Beijing Fluorescence Biotechnology Co., Ltd.

Safety Report
(SafeRed and DuGreen)

Mutagenicity and Environmental Safety Test Results from Two Independent Laboratories

Beijing, 10, 2017

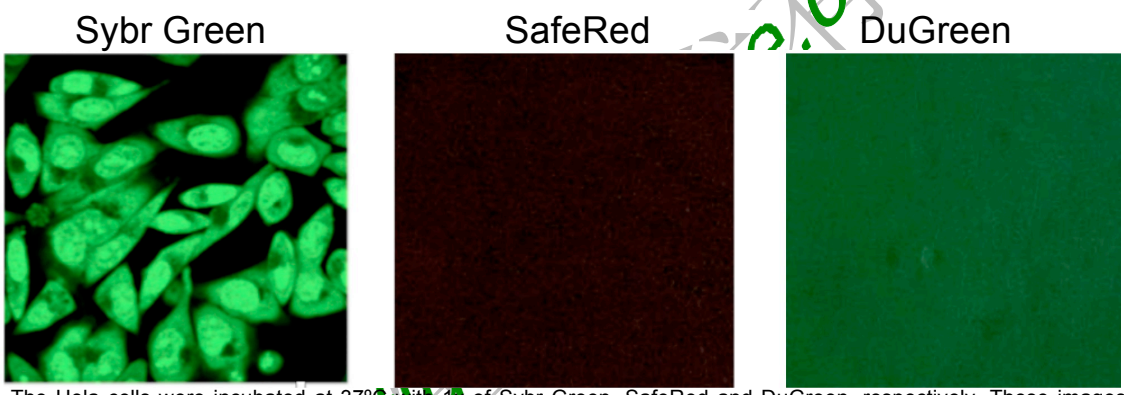
Cell Staining Test

Testing Laboratory
Feed Research Institute. Chinese Academy of Agricultural Sciences.

Purpose
The purpose of this test is to check if nucleic acid dyes: SafeRed and DuGreen can cross cell membranes to stain nuclear DNA.

Method
Mouse liver cells were incubated at 37◦C with SafeRed, DuGreen, 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 8 hours of incubation are shown in Figure 2. SYBR Green stained cell cytoplasm and nuclear DNA with bright green fluorescence in only a few minutes. SafeRed or DuGreen did not stain live cells even after 80 minutes of incubation and both dyes stained in dead cells.



The Hela cells were incubated at 37°C with 1× of Sybr Green, SafeRed and DuGreen, respectively. These images were taken after 30min incubation. Sybr Green entered into live cells clearly as evident from the bright green staining. However, SafeRed and DuGreen were unable to cross cell membranes of live cells as shown by the absence of fluorescence staining.

Conclusion
SafeRed and DuGreen do not penetrate live cell membranes.

Mutagenicity and Environmental Safety Test (Ames Test)

Testing Laboratory

Occupational Health and Poisoning Control Center, the Chinese Center for Disease Control and Prevention

Purpose

This Bacterial Reverse Mutation Screening Assay was performed to evaluate the ability of this test article to induce a mutagenic response in four strains of Salmonella typhimurium. A positive test indicates that the chemical is mutagenic and therefore may act as a carcinogen,because cancer is often linked to mutation. The test can be therefor performed to estimate the carcinogenic potential of a compound.

Test Specifications

Test starting time: 20.09. 2011 Test ending time: 01.01.2012

Test organism: 4 strains of *Salmonella typhimurium* (TA97, TA98, TA100 or TA102)

Regulatory guidelines: 《The Guidelines for the Testing of Chemicals 》（State Environmental Protection Administration of China, 2004）
《The guidelines for the hazard evaluation of new chemical substances》(State Environmental Protection Administration of China,2004)

Materials

Test Sample Description: Test sample was received as a red odourless powder vial labeled: “Cat: DCM2662”, Beijing Fluorescence Biotechnology Co. Ltd., For Research Use Only.” The SafeRed powder was dissolved in DMSO in different concentrations.

The *Salmonella* strains: The Salmonella strains used in this study were obtained from American Ames Lab. The strains were met the experimental standards tested by our lab.

Positive substance:

2,4,7-Trinitro-9-fluorenone and NaN_3 (From Fluka), MMS (from Merck); 2-AF and 1,8-Dihydroxyanthraquinone (From Shanghai Reagent)

Test Procedure

Dose Setting:

According to antibacterial experiment, the SafeRed Doses setting was as following: 0, 62.5, 125, 250,500 and 1000 μg/plate. We also set the Positive control, Solvent control and Blank control. The Solvent control was 100uL DMSO/plate. Without S9 the Positive control was setting as following: TA97 with 2,4,7-Trinitro-9-fluorenone, TA98 with 2,4,7-Trinitro-9-fluorenone, TA100 with NaN3 and TA102 with 1,8-Dihydroxyanthraquinone. When the S9 was added to the mixture, TA97, TA98 and TA100 were with 2-AF and the TA102 with 1,8-Dihydroxyanthraquinone.

Method:

The sterile culture tubes were inverted and incubated at 42ºC~45 ºC prior to test. The following was added to each tube containing 2.0 ml top agar: 0.1 ml of overnight cell culture, 0.1 ml of each test article concentration or control chemical, and either 0.3 ml of S9/Cofactor mix or 0.5 ml of phosphate buffered saline. 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 ºC for 48 hours. Triplicate plates were used for each dose with/without S9 metabolic activation. The automatic colony counter was used for counting revertant colonies every plate. If the revertant colonies number is more than two times of the initiative revertant colonies number, the test sample is positive.

Results:

SafeRed was negative in the Ames Test using *Salmonella* strain TA97, TA98, TA100 and TA102 (with/without S9 metabolic activation), as the revertant colonies number is less than two times of the initiative revertant colonies number with/without S9 metabolic activation. The same data was obtained by the repeated experiment, see table below:

The revertant colonies number with/without S9 metabolic activation in Ames test for SafeRed

Doses setting μg/plate	TA97		TA98		TA100		TA02	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
0	125±5	126±2	36±1	36±3	128±5	130±3	297±7	298±10
100μL/plate DMSO	123±6	126±3	35±3	35±3	127±6	129±7	299±4	297±2
1000	126±5	126±6	35±4	37±3	125±2	127±6	297±9	295±7
500	125±6	127±3	34±5	36±5	130±5	130±7	303±4	299±7
250	125±4	128±7	38±3	36±2	128±4	130±9	298±2	297±7
125	127±5	126±3	36±5	35±3	126±3	127±4	301±4	296±9
62.5	128±5	124±2	35±2	36±2	128±4	127±6	302±11	298±3
Control test	① 2011	② 2112	② 1461	② 1749	③ 2059	② 1504	④ 1963	⑤ 1051

*①2,4,7-Trinitro-9-fluorenone 0.2ug/plate; ②2-AF, 50ug/plate ③ NaN_3 2ug/plate ④MMS, 30uL/plate ⑤1,8-Dihydroxyanthraquinone 50ug/plate; ** The colonies number in the table is average number of the three parallel control test.

Conclusions:

According to the Guidelines for the Testing of Chemicals State Environmental Protection Administration of China, 2004, the Ames test of SafeRed was negative. The negative results show this assay of SafeRed is Non mutagen to the four strains (TA97, TA98, TA100 or TA102) under the experimental conditions

Aquatic Toxicity Test

Testing Laboratory
Feed Research Institute. Chinese Academy of Agricultural Sciences

Purpose
This test assesses the acute toxicity of SafeRed and DuGreen 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 starting time: 25.09.2017, 11:50
Test ending time: 29.09.2017, 17:45
Test organism: *Danio rerio* (Zebrafish)
Organism mean length/weight: 18.1 mm/69 mg
Test concentration: 250, 500, and 750 mg/L of each sample (SafeRed or DuGreen); Negative control
Replicates and number of fish: 2 replicates with 10 fish for each concentration (20 fish total in container of each 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 below. Both samples gave LC_{50} > 750 mg/L.

Sample	Dose(mg/L)	Survival rate (%)
Lab Control	0	100
SafeRed	250	100
	500	100
	750	100
DuGreen	250	100
	500	100
	750	100

Conclusion
Both SafeRed and DuGreen at 3X are classifed as nonhazardous to aquatic life, under CCR Title 22 regulation. Thus, SafeRed and DuGreen at 3X or lower concentrations can be safely released into the environment.