

## Comparison of Nucleic Acid Gel Stains

### Cell permeability, safety, and sensitivity of ethidium bromide alternatives

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#### Introduction

Ethidium bromide has been used for decades to detect nucleic acids in agarose gels because it is inexpensive, sensitive enough for routine applications, and simple to use. However, because ethidium bromide has been shown to be mutagenic in the Ames test, it is potentially hazardous to laboratory workers and the environment, and institutions commonly require it to be disposed of as hazardous waste. Because of this, many users seek to find safer alternatives to ethidium bromide for gel electrophoresis. Biotium developed red fluorescent GelRed™ and green fluorescent GelGreen™ as safer replacements for ethidium bromide. GelRed™ and GelGreen™ are non-toxic and non-mutagenic because they do not bind DNA in living cells due to their membrane impermeability.

A number of other alternative gel stains have been marketed as safer alternatives to ethidium bromide. We performed a comparison of several alternative gel stains to evaluate their live cell permeability and sensitivity in gel staining. We found that many of these gel stains are membrane permeable dyes that readily penetrate living cells and stain the nucleus and other cellular structures. Chemical analysis showed that several of these products contain dyes that are known to be cytotoxic and to potentiate DNA damage by mutagens. In contrast, GelRed™ and GelGreen™ do not readily penetrate the nuclei of living cells. Furthermore, GelRed™ and GelGreen™ provide superior sensitivity in gel staining compared to other so-called safe nucleic acid gel stains.

#### Materials and Methods

##### Gel stains

EZ Vision® In-Gel was purchased from VWR; GreenSafe Premium was purchased from Cedarlane; Midori Green Advance and RedSafe™ Nucleic Acid Staining Solution were purchased from BullDog Bio; SafeView™ FireRed was a gift from Lucien Orbai; other gel stains were purchased directly from the manufacturer (see Table 1).

##### Chemical analysis

Nucleic acid gel stains were analyzed by thin layer chromatography (TLC). Dye absorbance spectra were measured using a Beckman Coulter DU-800 UV-visible spectrophotometer. Dye fluorescence emission spectra were measured in the presence of double stranded DNA using a Hitachi F-4500 fluorescence spectrophotometer. Mass spectrometry (LC-MS) was performed on an Agilent 6130 Quadrupole System. C18 reverse phase HPLC was performed on an Agilent 1100 Series System. DAPI (catalog no. 40011), propidium iodide (catalog no. 40016), and acridine orange (catalog no. 40039) from Biotium were used as reference dyes. Acridine orange from Sigma-Aldrich (catalog no. 235474) also was used for reference.

##### Cell staining

HeLa cells were plated at  $2 \times 10^4$  cells/well in a coverglass bottom 96-well plate. On the day after plating, cells were incubated with gel stains in culture medium at the indicated concentrations indicated in Table 2 for 5 minutes or 30 minutes at 37°C. The medium was removed and replaced with fresh medium without dye and the cells were imaged on a Zeiss LSM 700 confocal microscope. For cell membrane-impermeable dyes, far-red fluorescent CF™640R wheat germ agglutinin (Biotium) was included with the dye for 30 minutes at 37°C to label the cell surface to allow the cells to be imaged.

##### Gel electrophoresis

Gels were stained by precast or post-electrophoresis staining according to the manufacturer's product protocols. GelRed™, GelGreen™, SYBR® Safe, Midori Green Advance, and EZ-Vision® In-Gel were used for post-electrophoresis staining. RedSafe™, SafeView™ Classic, and SafeView™ FireRed were used in precast gel protocols. Agarose gels (1% in TBE) were prepared with no dye added for post-electrophoresis staining. Dye was added to molten agarose before gel casting for precast gel staining. For SafeView™ FireRed, dye was also included in the running buffer per product instructions. A dilution series of 200 ng to 12.5 ng per lane of Biotium's 1 kb DNA ladder was loaded on each gel, and gels were run in 1X TBE buffer at 100 volts for 90 minutes. Gels were post-stained in 50 mL water with dye for 30 minutes at room temperature with rocking. Precast gels were imaged directly after electrophoresis. Gels were imaged on a UVP GelDoc-It® imaging system with a UV transilluminator and EtBr or SYBR® Green filter. Alternatively, gels were imaged using visible blue LED illumination (465-475 nm) and photographed.

Table 1. Gel Stains Chemical Analysis

| Product                                 | Manufacturer                 | Catalog number | Analytical methods/references   | Dyes identified   |
|---|------------------------------|----------------|---|---|
| EZ-Vision® In-Gel Dye                   | AMRESCO                      | N391           | TLC<br>Absorbance spectrum<br>LC-MS   | DAPI  |
| SafeView™ FireRed                       | Applied Biological Materials | G926           | TLC<br>LC-MS<br>Analytical HPLC   | Propidium iodide  |
| RedSafe™ Nucleic Acid Staining Solution | iNtRON Biotechnology         | 21141          | TLC<br>Absorbance spectrum<br>LC-MS   | Acridine orange   |
| SafeView™ Classic                       | Applied Biological Materials | G108           | TLC   | Acridine orange   |
| Midori Green Advance                    | Nippon Genetics              | MG 04          | TLC<br>Absorbance spectrum<br>Emission spectrum<br>LC-MS<br>Analytical HPLC | Acridine orange and DAPI  |
| GreenSafe Premium                       | NZYTech                      | MB13201        | TLC<br>LC-MS  | Acridine orange and DAPI  |
| SYBR® Safe, 10,000X in DMSO             | Thermo Fisher Scientific     | S33102         | Reference 1   | Thiazole orange derivative with N-propyl substitution on quinolinium ring |
| Nancy-520                               | Sigma-Aldrich                | 01494          | Reference 2   | SYBR dye derivative with hydroxyalkyl substitution on benzothiazole       |

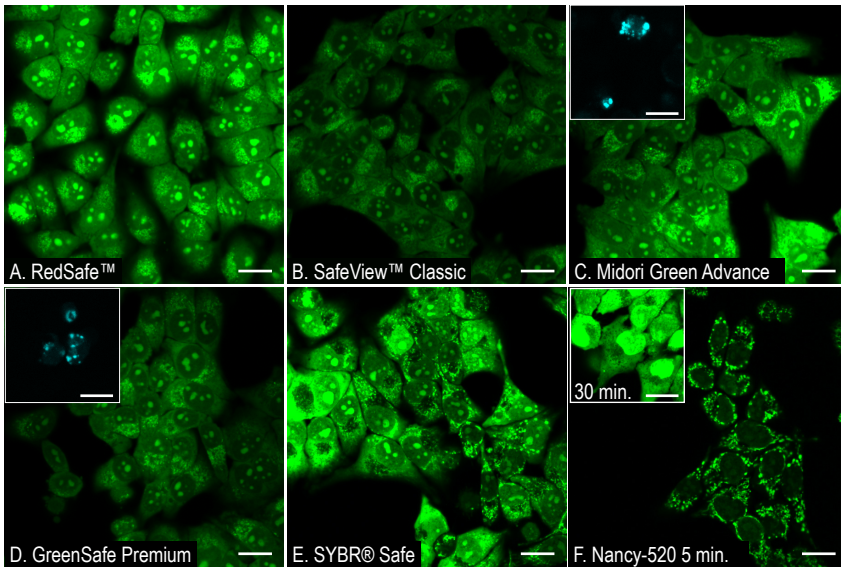


Figure 1. Acridine orange- and SYBR-based nucleic acid gel stains rapidly penetrate live cells to stain the nucleus, cytoplasm, and other organelles. HeLa cells were incubated with nucleic acid gel stains diluted in cell culture medium at the concentrations indicated in Table 2 for five minutes at 37°C. A. RedSafe. B. SafeView Classic. C. Midori Green Advance; inset shows a different field of view with blue fluorescent staining of sporadic dead cells on top of cell monolayer. D. GreenSafe Premium; inset shows a different field of view with blue fluorescent staining of sporadic dead cells on top of cell monolayer. E. SYBR Safe. F. Nancy-520; inset shows cells incubated for 30 minutes at 37°C. Green fluorescence was imaged using 488 nm excitation with 10% laser power and 490 nm longpass filter. Master gain was set at 500 for Midori Green Advance, GreenSafe Premium, Nancy-520. Master gain was set at 450 for RedSafe, SafeView Classic, and SYBR Safe. Blue fluorescence was imaged using 405 nm excitation with 10% laser power and 490 nm shortpass filter with master gain at 650. Scale bars: 20 μm.

## Results

### Chemical analysis of nucleic acid stains

RedSafe™, Midori Green Advance, and GreenSafe Premium were found to contain acridine orange, a cell membrane permeable dye commonly used for live cell staining. Acridine orange stains DNA with green fluorescence and RNA and acidic organelles with red fluorescence. Midori Green Advance and GreenSafe Premium were found to also contain low levels of DAPI, a commonly used blue fluorescent DNA counterstain. EZ-Vision® In-Gel was found to contain DAPI. At low concentrations, DAPI has poor cell membrane permeability, but can be used to stain live cells at high concentrations. SafeView™ FireRed contained propidium iodide, a cell membrane-impermeable red fluorescent dye that binds both DNA and RNA, and is widely used as a dead cell-specific vital dye. The structures of SYBR® Safe (1) and Nancy-520 (2) have been reported previously.

### Live cell staining

The acridine-orange based dyes Midori Green Advance, GreenSafe Premium, SafeView™ Classic, and RedSafe™ rapidly penetrated the membranes of living cells. After five minutes incubation with these dyes, cells showed intense staining of the nucleus, nucleoli, cytoplasm, and intracellular vesicular structures (Figure 1). Both green and red fluorescence was observed (not shown). These results are consistent with the chemical analysis of the gel stains. Acridine orange stains DNA with green fluorescence and RNA and acidic organelles with red fluorescence. Midori Green Advance and GreenSafe Premium also showed blue fluorescent staining of sporadic dead cells on top of the cell monolayer (Figure 1C and 1D, insets), which is consistent with the presence of low concentrations of DAPI, which has poor cell membrane permeability and preferentially stains dead cells with leaky plasma membranes.

Nancy-520 and SYBR® Safe showed bright staining in a pattern suggesting mitochondrial localization, as well as some cytoplasmic and nuclear staining after 5 minutes (Figure 1). After 30 minutes, Nancy-520 showed strong nuclear and cytoplasmic staining (Figure 1F inset), similar to what we've previously observed for SYBR® Safe (not shown).

SafeView™ FireRed, EZ-Vision® In-Gel, GelRed™ and GelGreen™ showed no staining of live cells after a five minute incubation, but only stained sporadic dead cells on top of the cell monolayer (not shown). After 30 minutes incubation at 37°C, no live cell staining was observed for SafeView™ FireRed, EZ-Vision® In-Gel, or GelRed™. These results are consistent with the chemical analysis of the gel stains. After 30 minutes incubation, weak mitochondrial staining was observed for GelGreen™ at higher gain settings, but no nuclear staining was observed. To facilitate imaging of cells stained with the membrane-impermeable dyes, far-red fluorescent CF™640R wheat germ agglutinin (WGA) was included with the dyes for 30 minutes. Identical staining results for the nucleic acid gel stains was observed without WGA (not shown). Cell staining results are summarized in Table 2.

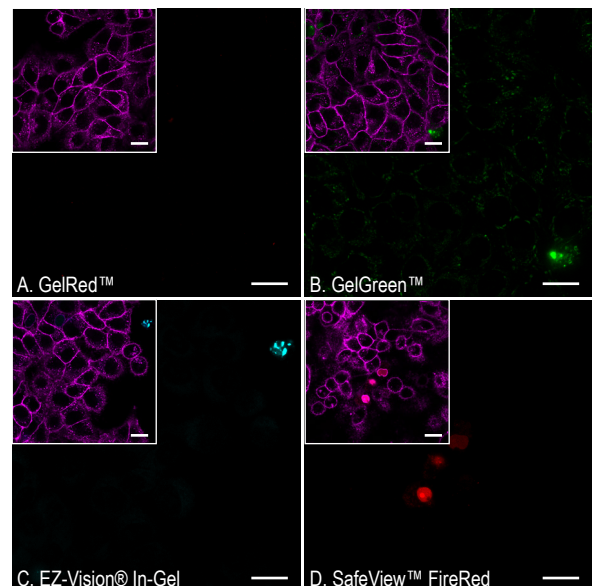


Figure 2. GelRed, GelGreen, EZ-Vision In-Gel, and SafeView FireRed do not stain the nucleus in living cells after 30 minutes incubation at 37°C. HeLa cells were incubated with nucleic acid gel stains diluted in cell culture medium at the concentrations indicated in Table 2. Insets show CF640R WGA staining (magenta) of the same field of view to visual cell outlines. Bright staining was observed only for sporadic dead cells on top of the cell monolayer. A. GelRed (red) imaged with 555 nm laser at 10% power and 640 nm shortpass filter with master gain 600. B. GelGreen (green) imaged with 488 nm laser at 10% power and 640 nm shortpass filter with master gain 600. C. EZ-Vision In-Gel (cyan) imaged with 405 nm laser at 10% power and 640 nm shortpass filter with master gain 550. D. SafeView FireRed imaged with 555 nm laser at 10% power and 640 nm shortpass filter with master gain 600. Scale bars: 20 μm.

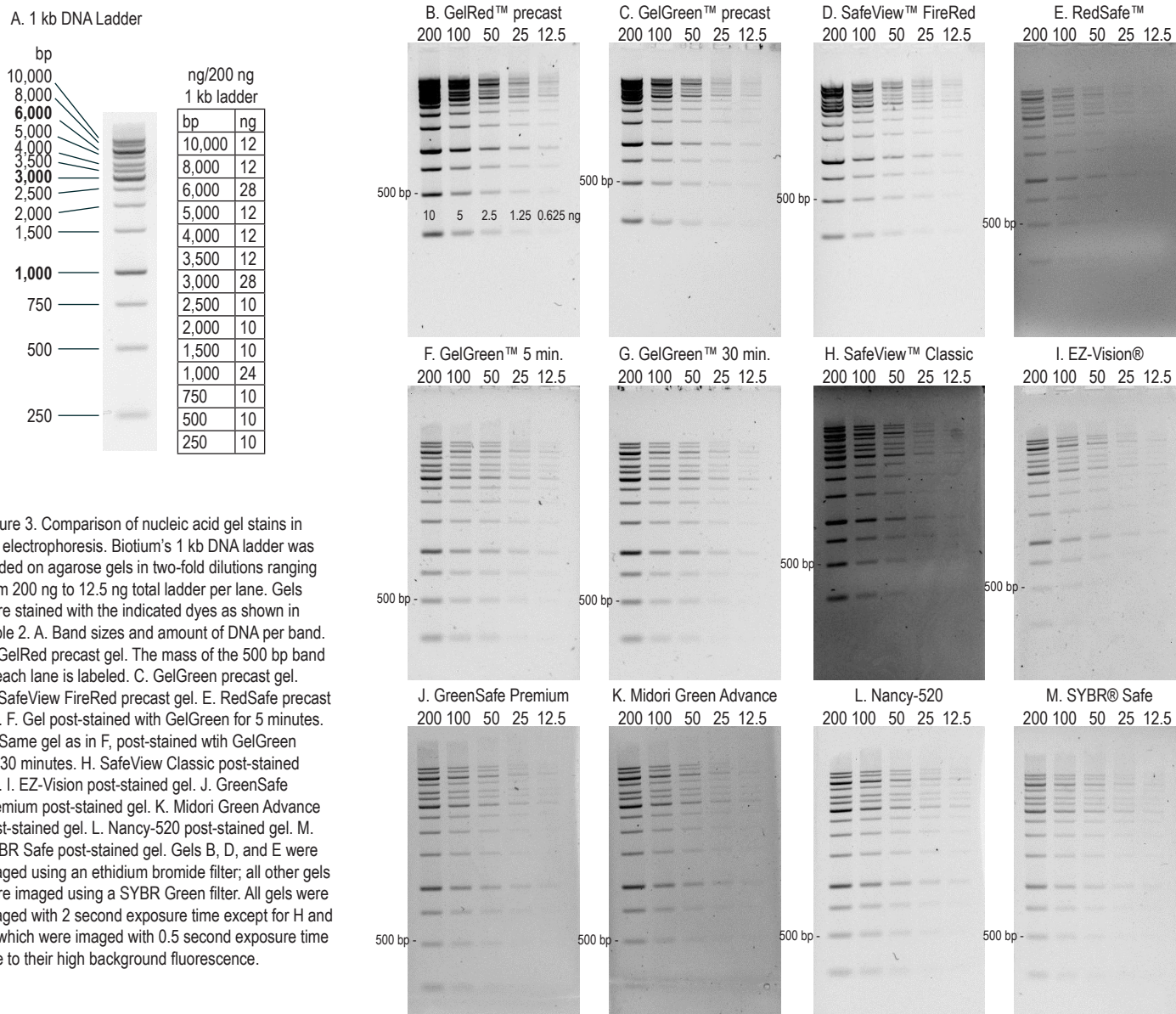


Figure 3. Comparison of nucleic acid gel stains in gel electrophoresis. Biotium's 1 kb DNA ladder was loaded on agarose gels in two-fold dilutions ranging from 200 ng to 12.5 ng total ladder per lane. Gels were stained with the indicated dyes as shown in Table 2. A. Band sizes and amount of DNA per band. B. GelRed precast gel. The mass of the 500 bp band in each lane is labeled. C. GelGreen precast gel. D. SafeView FireRed precast gel. E. RedSafe precast gel. F. Gel post-stained with GelGreen for 5 minutes. G. Same gel as in F, post-stained with GelGreen for 30 minutes. H. SafeView Classic post-stained gel. I. EZ-Vision post-stained gel. J. GreenSafe Premium post-stained gel. K. Midori Green Advance post-stained gel. L. Nancy-520 post-stained gel. M. SYBR Safe post-stained gel. Gels B, D, and E were imaged using an ethidium bromide filter; all other gels were imaged using a SYBR Green filter. All gels were imaged with 2 second exposure time except for H and K, which were imaged with 0.5 second exposure time due to their high background fluorescence.

#### Gel electrophoresis

In precast gels, bands containing between 0.625 and 1.25 ng dsDNA were detectable with GelRed™ (Fig. 2B) and GelGreen™ (Fig. 2C), depending on fragment length. For the 500 bp band, 1.25 ng was readily detected and 0.625 ng was barely visible. SafeView™ FireRed was able to detect 1.25 ng of the 500 bp band (Fig. 2D), but with dimmer fluorescence compared to GelRed™. With RedSafe, the 500 bp band was only detectable down to 2.5 ng (Fig. 2E), with high background compared to the other stains.

In post-electrophoresis stained gels, the 500 bp band was detectable at 1.25 ng with GelGreen™. While the recommended time for post-staining with GelGreen™ is 30 minutes, very similar results were obtained after only 5 minutes incubation (Fig. 2F) compared to 30 minutes (Fig. 2G). Nancy-520 (Fig. 2L) showed similar sensitivity as GelGreen™, but with dimmer signal. SafeView™ Classic (Fig. 2H), GreenSafe Premium (Fig. 2J), Midori Green Advance (Fig. 2K), and SYBR® Safe (Fig. 2M) were able to detect 2.5 ng of the 500 bp band. EZ-Vision® In-Gel (Fig. 2I) was only able to detect 5 ng of the 500 bp fragment. SafeView™ Classic (Fig. 2H), GreenSafe Premium (Fig. 2J), and Midori Green Advance (Fig. 2K) showed high background compared to the other gel stains. Gel electrophoresis results are summarized in Table 2.

One feature of green fluorescent gel stains like GelGreen™ is that they have absorbance around 500 nm (Fig. 4A), allowing them to be excited by visible blue LED light. Visible light illumination users to avoid harmful UV irradiation. Cloning efficiency with gel-purified DNA is reportedly improved with blue light illuminators as well, presumably because they avoiding UV damage to DNA during gel excision (3). Absorbance spectroscopy revealed that EZ-Vision® has absorbance peak near 350 nm, the same as DAPI (Fig. 4A). Consequently, unlike GelGreen™ (Fig. 4B), EZ-Vision® is incompatible with blue light excitation (Fig. 4C).

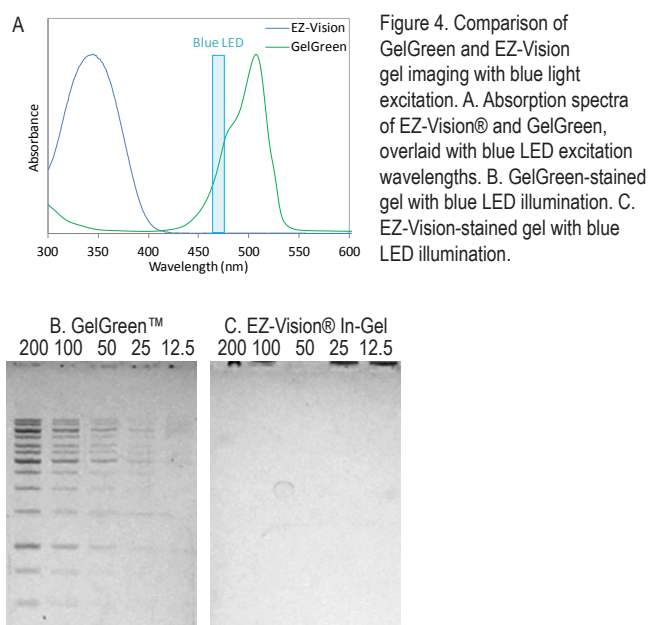


Figure 4. Comparison of GelGreen and EZ-Vision gel imaging with blue light excitation. A. Absorption spectra of EZ-Vision® and GelGreen, overlaid with blue LED excitation wavelengths. B. GelGreen-stained gel with blue LED illumination. C. EZ-Vision-stained gel with blue LED illumination.

Table 2. Gel Stains Comparison Summary

| Product                                 | Manufacturer                 | Catalog number | Dye identity               | Stains live cell nucleus? (concentration, dilution) | Gel staining method  | Sensitivity (500 bp dsDNA) |
|---|------------------------------|----------------|----------------------------|---|--|----------------------------|
| GelRed™, 10,000X in water               | Biotium                      | 41003          | GelRed                     | No (3X, 1:3,333)                                    | Pre-cast 1X in agarose                                     | 0.625-1.25 ng              |
| GelGreen™, 10,000X in water             | Biotium                      | 41005          | GelGreen                   | No (3X, 1:3,333)                                    | Post-stain 3X in water or precast 1X in agarose            | 0.625-1.25 ng              |
| EZ-Vision® In-Gel Dye                   | AMRESCO                      | N391           | DAPI                       | No (1X, 1:10,000)                                   | Post-stain 2.5X in 0.1 M NaCl Destain 2 x 10 min. in water | 5 ng                       |
| SafeView™ FireRed                       | Applied Biological Materials | G926           | Propidium iodide           | No (1X, 1:20,000)                                   | Precast 1:20,000 in agarose + 1:20,000 in running buffer   | 0.625-1.25 ng              |
| RedSafe™ Nucleic Acid Staining Solution | iNTRON Biotechnology         | 21141          | Acridine orange            | Yes (1X, 1:20,000)                                  | Precast 1:20,000 in agarose                                | 2.5 ng                     |
| SafeView™ Classic                       | Applied Biological Materials | G108           | Acridine orange            | Yes (1X, 1:20,000)                                  | Precast 1:20,000 in agarose                                | 2.5 ng                     |
| Midori Green Advance                    | Nippon Genetics              | MG 04          | Acridine orange + DAPI     | Yes (1X, 1:20,000)                                  | Post-stain, 1:4000 in TBE                                  | 2.5 ng                     |
| GreenSafe Premium                       | NZYTech                      | MB13201        | Acridine orange + DAPI     | Yes (1X, 1:20,000)                                  | Post-stain, 1:6,666 in TBE                                 | 2.5 ng                     |
| SYBR® Safe, 10,000X in DMSO             | Thermo Fisher Scientific     | S33102         | Thiazole orange derivative | Yes (1X, 1:10,000)                                  | Post-stain, 1X in TBE                                      | 2.5 ng                     |
| Nancy-520                               | Sigma-Aldrich                | 01494          | SYBR® derivative           | Yes (1X, 1:5,000)                                   | Post-stain, 1X in TBE, rinsed in TBE                       | 1.25 ng                    |

## Discussion

Nucleic acid gel stains have been marketed as being safer than ethidium bromide based on their low mutagenicity in experimental models like the Ames test, with many incorporating the word safe in their trade names. We have found that several of these dyes rapidly penetrate living cells to interact with the cell nucleus, increasing their likelihood of causing damage to living cells or their DNA. GelRed™ and GelGreen™ were designed to be excluded from living cells and therefore non-toxic, which has been confirmed by independent laboratories (4, 5).

Chemical analysis showed that a number of the gel stains tested contain the dye acridine orange. Acridine orange is highly membrane permeable and has been shown to potentiate DNA damage by mutagens (6), and has even been proposed as an anti-tumor agent due to its cytotoxicity (7). Others have also shown that the acridine orange-based RedSafe™ Nucleic Acid Staining Solution is highly membrane permeable (4) and far more cytotoxic than ethidium bromide in V79 cells, a cell line commonly used for chemical safety testing (8). In addition, acridine orange-based gel stains have much lower sensitivity and high background compared to GelRed™ and GelGreen™, which also has been shown by others (4).

SYBR® Safe is one of the original ethidium bromide alternatives to be marketed as safe. However, it has been reported to show mutagenicity after metabolic activation in the Ames test (9). While SYBR® Safe is reported to be non-mutagenic in Syrian hamster embryo (SHE) cells and L5178YTK +/- mouse lymphoma cells (10), it was tested at concentrations well below its 1X working concentration of 0.66 µg/mL (11) due to its excessive cytotoxicity (9). These results are consistent with the fact that SYBR® Safe rapidly penetrates cell membranes and stains the nucleus of live cells. Nancy-520, a SYBR derivative, also readily penetrates living cells to interact with DNA, suggesting that it has similar potential for cytotoxicity. Moreover, these dyes also showed no advantage in sensitivity compared to GelRed™ or GelGreen™.

SafeView™ FireRed was found to contain propidium iodide, which is commonly used to stain dead cells. Consistent with this, SafeView™ FireRed did not stain live cells. However, it has been reported that propidium iodide is cell permeable and cytotoxic to J774 cells with prolonged exposure (1 day) (5). In the same study, GelRed™ and GelGreen™ were found to be non-permeable and non-toxic for up to three days of incubation (5). EZ-Vision® In-Gel also was found to be cell membrane impermeable, consistent with the finding that it contains DAPI. However, EZ-Vision® has very low sensitivity, and the added disadvantage of being incompatible with visible blue gel imagers, unlike GelGreen™.

We recommend handling all laboratory reagents as potentially hazardous, using universal safety precautions such as gloves, lab coat, and eye protection. Risk of exposure to toxic or mutagenic substances can also be lessened by avoiding chemicals that readily penetrate membranes and interact with DNA in living cells, even if they are labeled as "safe."

GelRed™ and GelGreen™ are non-toxic, non-mutagenic dyes that do not readily penetrate living cells or gloves at working concentrations. They also have been classified as non-hazardous for waste disposal under CA Title 22 regulations (12). GelRed™ and GelGreen™ offer superior sensitivity compared to other ethidium bromide alternatives.

## References

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GelRed, GelGreen, and their uses are covered by US and international patents. EZ-Vision is a registered trademark of AMRESCO; SafeView is a trademark of Applied Biological Materials; RedSafe is a trademark of iNTRON Biotechnology. SYBR is a registered trademark of Thermo Fisher Scientific.