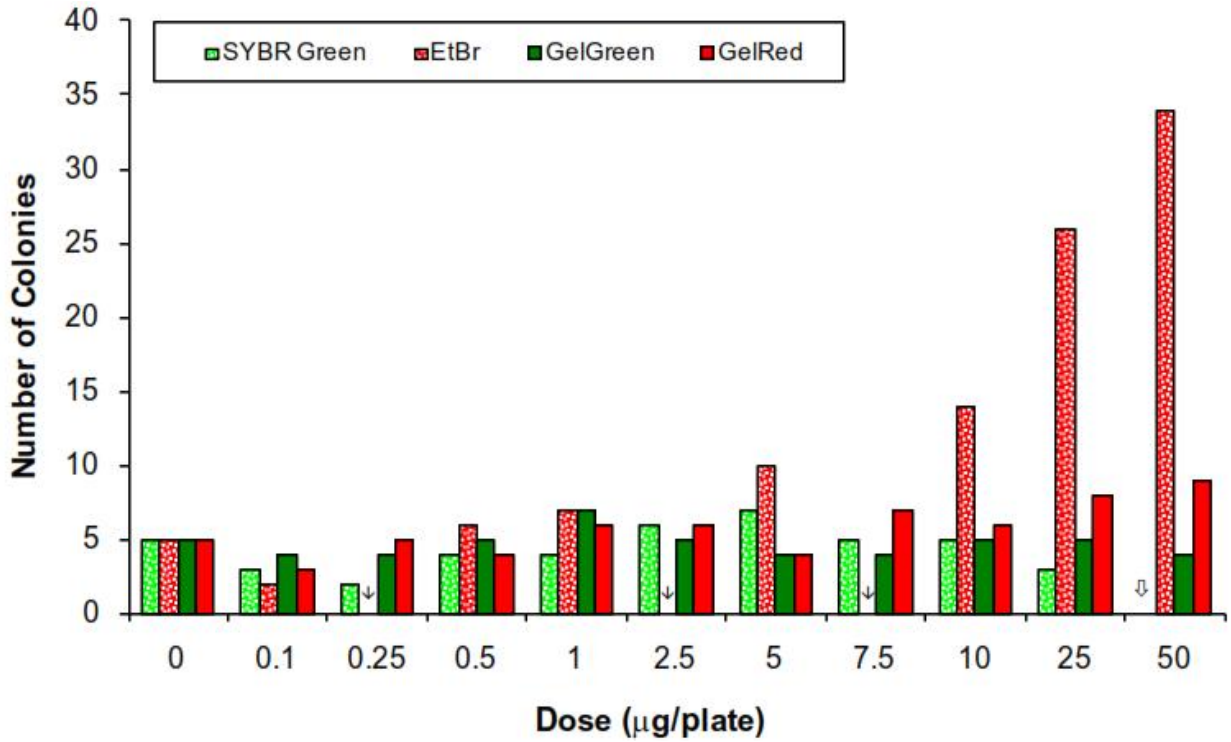

Figure 6. Comparison of mutagenicity among GelGreen™, GelRed™, SYBR Green I and EtBr in -1 frameshift Salmonella indicator strain TA1537 with the presence of S9 fraction. "↓" indicates EtBr was not tested at this concentration. "⇩" indicates SYBR Green I became cytotoxic at this concentration.

Conclusion

GelGreen™ and GelRed™ are nonmutagenic over the dose range from 0.1 µg/plate (or 40 ng/ml) to 50 µg/plate (or 18.5 µg/ml) in -1 Salmonella frameshift indicator strain TA1537 without S9 metabolic activation. The working concentration used in gel staining for both GelRed™ and GelGreen™ is 1-3 µg/ml (1X-3X), which is well within the safety range.

EtBr is highly mutagenic with S9 metabolic activation, consistent with the known toxicity of the dye.

SYBR Green is nonmutagenic at lower concentrations (0.1-25 µg/plate or 0.04-9.3 µg/ml), but becomes cytotoxic at higher concentrations (≥25 µg/plate or 9.3 µg/ml), consistent with an earlier report (Singer, et al. Mutat. Res. 439, 37(1999)).

Aquatic Toxicity Test

(Tests performed by Nautilus Environmental, San Diego, CA)

Purpose
This test assesses the acute toxicity of GelRed™ and GelGreen™ to aquatic life. The results of the test are used to determine if the dyes can be directly released into the environment for disposal.

Test Specifications
Test start date and time: 4/7/08, 09:30
Test end date and time: 4/11/08, 08:45
Test organism: *Pimephales promelas* (Fathead minnow)
Organism mean length/weight: 34 mm/0.34 g
Test concentration: 750, 500, and 250 mg/L sample (GelRed™ or GelGreen™ at 3X); 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 guidelines: CCR Title 22 Hazardous Waste Characterization
Passing requirements: Sample must result in greater than 50% survival at a concentration of 500 mg/L (LC > 500 mg/L) to be "not hazardous" to aquatic life.

Results
The results are summarized in Table 2 below. Both samples gave LC50 >750 mg/L.

Conclusion

Both GelRed™ and GelGreen™ at 3X are classified as nonhazardous to aquatic life, under CCR Title 22 regulation. Thus, GelRed™ and GelGreen™ at 3X or lower concentrations can be safely released into the environment.

Table 2. Summary of GelGreen™ and GelRed™ Aquatic Toxicity Test Results

Sample	Dose (mg/L)	% Survival
Lab Control		95
GelRed™	250	100
	500	100
	750	100
GelGreen™	250	100
	500	100
	750	100

Corrosivity, Reactivity and Ignitability Tests

(Tests performed by Curtis & Tompkins, Ltd., Analytical Laboratories, Berkeley, CA)

Purpose

The corrosivity, reactivity and ignitability of GelRed™ and GelGreen™ solutions are tested. These tests are designed to further assess the environmental safety of GelRed™ and GelGreen™ and safety associated with the shipping, handling and storage of the dyes.

Methods

All tests were conducted according to EPA guidelines or ASTM guideline as specified in the result table below.

Results

Results of the tests are summarized in Table 3 below.

Conclusion

Based on these results, GelRed™ and GelGreen™ at 3X or lower concentrations are classified as non-corrosive and non-hazardous materials.

Table 3. Summary of Environmental Safety Test Results

Test Name (test code)	GelGreen™, 3X	GelRed™, 3X
Reactive cyanide (SW-846 CH.7)	None detected	None detected
Reactive Sulfide (SW-846 CH.7)	None detected	None detected
pH (EPA 9040C)	4	5.3
Flash Point (ASTM D-93)	>150°F	>150°F