HDGreen® Plus Safe DNA Dye – Safety Test Reports

IDENTIFICATION OF THE PRODUCT AND OF THE COMPANY	
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INTRODUCTION

Ethidium bromide (EB) is the most commonly used nucleic acid stain in molecular biology laboratories. It has been proved to be highly mutagen and is therefore considered hazardous for laboratory personnel and the environment.

HDGreen® Plus Safe DNA Dye is a nucleic acid stain which can be used as a safer alternative to the traditional Ethidium bromide stain for detecting nucleic acid in agarose gels. It is as sensitive as Ethidium bromide and can be used exactly the same way in agarose gel electrophoresis with some extra possibilities.

The safety of **HDGreen® Plus Safe DNA Dye** has been controlled with the below three tests.

I AMES TEST

1. Test System:

The Ames test employed four Salmonella strains, TA97, TA98, TA100 and TA102. 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. 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.

HDGreen® Plus Safe DNA Dye was dissolved in sterile distilled water, the test volume was 0.1 ml per plate.
Preliminary assays were performed. The dosages were 0.5 , 1.0, 2.5 and 5 mg/plate, respectively.. The control groups included blank control plates, solvent control plates (sterile distilled water) and positive control plates. In the absence of S9 mix, the positive control reference for strains TA97 and TA98 was 9-fluorenone, for TA100 NaN_3 and for TA102 was Mitomycin C. In the presence of S9 mix, the positive control reference substance for strains TA97, TA98 and TA100 was 2-AF (Aminofluorene), and for TA102 was 1,8-Hydroxyanthraquinone.

2. Test Procedure:

The test substance (0.1 mL) and 0.1 mL bacterial suspension with 0.5 mL S9 mixture (+S9) or without S9 mixture (-S9) were mixed uniformly in test tubes with 1.5 mL overlay agar (liquid, 45℃). The mixture was uniformly poured on the prepared underlay agar plates. After solidification, the plates were incubated at 37℃ for 48 h. At the end of the incubation, revertant colonies per plate were counted. All plating was done in triplicate. If the number was more than twice the spontaneous revertant colonies counts and showed a dose-response relationship, positive result for mutagenicity could be concluded.

3. Test Result:

According to the results of the Ames test (Table 1), in the presence and absence of metabolic activator S9 the increase in the numbers of revertant colonies of strains TA97, TA98, TA100 and TA102 compared to spontaneous revertant colonies was less than 2 times, and there was no dose-response relationship.

Appropriate reference mutagens were used as positive controls and they showed a distinct increase of induced revertant colonies (Table 1).

Table 1: Results of HDGreen® Plus Safe DNA Dye Ames test ($\bar{x}$± SD)

Dose level	TA 97		TA 98		TA 100		TA 102	
[µg/Plate]	S9 -	S9 +	S9 -	S9 +	S9 -	S9 +	S9 -	S9 +
Water	132 ± 16	155 ± 2	30 ± 2	32 ± 1	146 ± 10	158 ± 19	248 ± 7	275 ± 15
Neg. control	135 ± 12	156 ± 5	30 ± 1	32 ± 1	136 ± 21	155 ± 11	247 ± 10	275 ± 14
Pos. control	1475±105[1]	1519±161[2]	2244±36[1]	2306±234[2]	1283±26[3]	1338±166[2]	1464±156[4]	1562±285[5]
500	121 ± 14	160 ± 19	34 ± 2	59 ± 7	178 ± 8	178 ± 7	268 ± 60	369 ± 28
1000	114 ± 9	170 ± 12	34 ± 3	50 ± 8	166 ± 14	167 ± 16	312 ± 10	553 ± 87
2500	123 ± 11	180 ± 23	32 ± 3	46 ± 6	124 ± 17	180 ± 8	296 ± 36	525 ± 86
5000	98 ± 8	174 ± 10	30 ± 2	49 ± 9	128 ± 38	173 ± 28	303 ± 25	524 ± 35

[1] 9-Fluorenone, 0.2 µg/plate
[2] 2-AF, 10 µg/plate.
[3] NaN_3, 2,5 µg/plate
[4] Mitomycin C, 4.0 µg/plate
[5] 1,8-Hydroxyanthraquinone, 50 µg/plate.
$\bar{x}$ is the average of revertant colony counts of the 6 plates.

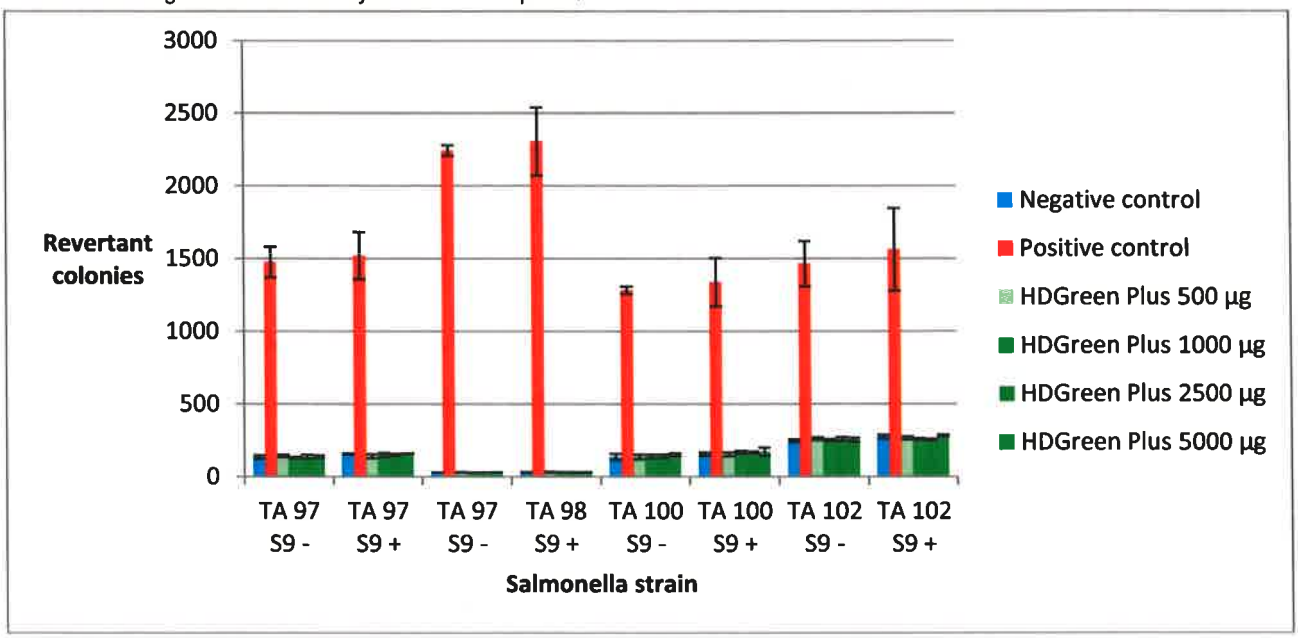

Diagram 1: Results of HDGreen® Plus Safe DNA Dye Ames test

4. Conclusion:

According to the guidelines, negative result was obtained and a significant mutagenic effect of HDGreen® Plus Safe DNA Dye could not be detected.

II MOUSE BONE MARROW MICRONUCLEUS TEST

1. Test System:

This test was performed to assess the mutagenic properties of HDGreen® Plus Safe DNA Dye on the incidence of micronuclei of bone marrow polychromatic erythrocytes of the mouse. The micronucleus test is one of the most widely applied short term test used in genetic toxicology and has become one of the most important tests to evaluate the genotoxic potential of new chemical compounds. The test detects agent-induced chromosomal damage or damage of the mitotic spindle apparatus.

Sample: Original liquid of HDGreen® Plus Safe DNA Dye.

Positive Control: Mitomycin C.

Animals: The animals used in the test were healthy Kunming mice (body weight from 25g - 30g).

2. Test Procedure:

Mice in test groups were administrated orally with the test item twice in 30 hours. The dosages of the test groups were 1000, 2000 and 5000 mg/kg, respectively. The negative control was administrated with distilled water and the positive control group was administrated with Mitomycin C (1.5 mg/kg). In each group, five males and five female mice were used. At the interval of 6h after the second administration, the mice were sacrificed.

The marrow in the sternum of the mice was taken, and the marrow suspension was brought onto microscopic slides.

The slides were stained with Giemsa's and examined under the microscope. 1000 polychromatic erythrocytes (PCE) were observed for each animal. The number of cells with micronucleus was counted.

3. Test Result:

Table 2: Data of micronucleus test in bone marrow polychromatic erythrocytes of the mouse with HDGreen® Plus Safe DNA Dye.

Sex	Test substance	Dose [mg/kg]	PCEs examined	PCEs with micronuclei	Incidence of micronuclei per 1000	P
male	HDGreen Plus	1000	5000	4	0.8 ± 0.45	>0.05
		2000		4	0.8 ± 0.45	>0.05
		5000		3	0.6 ± 0.55	>0.05
	Distilled water	-		4	0.8 ± 0.45	-
	Mitomycin C	1.5		135	27.0 ± 1.22	<0.01
female	HDGreen Plus	1000		4	0.8 ± 0.45	>0.05
		2000		4	0.8 ± 0.45	>0.05
		5000		3	0.6 ± 0.55	>0.05
	Distilled water	-		3	0.6 ± 0.55	-
	Mitomycin C	1.5		123	24.6 ± 3.05	<0.01

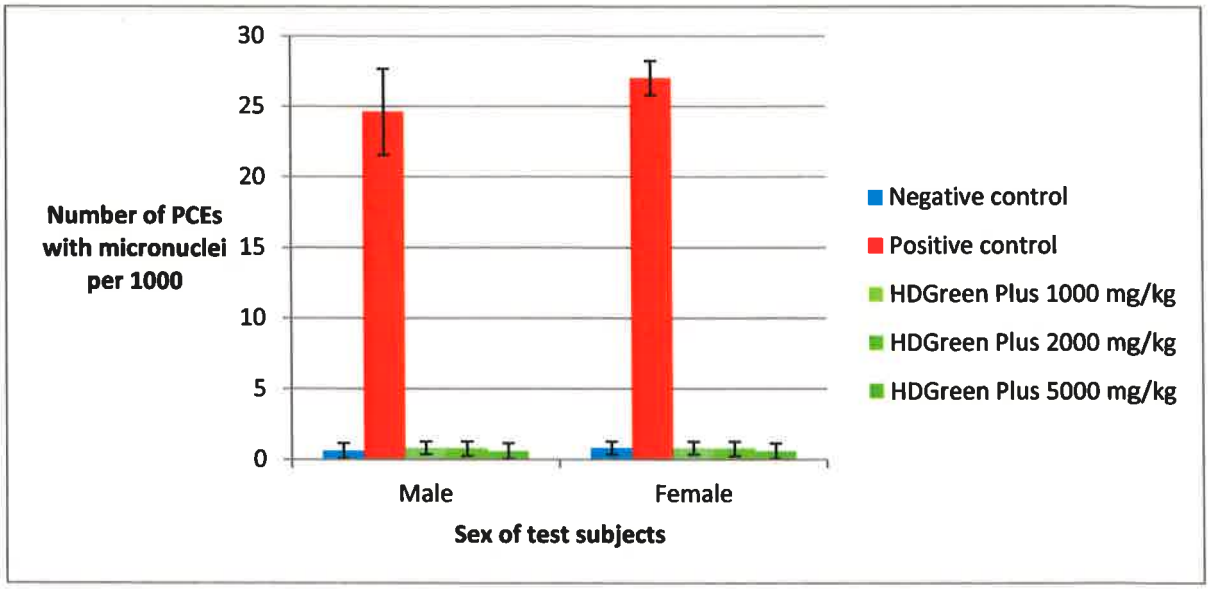

Diagram 2: Data of micronucleus test in bone marrow polychromatic erythrocytes of the mouse with HDGreen® Plus Safe DNA Dye.

4. Conclusion:

The results showed that there was no significant difference ($P>0.05$) in the incidence of micronuclei between the test groups and negative controls while there was a significant difference ($P<0.01$) between Mitomycin C groups and the negative controls. Therefore, according to the guidelines the mouse marrow chromophilous erythrocyte micronucleus test with HDGreen® Plus Safe DNA Dye was negative.

III THE *IN VITRO* MAMMALIAN CHROMOSOME ABBERATION TEST

1. Test System:

The purpose of the in vitro chromosomal aberration test is to identify agents that cause structural chromosome aberrations in cultured mammalian cells. This test was performed to assess the potential of HDGreen® Plus Safe DNA Dye to induce chromosomal aberrations in vitro

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Cell strains: Chinese hamster ovary line.

Metabolic activation system: S9 mixture, which is rat liver homogenate induced by both Phenobarbital sodium and Naphthaflavone and some appropriate cofactors.

Test compound: 5.0 mg/ml of MEM stock solution, separately diluted to different concentration by serum-free culture solution (3 hours, presence and absence of S9 mixture) and MEM medium supplemented with 10% fetal bovine serum (24 hours, absence of S9 mixture).

2. Test Procedure:

Culture solution: MEM medium supplemented with 10% fetal bovine serum and 100 IU/ml of penicillin and streptomycin.

Maximum final concentration determination: It has been shown that the metabolic activation way is +S9 and -S9, and that the action time is 3 hours through two preliminary experiments. We also confirmed the highest final concentration of the -S9 metabolic activation responding at 24 hours for the two different conditions. Both of the two preliminary experiments contained a test group and a blank control group. Measuring cell activity was done with Resazurin, following determination of the final concentration according to the cell inhibiting rate.

Chromosome aberration test: Two kinds of test conditions were used. The first one was metabolic activation +S9 and -S9, action time was 3 hours, the other one was -S9, action time was 24 hours.

Cell harvesting: 4 hours before harvesting, 1.0 μg/ml Colchicin was added followed by hypotonic treatment, fixation, tableting and dyeing with Giemsa. 200 normal metaphase cells were selected in the test compound and the blank control set, and 100 normal metaphase cells were selected in the positive control set. For the analysis the chromosome aberration types and numbers were recorded and the chromosome aberration rate was calculated.

Statistical analysis: ^{2}analysis was performed to compare the chromosome aberration rate of each test compound and of the negative control set.

3. Test Results:

Table 3: Results of the in vitro mammalian cell chromosome aberration test (3 hours, -S9).

Substance	Concentration [μg/ml]	Cells observed	Cells with chromosome aberration	Chromosome aberration rate
Negative control	-	200	3	1,5 %
Mitomycin C	1.0	100	11	11,0 %
HDGreen Plus	1250	200	2	1,0 %
	2500	200	4	2,0 %
	5000	200	1	0,5 %