

Safety Report of GelRed and GelGreen

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

Overview

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

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 at Biotium 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 us 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 3-11.

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 are impenetrable to both latex gloves and cell membranes. The dyes are noncytotoxic and nonmutagenic at concentrations well above the working concentrations used in gel staining. 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.

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	Cytotoxicity					
GelRed	impenetrable	impenetrable	noncytotoxic	nonmutagenic	nontoxic to aquatic life	unreactive	noncorrosive	nonflammable
GelGreen	impenetrable	impenetrable	noncytotoxic	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. If you wish to see the original test reports, you may contact Biotium Technical Support.

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 ug/mL salmon sperm dsDNA.

Results

The results of the test show that both GelRed and GelGreen are impenetrable to latex gloves (Figure 1).

Conclusion

Handling GelRed and GelGreen with latex gloves is safe.

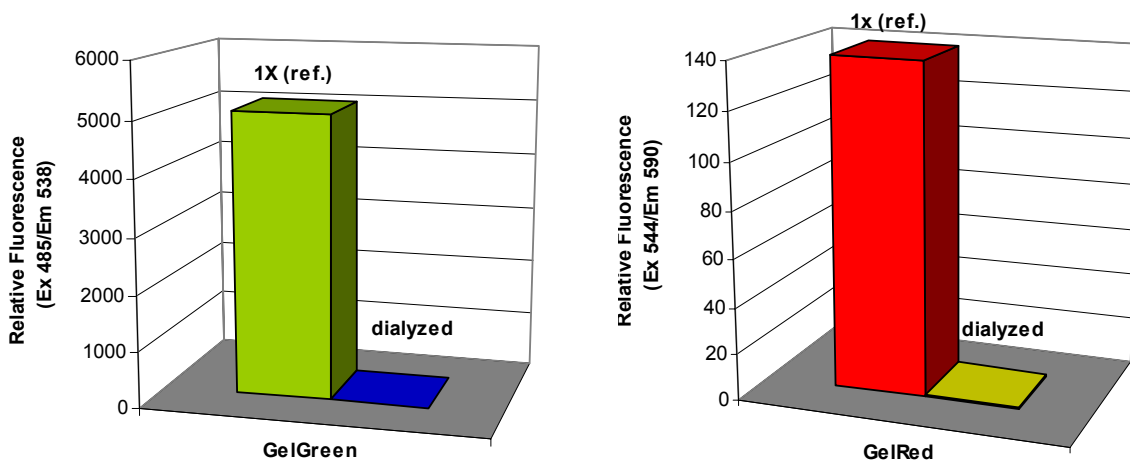


Figure 1. Relative fluorescence of dialyzed solutions for GelGreen (blue) and GelRed (yellow) and the relative fluorescence of the corresponding 1X dye solutions as references. The data show that the amount of the fluorescence for the dialyzed solutions are negligible, suggesting that both dyes can not penetrate latex gloves.

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 nuclear DNA brightly green in only a few minutes (only SYBR Green images are shown). GelRed or GelGreen did not stain cells even following 30 minutes of incubation.

Conclusion

GelRed and GelGreen are impenetrable to cell membranes.

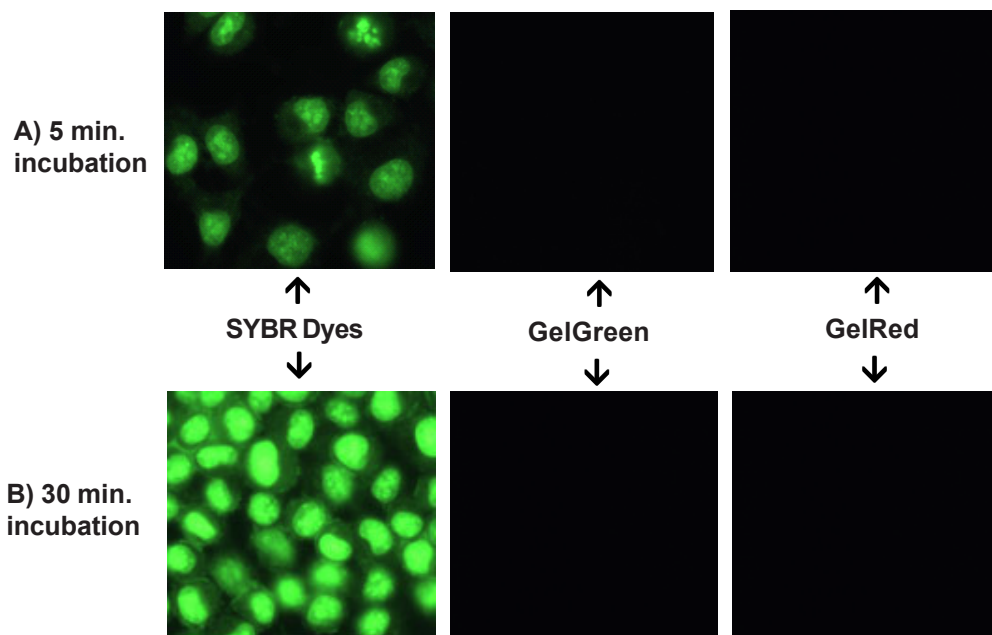


Figure 2. HeLa cells were incubated at 37 °C with 1X of SYBR Green I, SYBR Safe, GelGreen and GelRed, respectively. Images were taken following incubation for 5 min (panel A) and 30 min (panel B), respectively. SYBR Green I and SYBR Safe entered into cells rapidly as evident from the bright green nuclear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

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

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

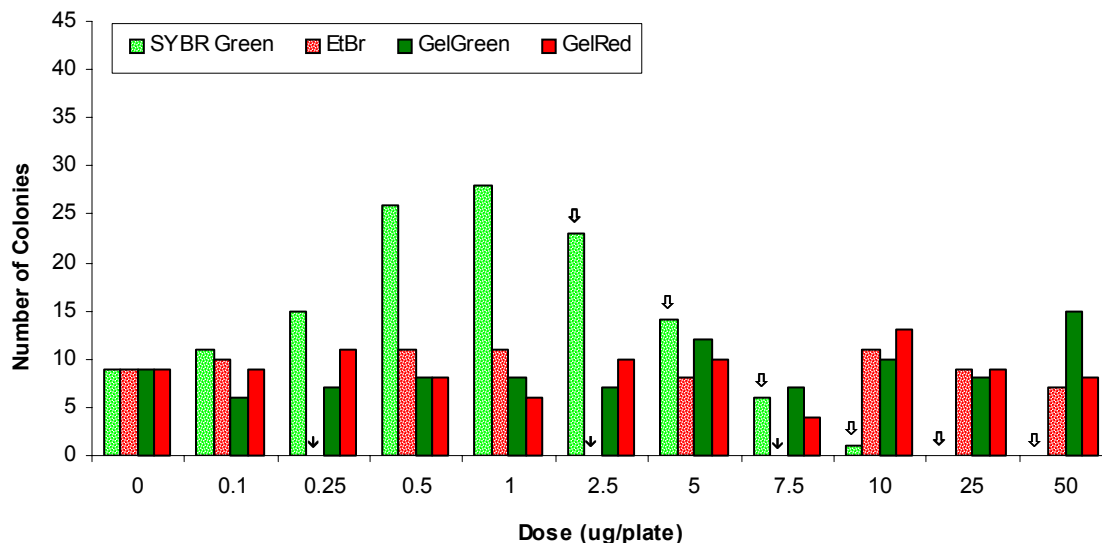


Figure 3. Comparison of mutagenicity among GelGreen™, GelRed™, SYBR® Green I and EB in +1 frameshift *Salmonella* indicator strain TA98 without the presence of S9 fraction. "↓" indicates EB was not tested at this concentration. "∩" indicates SYBR® Green I became cytotoxic at this concentration.

Conclusion

- GelGreen and GelRed are nonmutagenic over the dose range from 0.1 ug/plate (or 40 ng/mL) to 50 ug/plate (or 18.5 ug/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 ug/mL (1X-3X), which is well within the safety range.*
- EB is nonmutagenic without S9 metabolic activation, consistent with and 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 ug/plate (or 0.37 ug/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)

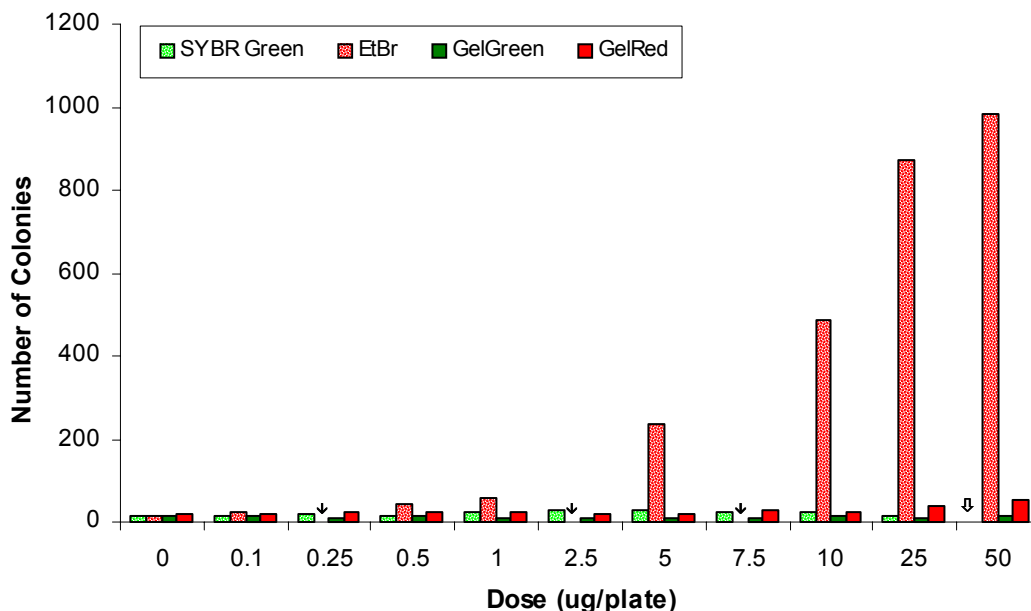


Figure 4. Comparison of mutagenicity among GelGreen™, GelRed™, SYBR® Green I and EB in +1 frameshift *Salmonella* indicator strain TA98 with the presence of S9 fraction. "↓" indicates EB was not tested at this concentration. "⚡" indicates SYBR® Green I became cytotoxic at this concentration.

Conclusion

- GelGreen is nonmutagenic over the dose range from 0.1 ug/plate (or 40 ng/mL) to 50 ug/plate (or 18.5 ug/mL) in +1 frameshift *Salmonella* indicator strain TA98 with S9 metabolic activation. *GelGreen* working concentration used in gel staining is 1-3 ug/mL (1X-3X), which is well within the safety range.
- GelRed is only weakly mutagenic at very high dose (50 ug/plate or 18.5 ug/mL) with S9 metabolic activation. *GelRed* working concentration used in gel staining is 1-3 ug/mL (1X-3X), which is well within the safety range.
- SYBR Green I is nonmutagenic at lower concentrations (0.1-25 ug/plate or 0.04-9.3 ug/mL), but becomes cytotoxic at higher concentrations (≥ 25 ug/plate or 9.3 ug/mL), consistent with an earlier report (Singer, et al. *Mutat. Res.* **439**, 37(1999))
- EB 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)

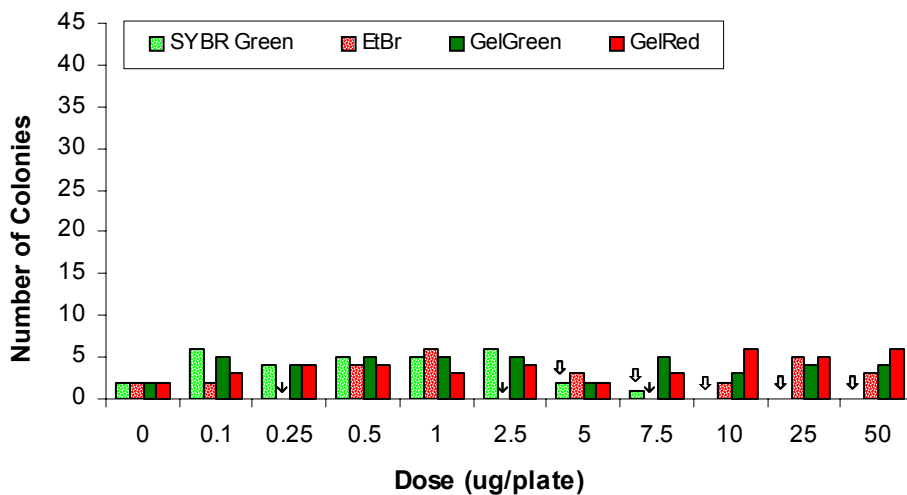


Figure 5. Comparison of mutagenicity among GelGreen™, GelRed™, SYBR® Green I and EB in -1 frameshift *Salmonella* indicator strain TA1537 without the presence of S9 fraction. "↓" indicates EB was not tested at this concentration. "↓" indicates SYBR® Green I became cytotoxic at this concentration.

Conclusion

- GelGreen and GelRed are nonmutagenic over the dose range from 0.1 ug/plate (or 40 ng/mL) to 50 ug/plate (or 18.5 ug/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 ug/mL (1X-3X), which is well within the safety range.*
- SYBR Green is nonmutagenic at lower concentrations (0.1-2.5 ug/plate or 0.04-0.93 ug/mL), but becomes cytotoxic at higher concentrations (≥ 2.5 ug/plate or 0.93 ug/mL), consistent with an earlier report (Singer, et al. *Mutat. Res.* **439**, 37(1999)).
- EB 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)

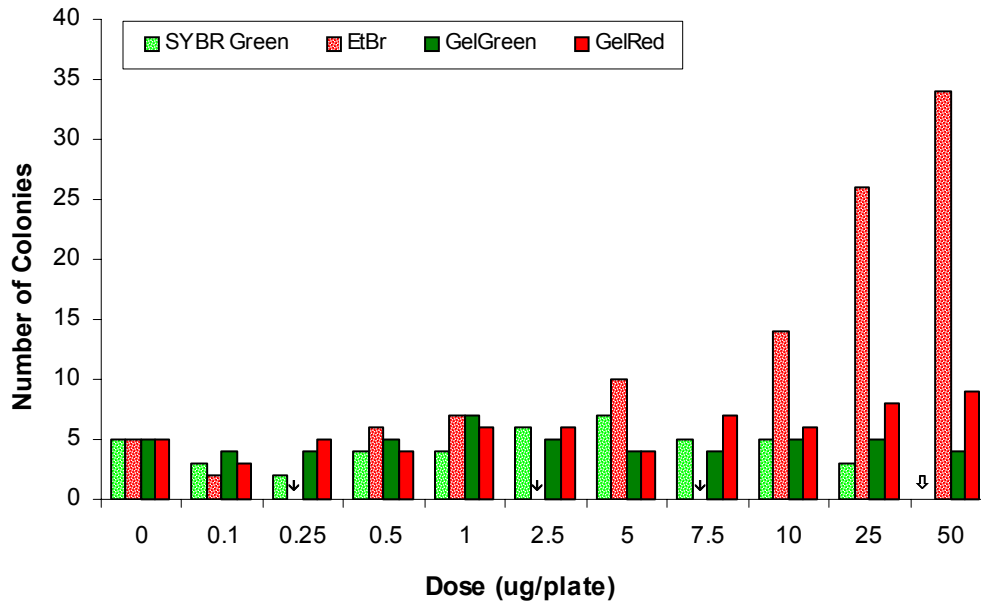


Figure 6. Comparison of mutagenicity among GelGreen™, GelRed™, SYBR® Green I and EB in *Salmonella* -1 frameshift indicator strain TA1537 with the presence of S9 fraction. "↓" indicates EB was not tested at this concentration. "⚡" indicates SYBR® Green I became cytotoxic at this concentration.

Conclusion

- GelGreen and GelRed are nonmutagenic over the dose range from 0.1 ug/plate (or 40 ng/mL) to 50 ug/plate (or 18.5 ug/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 ug/mL (1X-3X), which is well within the safety range.*
- EB 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 ug/plate or 0.04-9.3 ug/mL), but becomes cytotoxic at higher concentrations (≥25 ug/plate or 9.3 ug/mL), consistent with an earlier report (Singer, et al. *Mutat. Res.* **439**, 37(1999)).

Aquatic Toxicity Test

(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 ($LC_{50} > 500$ mg/L) to be "not hazardous" to aquatic life.

Results

The results are summarized in Table 2 below. Both samples gave $LC_{50} > 750$ 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

(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.

Conclusion

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

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.

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)	4.0	5.3
Flash Point (ASTM D-93)	>150 deg. F	>150 deg. F