Midori Green Xtra DNA Stain – Safety Report

IDENTIFICATION OF THE PRODUCT AND OF THE COMPANY	
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Introduction

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

Midori Green Xtra DNA Stain 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 **Midori Green Xtra DNA Stain** has been controlled with two tests:

I. Ames Test
II. Cytotoxicity Test

The result of the Ames test does not show a mutagenic effect of Midori Green Xtra. Additionally, based upon the observed results of the Cytotoxicity test Midori Green Xtra is considered to have no cytotoxic effects either.

I AMES TEST

1. Test System:

The Ames test employed four Salmonella strains, TA97a, TA98, TA100, TA102 and TA1535. 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 metabolised 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.

Midori Green Xtra DNA Stain was dissolved in DMSO, and the concentrations were 0.313, 0.625, 1.25, 2.5 and 5 µL/plate, respectively.

The control groups included solvent control plates (DMSO) and positive control plates. Different positive control articles are used based on different bacteria strains. The description of bacteria strains and individual concentration of mutagens are shown below:

Mutagen	S9	Concentration (µg/plate)	Strain
9-aminoacridine	-	50	TA97a
2-nitrofluorence	-	5	TA98
sodium azide	-	0.4	TA100, TA1535
mitomycin C	-	0.5	TA102
2-aminoanthracene	+	4.0	TA1535, TA102
benzo [a] pyrene	+	4.0	TA98
2-aminofluorene	+	4.0	TA97a, TA100

+: with S9; -: without S9

2. Test Procedure:

2.1. Spot test:

The test was performed by adding test article solution on sterile paper discs, which were placed on *S. typhimurium* plates. Plates were incubated at 37±2°C for 24 to 48 hours and the bacteriostasis ring (cell toxicity) or circularity formed by large number of colonies (mutation) was checked.

2.2. Plate incorporation test:

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 2 mL overlay agar (liquid, 45°C, containing 0.5 mM histidine/biotin). The mixture was uniformly poured on the prepared underlay agar plates. After solidification, the plates were incubated at 37°C for 48 h. At the end of the incubation, revertant colonies per plate were counted. All plating was done in triplicate. The spontaneous mutation colonies of negative control group must be in the reasonable range and the reverse mutation colonies of each positive control must be two times higher than the average of the negative control group.

3. Test Result:

In the spot test there were no obvious bacteriostasis ring (cell toxicity) or circularity formed by large numbers of colonies (mutation) around the disc in each test group, so the 5µL/plate, 2.5µL/plate, 1.25 µL/plate, 0.625 µL/plate and 0.313 µL/plate dosage were used in plate incorporation test. This test showed that whether the rat liver enzyme metabolic system treated or not, all test data were within effective range. Furthermore, the revertant numbers of test article did not appear to be two times more than that of the negative contol groups and did not reach positive reaction criteria.

Test Strain			TA97a		TA98		TA100		TA102		TA1535	
S9			+	-	+	-	+	-	+	-	+	-
Colony Number (CFU/plate)	Negative Control (NC)	plate 1	172	84	50	57	104	135	504	492	10	11
		plate 2	187	85	55	43	114	110	528	412	11	12
		plate 3	205	93	53	49	120	91	736	388	12	16
		Avg	188	87,3	52,7	49,7	112	112	589,3	430,7	11	13
		SD	16,5	4,9	2,5	7	22,1	22,1	127,6	54,5	1	2,6
	2X Avg. Of NC		376	174,7	105,3	99,3	224	224	1178,7	861,3	22	26
	Positive Control	plate 1	412	576	168	640	300	300	1352	1648	171	246
		plate 2	418	624	161	512	272	272	1304	1480	150	262
		plate 3	426	822	152	496	264	264	1256	1464	137	269
		Avg	418,7	666,7	160,3	549,3	278,7	278,7	1304	1530,7	152,7	259
		SD	7	117,9	8	78,9	18,9	18,9	48	101,9	17,2	11,8
	Test Article Solution (µL/plate) 5	plate 1	187	107	54	51	92	92	608	488	16	10
		plate 2	208	101	46	38	93	93	664	428	12	10
		plate 3	209	91	49	35	94	94	800	380	11	10
		Avg	201,3	99,7	49,7	41,3	93	93	690,7	432	13	10
		SD	12,4	8,1	4	8,5	1	1	98,7	54,1	2,6	0
	2,50	plate 1	207	87	46	36	100	53	720	488	13	18
		plate 2	201	85	52	45	106	65	732	416	13	16
		plate 3	195	84	56	47	119	65	752	404	16	16
		Avg	201	85,3	51,3	42,7	108,3	61	734,7	436	14	16,7
		SD	6	1,5	5	5,9	9,7	6,9	16,2	45,4	1,7	1,2
	1,25	plate 1	189	52	26	30	96	65	584	484	22	10
		plate 2	148	54	30	30	96	53	608	480	16	10
		plate 3	131	70	31	34	78	50	680	464	15	11
		Avg	165	58,7	29	31,3	90	56	624	476	17,7	10,3
		SD	29,8	9,9	2,6	2,3	10,4	7,9	50	10,6	3,8	0,6
	0.625	plate 1	210	66	26	30	106	55	592	516	8	10
		plate 2	226	64	26	23	97	57	776	464	9	12
		plate 3	223	56	24	22	89	65	816	460	11	13
		Avg	219,7	62	25,3	25	97,3	59	728	480	9,3	11,7
		SD	8,5	5,3	1,2	4,4	8,5	5,3	119,5	31,2	1,5	1,5
	0.313	plate 1	181	68	24	26	75	59	704	382	14	14
		plate 2	203	64	24	23	90	57	656	384	13	14
		plate 3	235	62	26	20	103	52	624	408	13	14
		Avg	206,3	64,7	24,7	23	89,3	56	661,3	388	13,3	14
		SD	27,2	3,1	1,2	3	14	3,6	40,3	18,3	0,6	0

4. Conclusion:

The results showed that the revertant numbers of the tested reagent „Midori Green Xtra“ appeared to be two times less than that of the negative control group in *Salmonella typhimurium* TA97a, TA100 and TA1535. Midori Green Xtra causes no mutagenic effects to these *Salmonella typhimurium* strains.

II CYTOTOXICITY TEST

1. Test System

The cytotoxicity test is performed according to the requirements described in Biological evaluation of medical devices – Part 5: Tests for *in vitro* cytotoxicity (ANSI/AAMI/ISO 10993-5).
The test is designed to evaluate the acute adverse biological effects of chemical compounds. Cytotoxicity is preferred as a pilot project test and an important indicator for toxicity evaluation as it is simple, fast and has a high sensitivity. This test saves animals from toxicity.

The cell line use for the test is L-929 cell (NCTC clone 929, BCRC RM60091). This cell line was cultured in Eagle's minimum essential medium (MEM) containing 10% fetal bovine serum (FBS) and 2.0 mM L-Glutamine at 37°C and 5% CO_2.

As negative control the cells were treated only with medium. The positive control group was treated with phenol. The test item (Midori Green Xtra was diluted 10,000 fold).

2. Test Procedure

L-929 cells were treated with the test item, negative control or positive control. Triplicate plates are prepared for each treatment.
The cells were incubated for 24 hours and observed microscopically for cytotoxic effects. Cultures were observed under microscopy and grade for reactivity using a 0 to 4 scale.

0= no reactivity	Discrete intracytoplasmatic granules; no cell lysis.
1= slight reactivity	Not more than 20% of the cells are round, loosely attached and without intracytoplasmatic granules; occasional lysed cells are present.
2= mild reactivity	Not more than 50% of the cells are round and devoid of intracytoplasmatic granules; no extensive cell lysis and empty areas between cells.
3= moderate reactivity	Not more than 70% of the cell layers contain rounded cells or are lysed.
4= severe reactivity	Nearly complete destruction of the cell layers.

MTT quantitative analysis:
Culture medium from L929 cells was replaced with culture medium containing the test item, negative control or positive control. After incubation for 24 hours, MTT was added in all wells and incubated for 2 ± 0.5 hours. After incubation, DMSO was added to the wells and read at 570 nm using a spectrophotometer. Mean value of growth inhibition was calculated by the following formula:
Mean value growth inhibition = $100\% + A570_{(\text{Negative control})} - A570_{(\text{positive control or test item})} / A570_{(\text{negative control})}$

3. Results:

Qualitative analysis: Results of morphology of cells after 24 hrs treatment

Figure 1: Morphology of cells treated for 24 hrs. (A) Negative control, culture medium containing 10% FBS. (B) Positive control, culture medium containing phenol. (C) Test item, culture medium containing 10,000 fold diluted test substance. Cells treated with negative control and test item displayed no lysis. Cells treated with positive control were nearly complete destructed of the cell layer.

Result of microscopical evaluation

Treatment group	Treatment duration (hr)	Morphology of cells[a]	Score values[b]
Negative control[c]	24±1	Discrete intracytoplasmaic granules, no cell lysis.	0
Positive control[d]	24±1	Nearly complete destruction of the cell layer.	4
Test item[e]	24±1	Discrete intracytoplasmaic granules, no cell lysis.	0

[a]Triplicate experiments were analyzed for each treatment
[b]Definition of Score value based on ISO10993-5 : 2009
[c]culture medium containing 10% FBS
[d]culture medium containing phenol
[e]culture medium containing 10,000 fold diluted test substance

Results of growth inhibition

Result of MTT quantitative analysis

Treatment group	Absorbance (570 nm)[a]	Mean value growth inhibition [%][b]
Negative control[c]	1.136±0.024	0.0.
Positive control[d]	0.028±0.001*	97.5
Test item[e]	1.049±0.015*	7.6

[a]Triplicate experiments were analyzed for each treatment, absorbance results were shown in Mean ± SD
[b]Mean value growth inhibition = 100% x $A570_{(Negative\ control)}$ – $A570_{(Positive\ control\ or\ Test\ istem)}$ / $A570_{(Negative\ control)}$
If the mean value of test item was less than 0%, data is presented as 0%.
[c]culture medium containing 10% FBS
[d]culture medium containing phenol
[e]culture medium containing 10,000 fold diluted test substance
* Significant different to negative control group (One-Way ANOVA, $p<0.005$)

4. Conclusion

Due to the high sensitivity of the mouse fibroblast growth inhibition test, it is assumed that a mean growth inhibition of up to 30% does not indicate a significant risk of cytotoxicity. Based upon the observed results and under the test-conditions chosen, the test substance “Midori Green Xtra” is considered to have no cytotoxic effects since the grade was zero in microscopical evaluation and mean growth inhibition was 7.6% in the growth inhibition test with L929 mouse fibroblasts.