Midori Green Direct DNA Stain Safety Test Report

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 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 EB, NIPPON GENETICS developed MIDORI GREEN DIRECT nucleic acid gel stains as superior alternatives. Extensive tests demonstrate this dye has significantly improved safety profiles over EB.

Dye Design Principle
At the very beginning of MIDORI GREEN DIRECT 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, NIPPON GENETICS incorporated structural features into the dye 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 able to metabolize to form compounds that have no or minimal interaction with DNA.

Safety Tests
MIDORI GREEN DIRECT were subjected to a series of tests both by us and by three independent testingservices 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
4) Environmental safety tests.

Results of the tests are summarized in Table 1 below. The data show that MIDORI GREEN DIRECT has passed all of the tests, thus validating the dye design principle. Detailed test results are described on in further pages.

Conclusion
MIDORI GREEN DIRECT stain is 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 non-cytotoxic and non-mutagenic at concentrations well above the working concentrations used in gel staining. Furthermore, MIDORI GREEN DIRECT have successfully passed environmental safety tests in compliance with CCR Title 22 Hazardous Waste Characterization. As a result, MIDORI GREEN DIRECT 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.

Latex Glove Penetration	Cell staining		Ames Test	Hazard-ous Waste Screening (aquatic toxicity test)	Reactivity test	Corrosivity test	Ignitability test
	Cell Membrane Permeability	Cytotoxicity					
impenetrable	impenetrable	noncytotoxicity	nonmutagenic	Nontoxic to aquatic life	unreactive	noncorrosive	nonflammable

Table 1: Summary of MIDORI GREEN DIRECT Safety test results

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Glove Penetration Test

Purpose: Latex gloves are commonly worn by researchers in laboratories as protective gear. Thus, it is important to show MIDORI GREEN DIRECT do not diffuse through the latex material.

Method: A finger of a latex glove containing TAE buffer was dialyzed against TAE buffer containing 5x MIDORI GREEN DIRECT for 48 hours. The solution in the finger was then analysed 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 MIDORI GREEN DIRECT is impenetrable to latex gloves (Fig. 1).

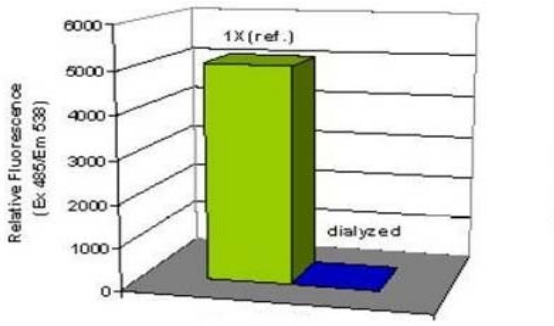

Figure 1: Relative Fluorescence of solutions dialyzed in latex glove fingers against 5X MIDORI GREEN DIRECT(blue) and the relative fluorescence of the corresponding 1X dye solution as a reference. The data show that the amount of the fluorescence for the dialyzed solutions is negligible, suggesting that no dye penetrates latex gloves at 5X concentration.

Conclusion: Handling MIDORI GREEN DIRECT with latex gloves is safe.

Cell Staining Test

Purpose: The purpose of this test is to see if MIDORI GREEN DIRECT can cross cell membranes to stain nuclear DNA.

Method: Hela cells were incubated at 37℃ with MIDORI GREEN DIRECT, 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). MIDORI GREEN DIRECT did not stain cells even following 30 minutes of incubation.

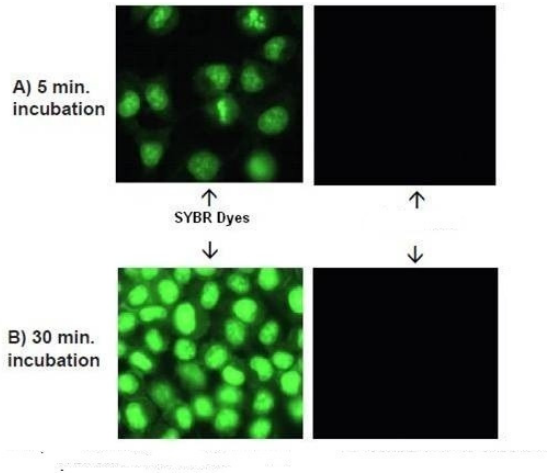

Figure 2: HeLa cells were incubated at 37℃ with 1X of SYBR Green I, SYBR Safe, and MIDORI GREEN DIRECT, respectively. Images were taken following incubation for 5 min (panel A) and 30 min (panel B), respectively. SYBR Green I and SYBR Safe entered into cells rapidly as evident from the bright green nuclear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

Conclusion: MIDORI GREEN DIRECT stain is are impenetrable to cell membranes.

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: MIDORI GREEN DIRECT 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 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 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)

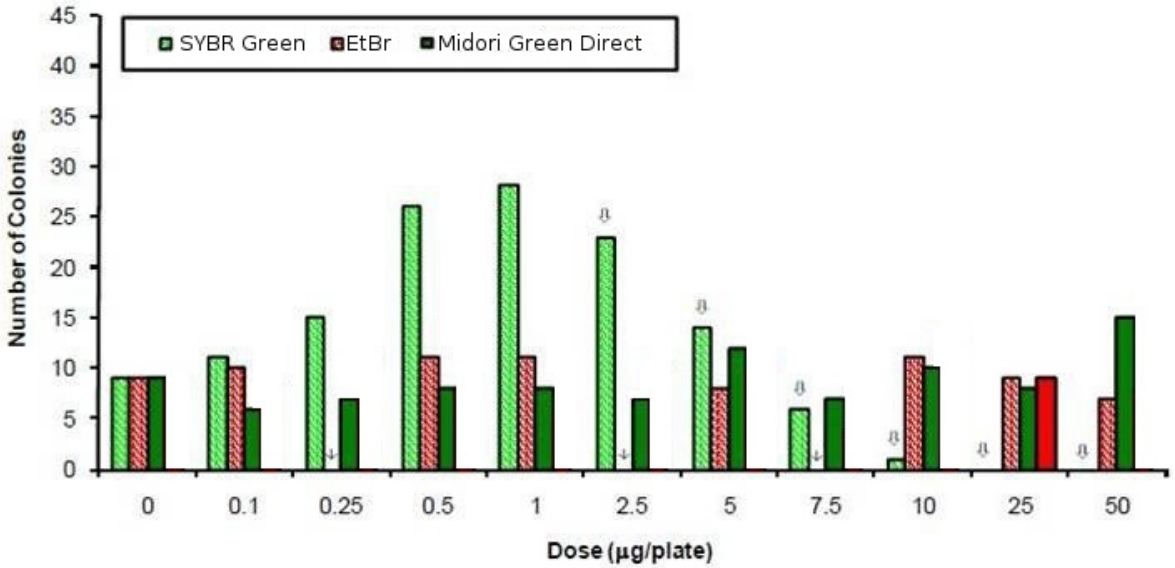

Figure 3: Comparison of mutagenicity among MIDORI GREEN DIRECT, MR100, SYBR Green I and EB in +1 frameshift Salmonella indicator strain TA98 without the presence of S9 fraction. "↓" indicates EB was not tested at this concentration. "⇩" indicates SYBR Green I became cytotoxic at this concentration.

Conclusion: MIDORI GREEN DIRECT are non-mutagenic over the dose range from 0.1 µg/plate (or 40 ng/mL) to 50 µg/plate (or 18.5 µg/mL) in +1 frame shift Salmonella indicator strain TA98 without S9 metabolic activation. The working concentration used in gel staining for MIDORI GREEN DIRECT is well within the safety range.
EB is non-mutagenic 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)

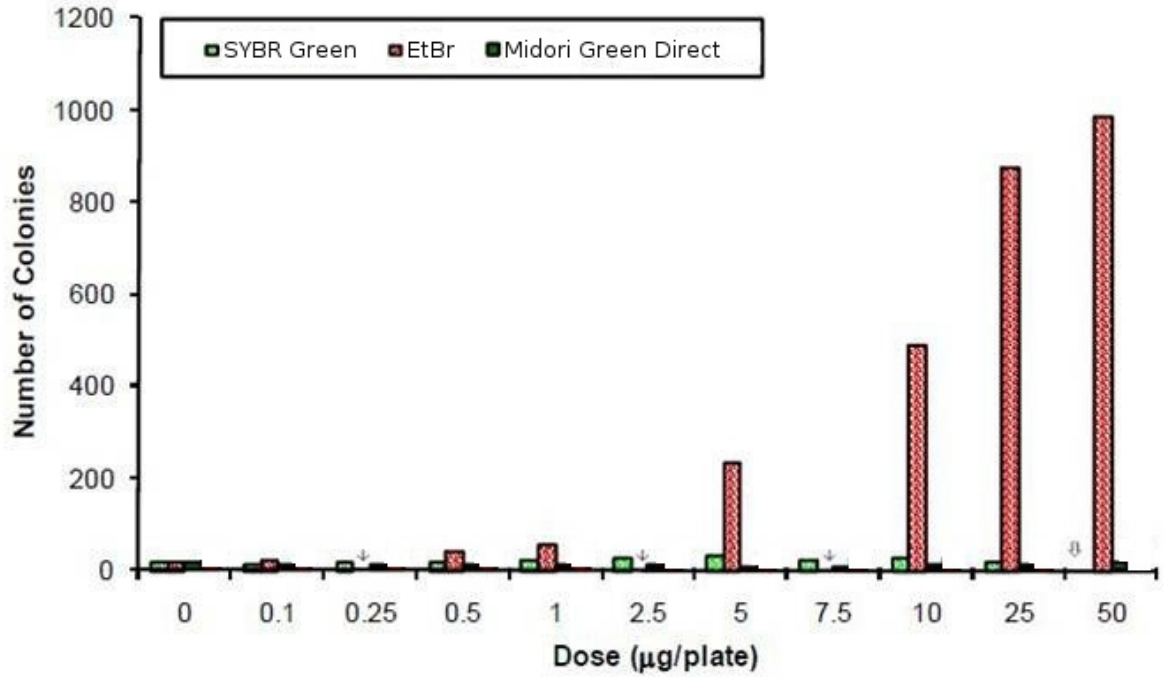

Figure 4: Comparison of mutagenicity among MIDORI GREEN DIRECT, MR100, SYBR Green I and EB in +1 frame shift Salmonella indicator strain TA98 with the presence of S9 fraction. "↓" indicates EB was not tested at this concentration. "⇩" indicates SYBR Green I became cytotoxic at this concentration.

Conclusion: MIDORI GREEN DIRECT is non-mutagenic over the dose range from 0.1 µg/plate (or 40 ng/mL) to 50 µg/plate (or 18.5 µg/mL) in +1 frame shift Salmonella indicator strain TA98 with S9 metabolic activation. MIDORI GREEN DIRECT working concentration used in gel staining is well within the safety range. SYBR Green I is non-mutagenic 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)). 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)

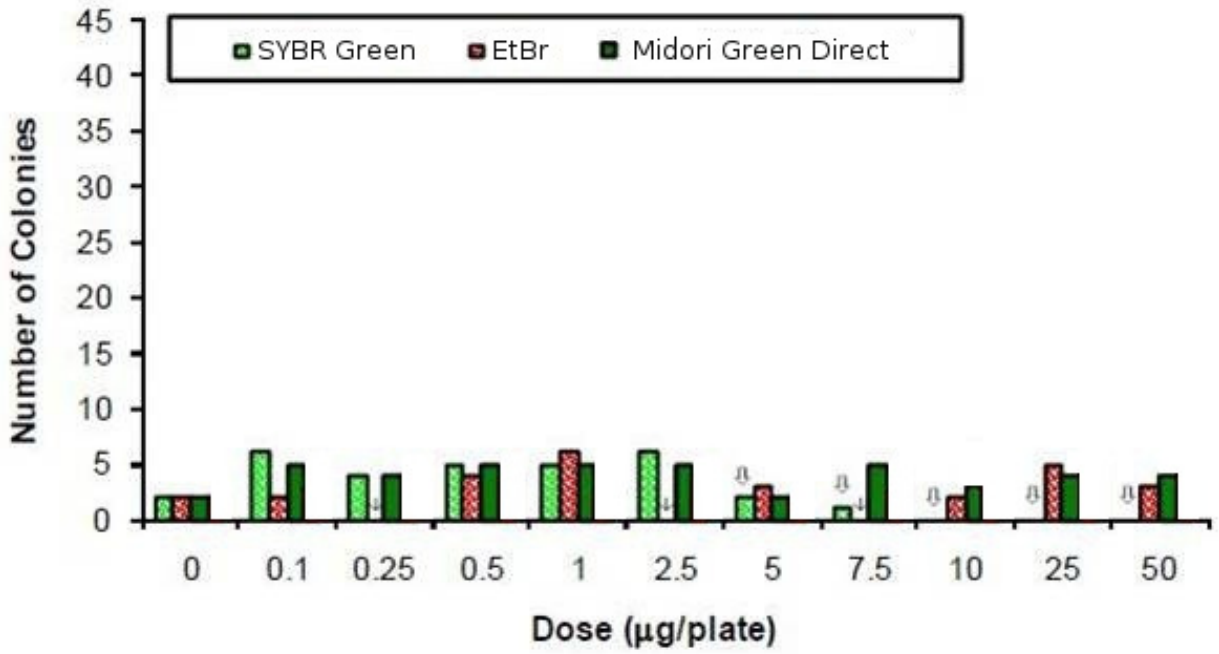

Figure 5: Comparison of mutagenicity among MIDORI GREEN DIRECT, MR100, SYBR Green I and EB in -1 frame shift Salmonella indicator strain TA1537 without the presence of S9 fraction. "↓" indicates EB was not tested at this concentration "⇩" indicates SYBR Green I became cytotoxic at this concentration.

Conclusion: MIDORI GREEN DIRECT are non-mutagenic over the dose range from 0.1 µg/plate (or 40 ng/mL) to 50 µg/plate (or 18.5 µg/mL) in -1 frame shift Salmonella indicator strain TA1537 without S9 metabolic activation. The working concentration used in gel staining for MIDORI GREEN DIRECT is 1-3 µg/mL (1X-3X), which is well within the safety range. SYBR Green is non-mutagenic 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)). 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)

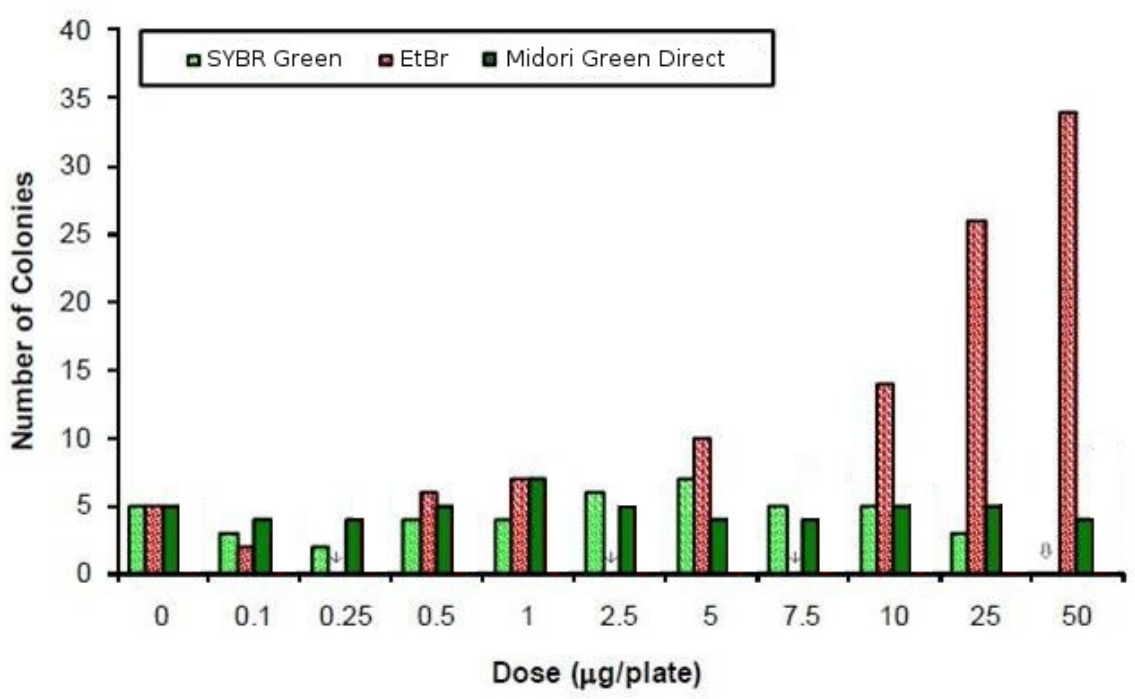

Figure 6: Comparison of mutagenicity among MIDORI GREEN DIRECT, MR100, SYBR Green I and EB in Salmonella -1 frame shift indicator strain TA1537 with the presence of S9 fraction. "↓" indicates EB was not tested at this concentration. "⇩" indicates SYBR Green I became cytotoxic at this concentration.

Conclusion: MIDORI GREEN DIRECT are non-mutagenic 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 frame shift indicator strain TA1537 without S9 metabolic activation. The working concentration used in gel staining for MIDORI GREEN DIRECT is 1-3 µg/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 non-mutagenic 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 (Performed by Nautilus Environmental, San Diego, CA)

Purpose: This test assesses the acute toxicity of MIDORI GREEN DIRECT 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 (MIDORI GREEN DIRECT 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 (LC50 > 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.

Sample	Dose (mg/ml)	% Survival
Lab Control		95
Midori Green Direct	250	100
	500	100
	750	100

Conclusion: MIDORI GREEN DIRECT at 3X are classified as non- hazardous to aquatic life, under CCR Title 22 regulation. Thus, MIDORI GREEN DIRECT at 3X or lower concentrations can be safely released into the environment.

Corrosivity, Reactivity and Ignitability Tests: (Performed by Curtis & Tompkins, Ltd., Analytical Laboratories, Berkeley, CA)

Purpose: The corrosivity, reactivity and ignitability of MIDORI GREEN DIRECT solutions are tested. These tests are designed to further assess the environmental safety of MIDORI GREEN DIRECT 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.

Test Name (test code)	Midori Green Direct
Reactive cyanide (SW-846 CH. 7)	None detected
Reactive Sulfate (SW-846 CH. 7)	None detected
pH (EPA 9040C)	4.0
Flash Point (ASTM D-93)	>150 deg F

Conclusion: Based on these results, MIDORI GREEN DIRECT at 3X or lower concentrations are classified as non-corrosive and non-hazardous materials.