Research Article

Miniaturization of the Microsuspension Salmonella/Microsome Assay in Agar Microplates

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The Salmonella/microsome assay (Ames test) is the most widely used mutagenicity test for the evaluation of pure chemicals and environmental samples. There are several versions of protocols available in the literature, including those that reduce the amount of sample needed for testing with liquid and agar media. The microsuspension version of the Salmonella/microsome assay is more sensitive than the standard protocol. It is performed using 5-times concentrated bacteria and less sample and S9 mixture, but still uses conventional Petri dishes (90 × 15 mm). It has been extensively used for environmental sample testing, including in effect-directed analysis (EDA). The objective of this study was to miniaturize the microsuspension assay using 12-well microplates instead of the conventional plates. For validation of this miniaturization, thirteen mutagenic compounds were tested using three Salmonella strains that were selected based on their different spontaneous reversion frequencies (low, medium, and high). The conditions of the miniaturized procedure were made as similar as possible to the microsuspension protocol, using the same testing design, metabolic activation, and data interpretation, and the tests were conducted in parallel. The miniaturized plate assay (MPA) and microsuspension procedures provided similar sensitivities although MPA is less laborious and require less sample and reagents, thereby reducing overall costs. We conclude that the MPA is a promising tool and can be particularly suitable for environmental studies such as EDA or monitoring programs. Environ. Mol. Mutagen. 00:000–000, 2018. © 2018 Wiley Periodicals, Inc.

Key words: mutagenicity; effect-directed analysis; monitoring studies; Ames test; MPA; validation

INTRODUCTION

The Salmonella/microsome assay is the most widely used mutagenicity test both for evaluation of pure chemicals and environmental samples and is considered a reliable tool in the toxicology of the 21st century [Claxton et al., 2010]. Some researchers have modified the test conditions and developed reduced volume protocols that are available in the literature [Kado et al., 1983; Brooks, 1995; Flamand et al., 2001; Pant et al., 2016; Proudlock and Evans, 2016]. These modifications provide clear advantages, such as cost-effectiveness, reduced hands-on time, and especially the reduction in S9 mix and sample volume needed.

A reduced version of the standard assay using liquid media, called the microplate fluctuation protocol (MPF), was developed and provided higher sensitivity than the

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standard Ames for chemical substances [Flückiger-Isler and Kamber, 2012], environmental samples [Umbuzeiro et al., 2010], and in effect-directed analysis (EDA) [Muz et al., 2017]. MPF showed good performance in interlaboratory studies [Reifferscheid et al., 2012].

Several other reduced protocols using agar media have been developed [Brooks, 1995; Flamand et al., 2001] which used 2.5 times less sample and 5 times less S9 mix per plate in comparison with the standard protocol [Mortelmans and Zeiger, 2000]. Pant et al. [2016] used five times less sample and Proudlock and Evans [2016] used 20 times less sample and S9 per plate. All agar-based reduced protocols provided good agreement with the standard protocol.

Another reduced version of the Ames test is the microsuspension assay [Kado et al., 1983]. The authors showed that the assay was 10 to 13 times more sensitive than the standard protocol. Agurell and Stensman [1992] tested single chemicals and particulate matter extracts and showed that the microsuspension procedure was 3 to 37 times more sensitive than the standard protocol. Watanabe et al. [1995] observed a greater variation in the sensitivity of the microsuspension procedure (2 to 4,400 times more sensitive) for a specific class of nitro compounds. Černá et al. [1999] found the microsuspension procedure 4 to 8 times more sensitive when testing atmospheric particulate extracts. It is clear that the microsuspension protocol is more sensitive than the standard Ames test protocol but how much depends on the type/class of compound and the conditions/strains employed in the test. It uses fivefold-concentrated bacterial culture, 10 times less sample and S9 mixture, but is performed with conventional Petri dishes (90 × 15 mm). This protocol has been extensively used for environmental testing [Umbuzeiro et al., 2004; Crebelli et al., 2005; Di Giorgio et al., 2011; Alves et al., 2016], and in EDA [Umbuzeiro et al., 2011; Brack et al., 2016].

The aim of this study was to develop a miniaturized version of the Salmonella/microsome microsuspension assay using agar microplates, which would work equally well with strains having low, medium, and high spontaneous mutation frequencies.

MATERIALS AND METHODS

The strategy used was to evaluate a number of mutagenic compounds in parallel in the new protocol, called microplate agar (MPA) and in the microsuspension procedure developed by Kado et al. [1983]. We used the same compounds, tester strains, S9 concentration and pre-incubation conditions in both assays, the difference being in the type of agar plates used; 90 × 15 mm plates for the microsuspension and 12-well microplates (21.4 × 17.5 mm) for the MPA.

Selection of Bacterial Strains, Test Compounds, Controls

Three different strains of *Salmonella enterica* serovar Typhimurium were selected based on their spontaneous mutant frequencies. We wanted to develop a protocol that could be used with strains with low, medium, and high spontaneous frequencies. Typically, in our laboratory TA1538 provides 10–20 colonies per plate, TA98, 20 – 40, and YG1041, 100 – 200 in the microsuspension protocol. TA1538 and TA98 were kindly supplied by Dr. Larry Claxton and, YG1041 by Dr. Takehiko Nohmi.

To compare the potency of both assays we selected thirteen variably mutagenic compounds. Six were tested only without metabolic activation (S9), because they provide higher potencies in this condition and the other seven were tested only with S9 because they require S9 to be mutagenic or provide higher responses under this condition (Table I). We also included some dyes that were expected to provide negative or weak responses with some of the strains used in this study (TA1538 and TA98) to verify the qualitative agreement of both protocols. All compounds were diluted in dimethyl sulfoxide (DMSO) and tested over a series of concentrations which were selected based on the literature, and expressed in ng/μL of the pre-incubation mixture.

The negative control consisted of DMSO and positive controls were included (4-nitroquinoline-1-oxide at 1.25 ng/μL for TA98 and 4-nitro-*o*-phenylenediamine at 25 ng/μL for TA1538 and YG1041 without S9, and 2-aminoanthracene at 6.25 ng/μL for TA1538, TA98 and at 0.3125 ng/μL for YG1041 with S9).

Microsuspension Assay

The microsuspension assay was performed using the protocol developed by Kado et al. [1983] using the buffer concentration recommended by DeMarini et al. [1989]. Overnight cultures were concentrated fivefold

TABLE I. Name, CAS Number, Supplier, and Purity of the Compounds Selected

Compounds		CAS	Supplier	Purity
-S9	1,6-dinitropyrene	42397-64-8	Sigma-Aldrich Co.	98%
	3,6-dinitrobenzo(a)pyrene	847862-64-0	NARD Chemical Ltd.	99%
	1-nitropyrene	5522-43-0	Sigma-Aldrich Co.	99%
	4-nitropyrene	57835-92-4	Tokyo Chemical Industry	97%
	3-nitrofluoranthene	892-21-7	Santa Cruz Biotechnology	94%
	4-nitroquinoline 1-oxide	56-57-5	Sigma-Aldrich Co.	98%
+S9	2-aminoanthracene	613-13-8	Aldrich Chem Co.	96%
	Benzo(a)pyrene	50-32-8	Aldrich Chem Co.	97%
	C.I. Disperse Blue 373	51868-46-3	Shanghai Orgchem Co. Ltd.	95%
	C.I. Disperse Orange 30	12223-23-3	Shanghai Orgchem Co. Ltd.	95%
	C.I. Disperse Orange 37	13301-61-6	Sigma-Aldrich Co.	96%
	C.I. Disperse Violet 93	52697-38-8	Shanghai Orgchem Co. Ltd.	95%
	C.I. Disperse Yellow 3	2832-40-8	Sigma-Aldrich Co.	96%

by centrifugation at 10,000 g at 4°C for 10 min and resuspended in 0.015 M sodium phosphate buffer. Volumes of 50 μL of cell suspension (0.5 to 2 × 10^{10} cells per mL), 50 μL of S9 mix or 0.015 M sodium phosphate buffer, and 2 μL of the sample were added to a tube, mixed by vortexing, and incubated with shaking at 180 rpm at 37°C for 90 min. After pre-incubation, 2 mL of molten agar was added, and the mixture was poured onto a minimal agar plate. Colonies were counted after 66 hr of incubation at 37°C, using an automatic counter. The S9 mix was prepared according to Mortelmans and Zeiger [2000] at a concentration of 5% v/v lyophilized Aroclor-1254-induced rat liver S9 fraction (Moltox Inc, NC). Toxicity was evaluated by observing the background densities of the agar plates. Duplicate plates were used for each concentration tested.

Microplate Agar (MPA) Assay

The microplate Salmonella/microsome assay was developed using 12-well microplates. A scheme with the key steps of the test, including a link to a video, is presented in Figure 1. Overnight cultures were concentrated fivefold by centrifugation at 10,000 g at 4°C for 10 min and resuspended in 0.015 M sodium phosphate buffer. Aliquots of 50 μL of cell suspension (0.5 to 2 × 10^{10} cells per mL) were added to a tube containing 2 μL of the sample then this volume was split into halves, by transferring 26 μL to a new tube. In one tube 25 μL of S9 mix was added and in the other 25 μL 0.015 M sodium phosphate buffer. Both tubes were incubated with shaking at 180 rpm at 37°C for 90 min. After pre-incubation 1 mL of molten agar was added to each tube, and 250 μL of the mixture was distributed in 4 wells (21.4 × 17.5 mm) containing 2.8 mL of minimal agar. Colonies were counted by hand using a stereomicroscope after 66 hr of incubation at 37°C. The maximum number of colonies we could count per plate was 150 due to the small size of the wells. The negative control and the S9 mix used in the experiments were the same as described above. Toxicity was also evaluated using a stereomicroscope. The assay is performed in only one tube that later on is divided into four aliquots and poured into four wells. Therefore four counts are obtained for each concentration tested. We decided to use only one tube to reduce as much as possible the quantity of sample used in the assay. This test design was based on the miniaturized pre-incubation protocol described by Proudlock and Evans [2016] except that they used 3 wells derived from a single treatment and we used 4 wells.

Data Analysis

The data from both assays were analyzed using the Salanal computer program (Integrated Laboratory Systems, Research Triangle Park, NC). An ANOVA including a post hoc test was performed to compare each tested concentration with the respective negative control. Then a linear regression was performed using the Bernstein model [Bernstein et al., 1982]. The linear equation was used to calculate the minimal effective concentration (MEC_2) that provided twice the number of revertants of the negative control and expressed in ng/μL. The lower this value, the more mutagenic is the tested sample. A substance was considered positive when both the ANOVA and linear regression provided significant responses ($P < 0.05$).

RESULTS AND DISCUSSION

The protocol was refined to correct for the problem of using small amounts of sample (no less than 2 μL) and the best result was obtained with 12-well microplates (Fig. 2). 24-well microplates were also tested but they provided a high incidence of zero mutant frequency, especially in the strains with lower reversion frequencies (data not shown). In the 12-well microplates, although we obtained 3.4% wells for TA1538, 2.4% wells for TA98 and 0% for YG1041 with zero revertants, we have not observed more than one well with a zero count for any tested concentration. We also observed that the microplates get less microbial contamination in comparison with the regular Petri dishes, which is an advantage of the miniaturized method.

The MPA and microsuspension protocols showed 100% agreement of the qualitative responses in the 39-paired tests (13 for each of the 3 strains tested). All substances were positive in strains TA98 and YG1041, and 5 were negative in TA1538 in both protocols (Tables II and III and Supporting Information). The concentration response curves showed a similar behavior for each compound in both protocols (Figs. 3–15).

MPA was more sensitive than the microsuspension assay in 12 of the 34 positive responses and less in 15. Identical values were observed in the 7 remaining comparisons, (Tables II and III). The differences between the MEC_2 values obtained in each protocol were in the range of the expected variation from intra- and inter-laboratory studies reported in the literature when the laboratories used the same protocol for the same substance [Claxton et al., 1991a,1991b]. Myers et al. [1987] when analyzing a interlaboratory study found that only slopes that differ by at least one order of magnitude (>10) could be considered statistically different. Therefore, we can't claim that MPA is more or less sensitive than the microsuspension protocol.

The MPA protocol uses 4 times less sample and 4 times less S9 than the microsuspension protocol because although the concentration tested in the preincubation tube is the same in both methods, we use only one tube with half of volume and split it in two for the test with and without S9 in contrast with the microsuspension where 2 tubes with and 2 tubes without S9 are used per concentration. This was done to allow for the maximum reduction of sample quantity to be used in the assay. Because the quantity of sample available for test can be very small in environmental studies, Daiber et al. [2016] used single plates for each dose when testing disinfection byproducts. The reduction of sample volume allows analyzing more sampling sites and the use of different diagnostic strains with the same amount of resources. This is particularly important in monitoring studies, where we want to find hot spots or specific sources of mutagenic discharges [Alves et al., 2016; Umbuzeiro et al., 2016]. Another advantage of the MPA is anticipated in EDA studies where several fractions need to be tested with the objective to find the most potent ones to perform chemical characterizations [Brack et al., 2016]. The MPA protocol could also be applied in specific situations such as drug impurity testing when only few mg of sample are available, with the limitations already pointed out by

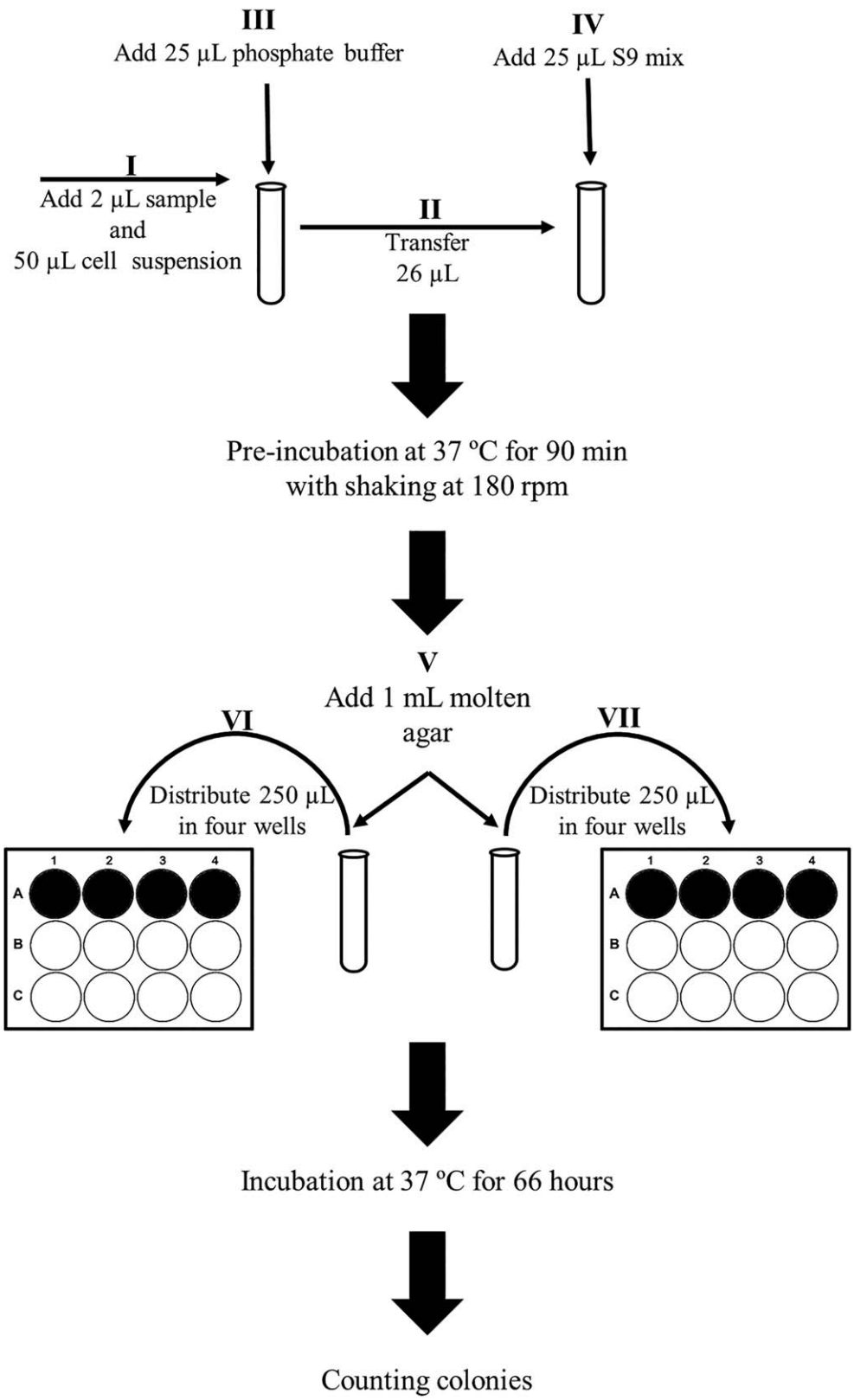

Fig. 1. Diagram depicting the steps to Microplate Agar (MPA) assay. For details watch the video https://www.youtube.com/watch?v=nZFcP_ooqwY.

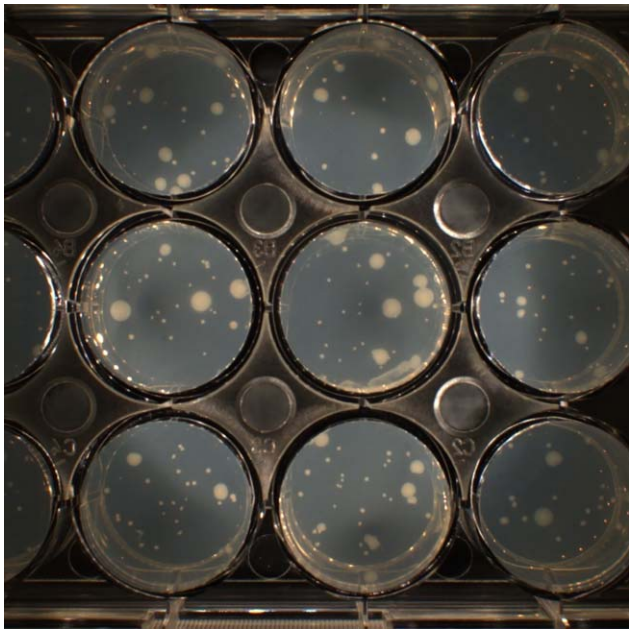

Fig. 2. Picture showing the revertant colonies in the 12-well microplates after 66 hr incubation.

Proudlock and Evans [2016]. The MPA protocol allows for the application of more strains with the same amount of sample used in the microsuspension protocol, enhancing the ability of detection of a mutagenic response for chemicals like C.I. Disperse Yellow 3 (Table III), which are not detected in the standard Salmonella strains. The use of YG1041 in this case allowed the detection of a clear positive response in comparison with TA1538 (negative) and TA98 (weak response).

The negative control means observed for the MPA protocol were one order of magnitude lower than for the microsuspension because of the miniaturization procedure (Fig. 16). In general, lower counts will produce a higher variance in the coefficient of the mean. Indeed, we observed increased coefficient of variation of the mean of the colony counts for the MPA when compared to the microsuspension protocol, especially for the strains with low and medium spontaneous mutation frequencies (Fig. 17). However, this did not influence the qualitative and quantitative responses obtained for the tested compounds (Tables II and III), although the MPA protocol might have a reduced ability to detect weak mutagens when

TABLE II. Minimal Effect Concentration (MEC_2) in ng/μL and MEC_2 Ratio (Microsuspension/MPA) for the Tested Compounds in the Absence of Metabolic Activation (-S9) for MPA and Microsuspension Assay

	Strains/Protocols								
	TA1538			TA98			YG1041		
MEC_2 ng/μL Compounds	MPA	Microsuspension	Ratio	MPA	Microsuspension	Ratio	MPA	Microsuspension	Ratio
1,6-dinitropyrene	0.002	0.005	2.5	0.001	0.001	1.0	0.00003	0.00005	1.7
3,6-dinitrobenz(a)pyrene	0.008	0.007	0.9	0.002	0.004	2.0	0.00005	0.0001	2.0
1-nitropyrene	0.02	0.01	0.5	0.001	0.002	2.0	0.0002	0.0001	0.5
4-nitropyrene	0.005	0.002	0.4	0.004	0.002	0.5	0.0001	0.00009	0.9
3-nitrofluoranthene	0.009	0.002	0.2	0.001	0.001	1.0	0.00001	0.00004	4.0
4-nitroquinoline 1-oxide	0.003	0.003	1.0	0.003	0.003	1.0	0.007	0.006	0.9

TABLE III. Minimal Effect Concentration (MEC_2) in ng/μL and MEC_2 Ratio (Microsuspension/MPA) for the Tested Compounds in the Presence of Metabolic Activation (+S9) for MPA and Microsuspension Assay

	Strains/Protocols								
	TA1538			TA98			YG1041		
MEC_2 ng/μL Compounds	MPA	Microsuspension	Ratio	MPA	Microsuspension	Ratio	MPA	Microsuspension	Ratio
2-aminoanthracene	0.4	0.4	1.0	0.9	1.2	1.3	0.1	0.3	3.0
Benzo(a)pyrene	2.0	1.6	0.8	3.0	4.0	1.3	0.5	0.1	0.2
C.I. Disperse Blue 373	Negative	Negative		0.2	0.16	0.8	0.16	0.12	0.8
C.I. Disperse Orange 30	Negative	Negative		1,100	1,100	1.0	150	180	1.2
C.I. Disperse Orange 37	Negative	Negative		10	17	1.7	2.7	2.0	0.7
C.I. Disperse Violet 93	Negative	Negative		0.4	1.0	2.5	0.7	0.2	0.3
C.I. Disperse Yellow 3	Negative	Negative		>10,000[a]	>10,000[a]		620	460	0.7

[a]Not possible to calculate MEC_2 because the maximum concentration tested did not provided twice the negative control, although ANOVA indicated a positive response.

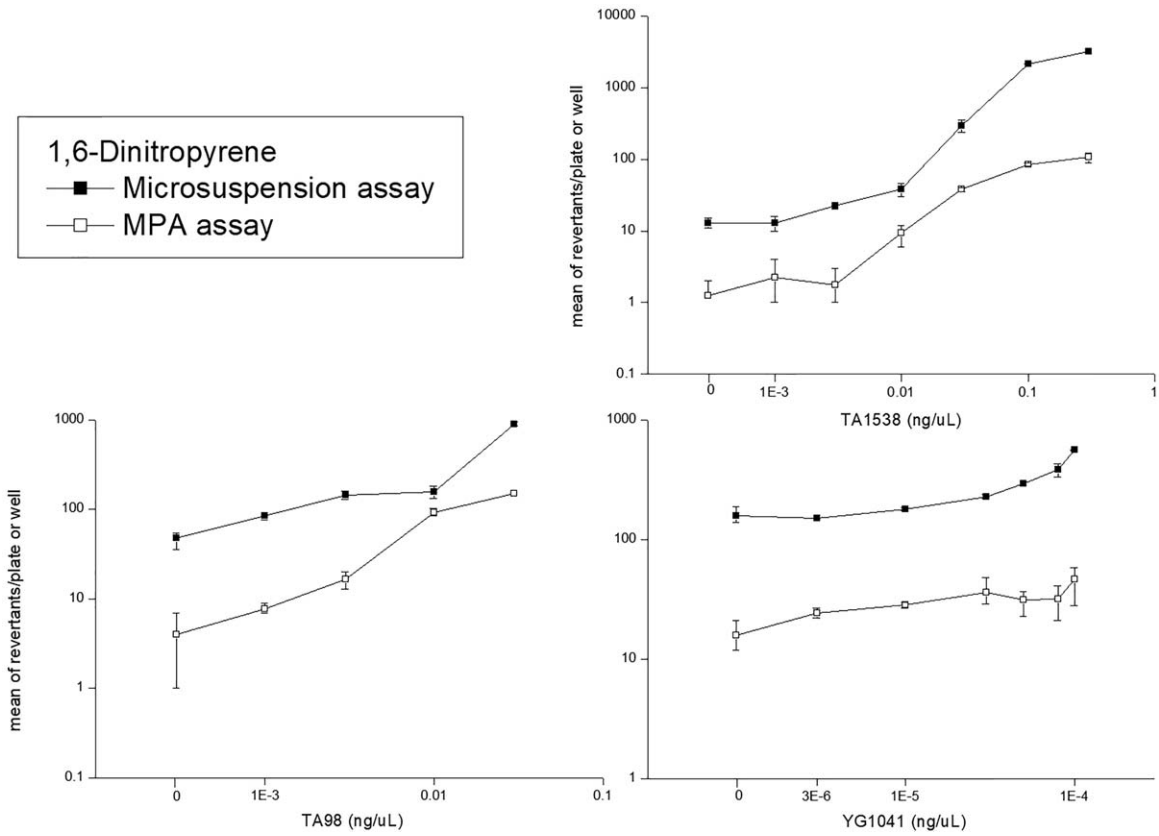

Fig. 3. Response curve, in log-scale, for 1,6-Dinitropyrene using strains TA1538, TA98, and YG1041 without S9 in the microsuspension and MPA protocols.

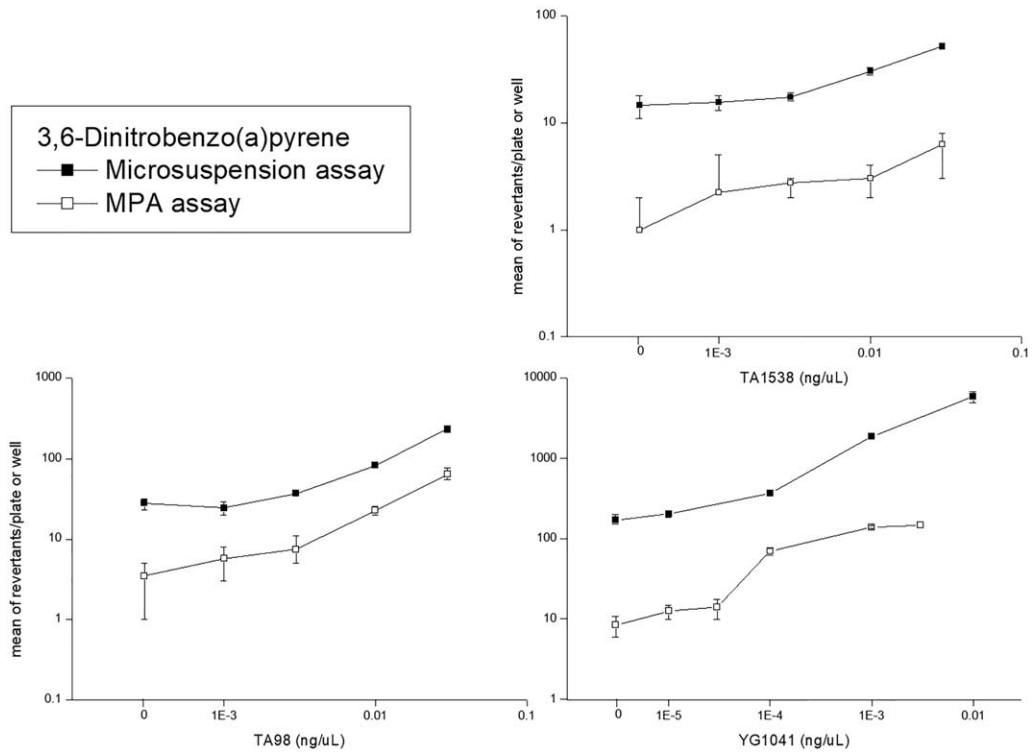

Fig. 4. Response curve, in log-scale, for 3,6-Dinitrobenzo(a)pyrene using strains TA1538, TA98, and YG1041 without S9 in the microsuspension and MPA protocols.

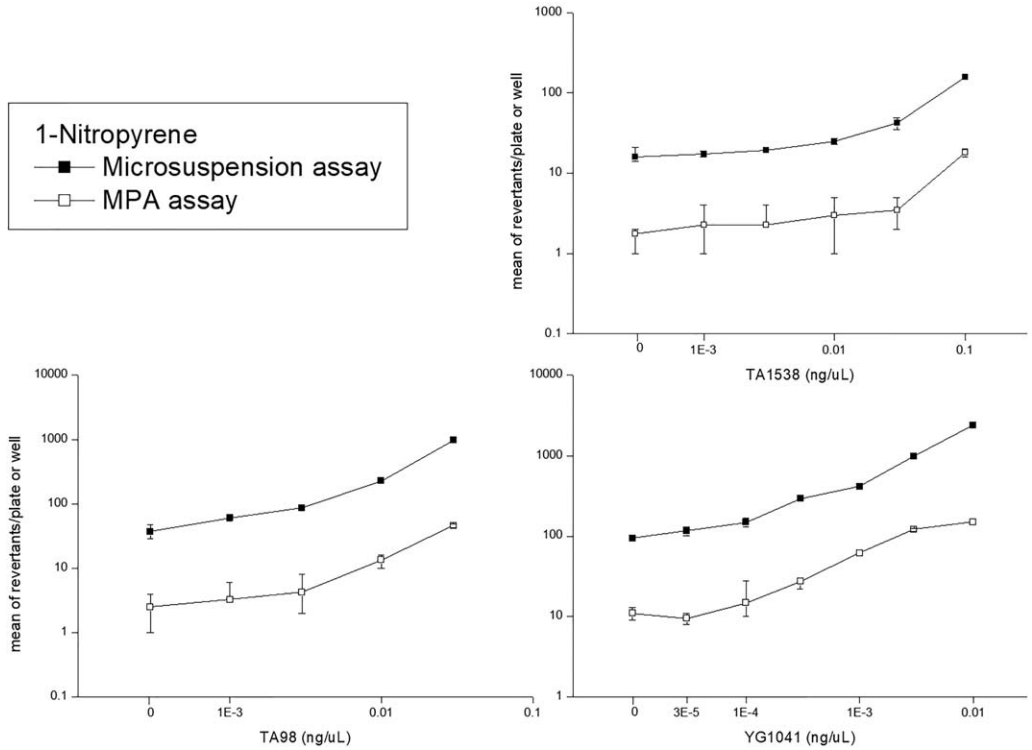

Fig. 5. Response curve, in log-scale, for 1-Nitropyrene using strains TA1538, TA98, and YG1041 without S9 in the microsuspension and MPA protocols.

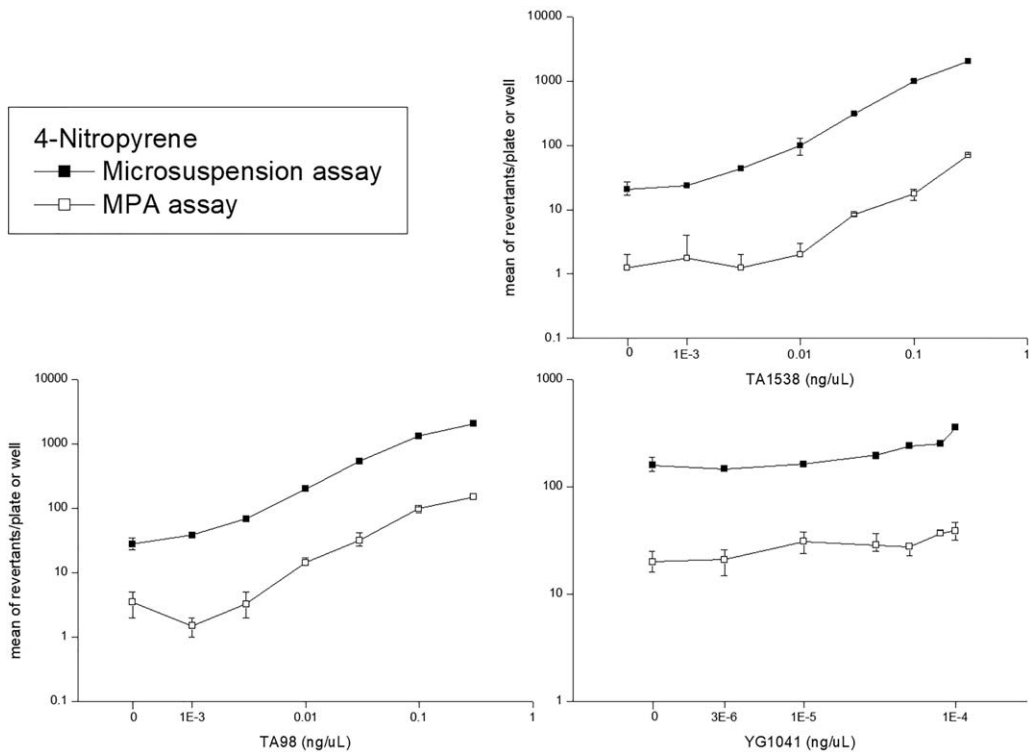

Fig. 6. Response curve, in log-scale, for 4-Nitropyrene using strains TA1538, TA98, and YG1041 without S9 in the microsuspension and MPA protocols.

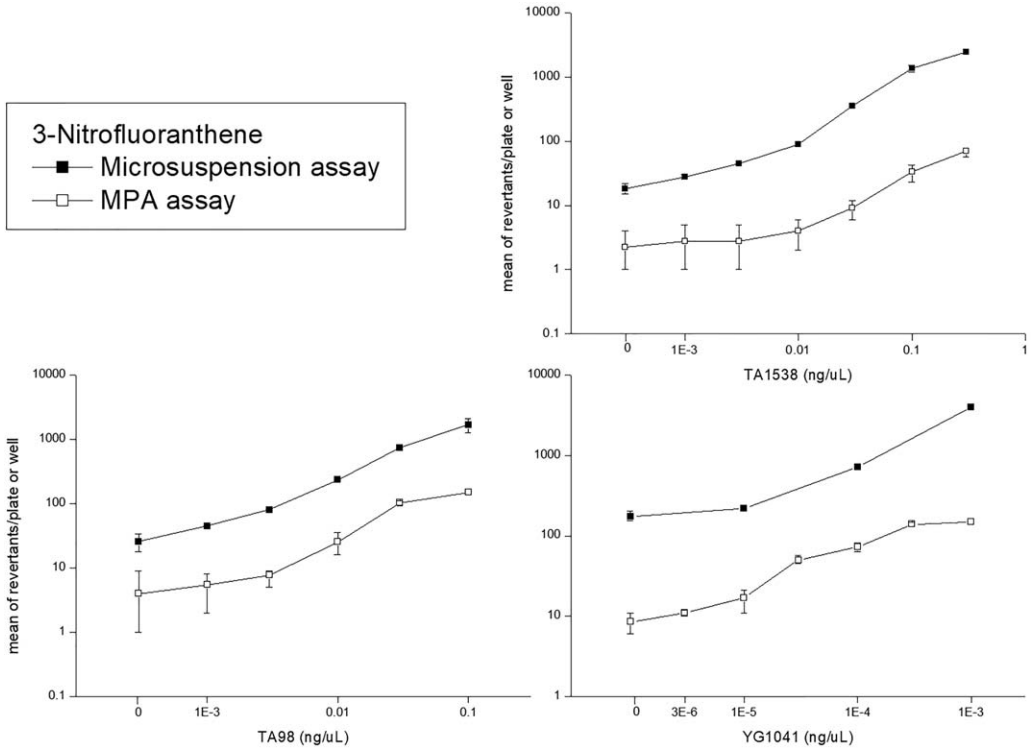

Fig. 7. Response curve, in log-scale, for 3-Nitrofluoranthene using strains TA1538, TA98, and YG1041 without S9 in the microsuspension and MPA protocols.

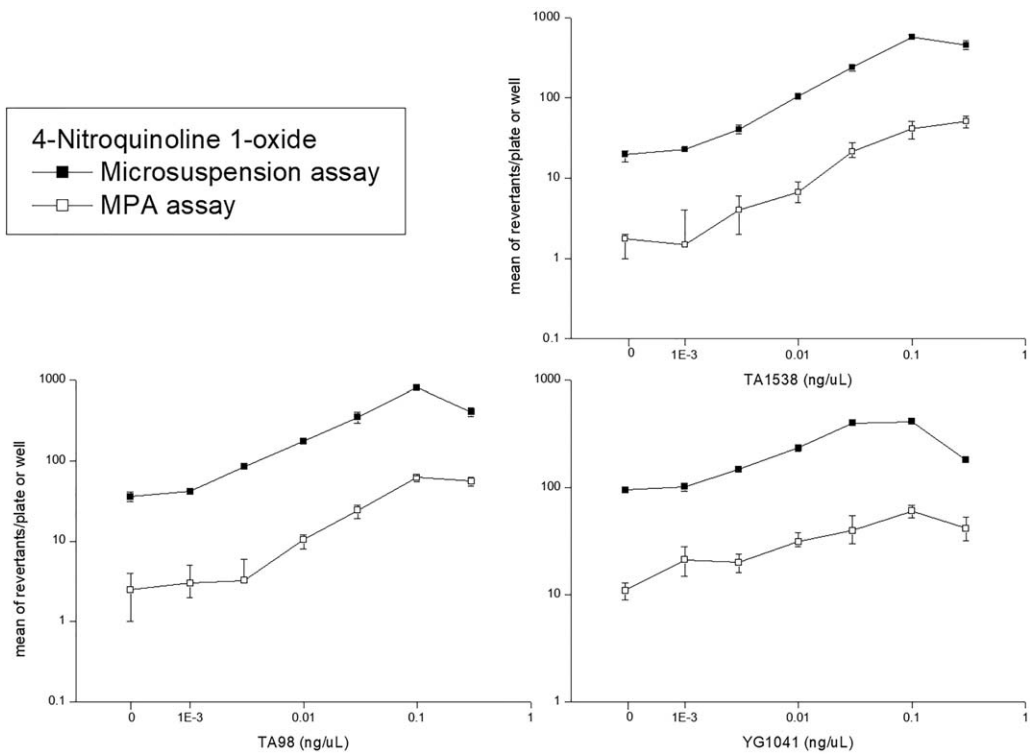

Fig. 8. Response curve, in log-scale, for 4-Nitroquinoline 1-oxide using strains TA1538, TA98, and YG1041 without S9 in the microsuspension and MPA protocols.

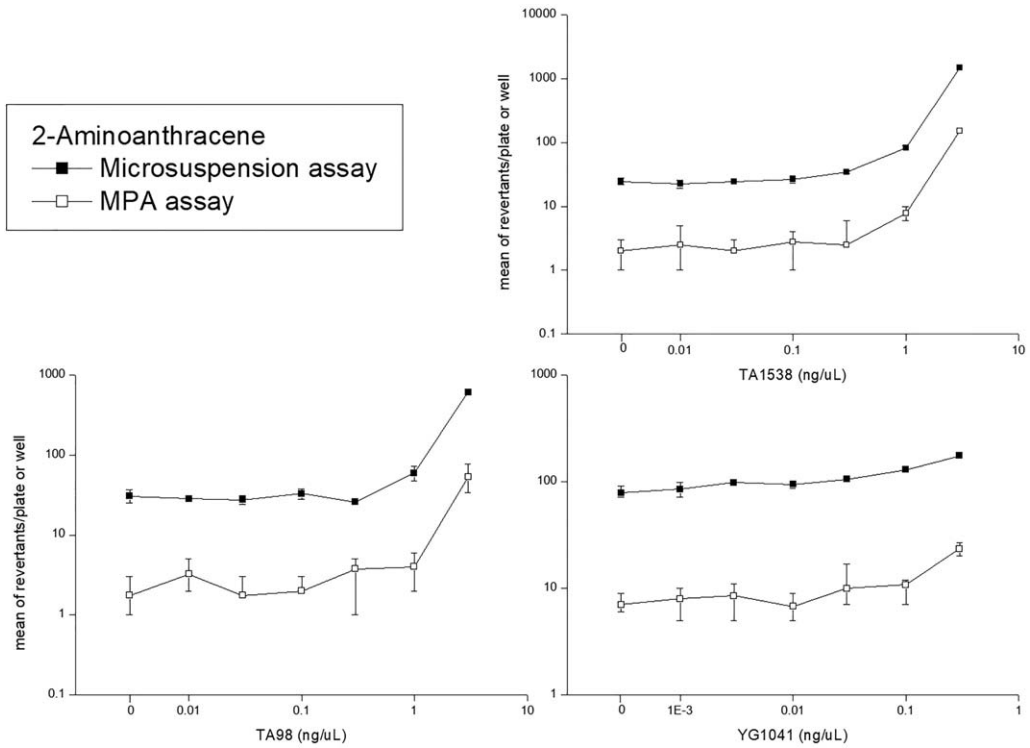

Fig. 9. Response curve, in log-scale, for 2-Aminoanthracene using strains TA1538, TA98, and YG1041 with S9 in the microsuspension and MPA protocols.

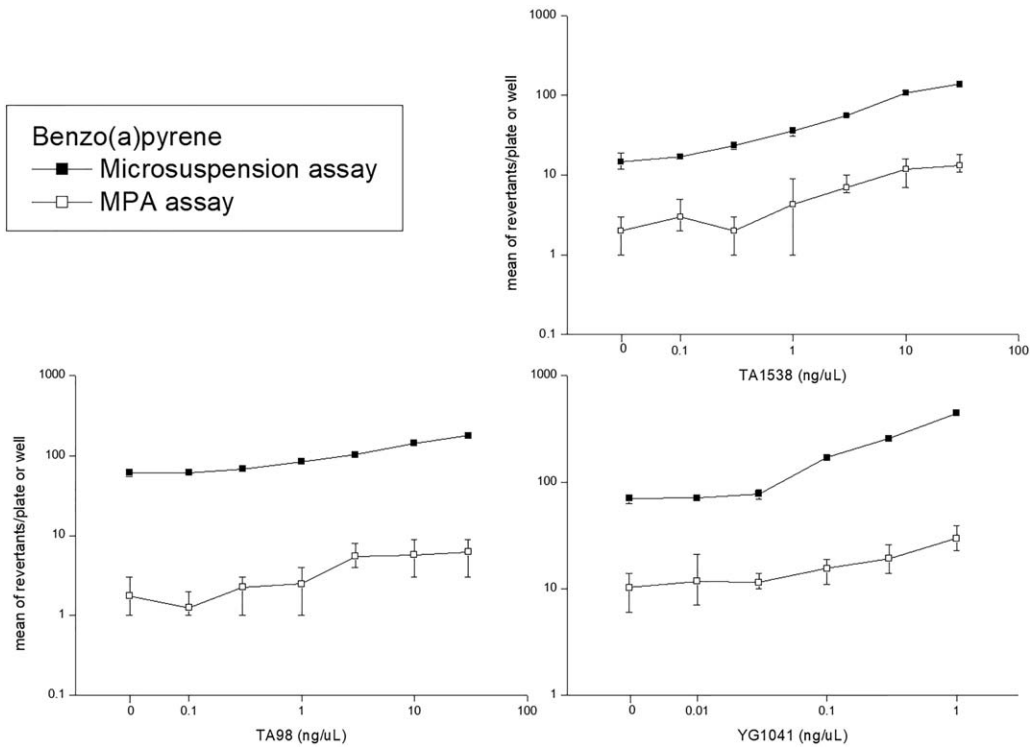

Fig. 10. Response curve, in log-scale, for Benzo(a)pyrene using strains TA1538, TA98, and YG1041 with S9 in the microsuspension and MPA protocols.

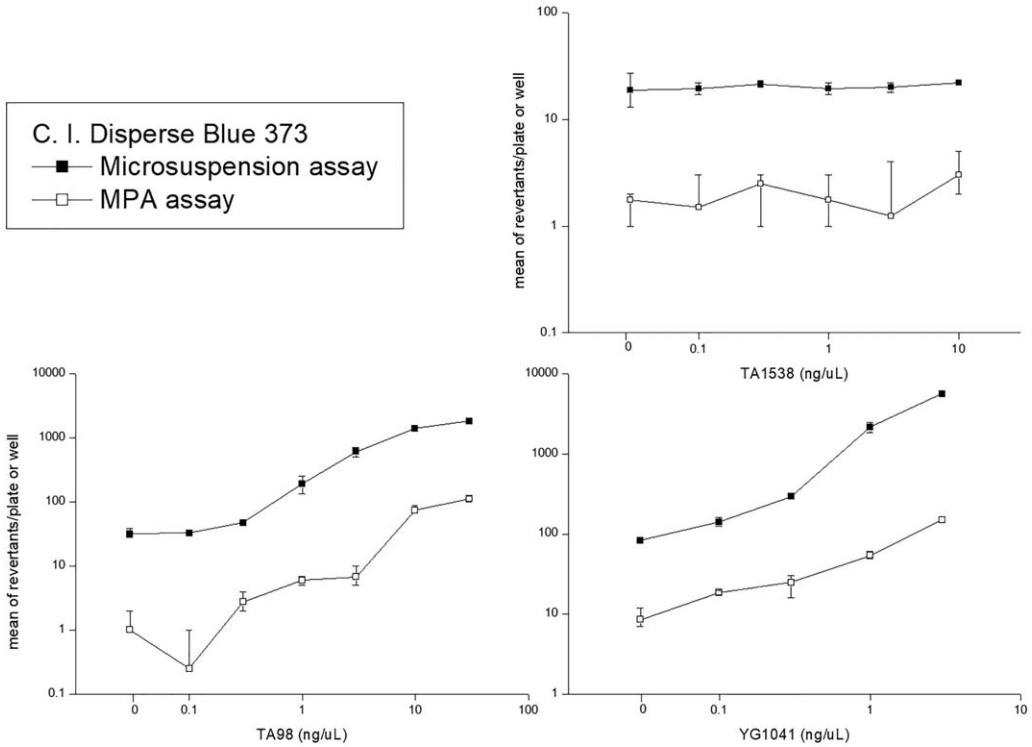

Fig. 11. Response curve, in log-scale, for C.I. Disperse Blue 373 using strains TA1538, TA98, and YG1041 with S9 in the microsuspension and MPA protocols.

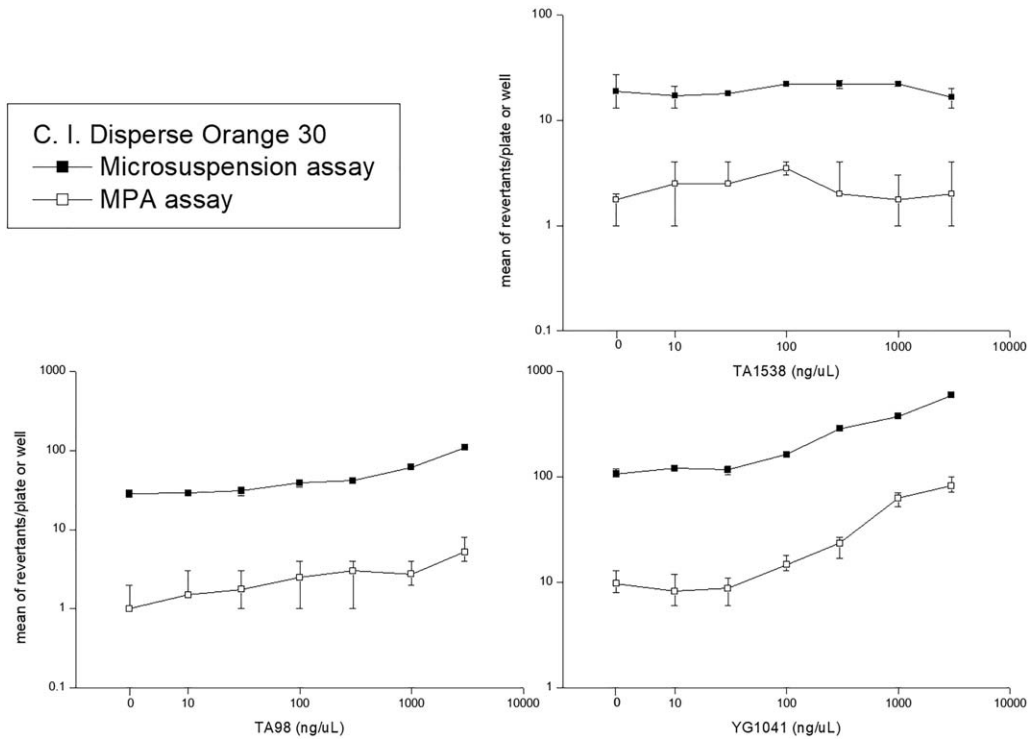

Fig. 12. Response curve, in log-scale, for C.I. Disperse Orange 30 using strains TA1538, TA98, and YG1041 with S9 in the microsuspension and MPA protocols.

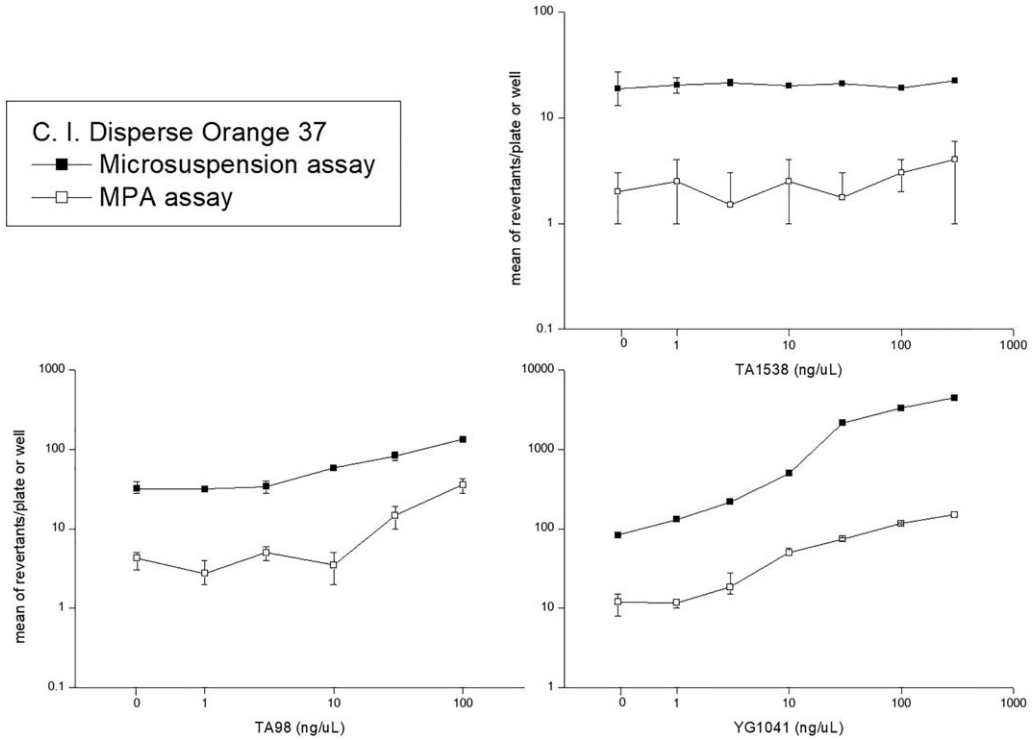

Fig. 13. Response curve, in log-scale, for C.I. Disperse Orange 37 using strains TA1538, TA98, and YG1041 with S9 in the microsuspension and MPA protocols.

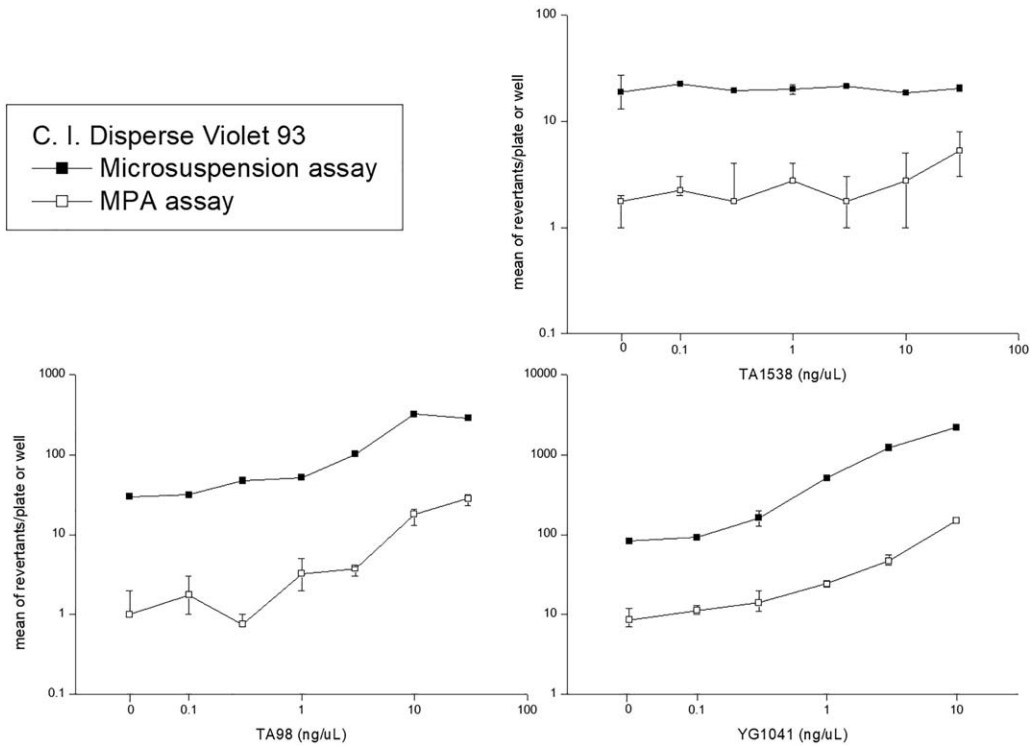

Fig. 14. Response curve, in log-scale, for C.I. Disperse Violet 93 using strains TA1538, TA98, and YG1041 with S9 in the microsuspension and MPA protocols.

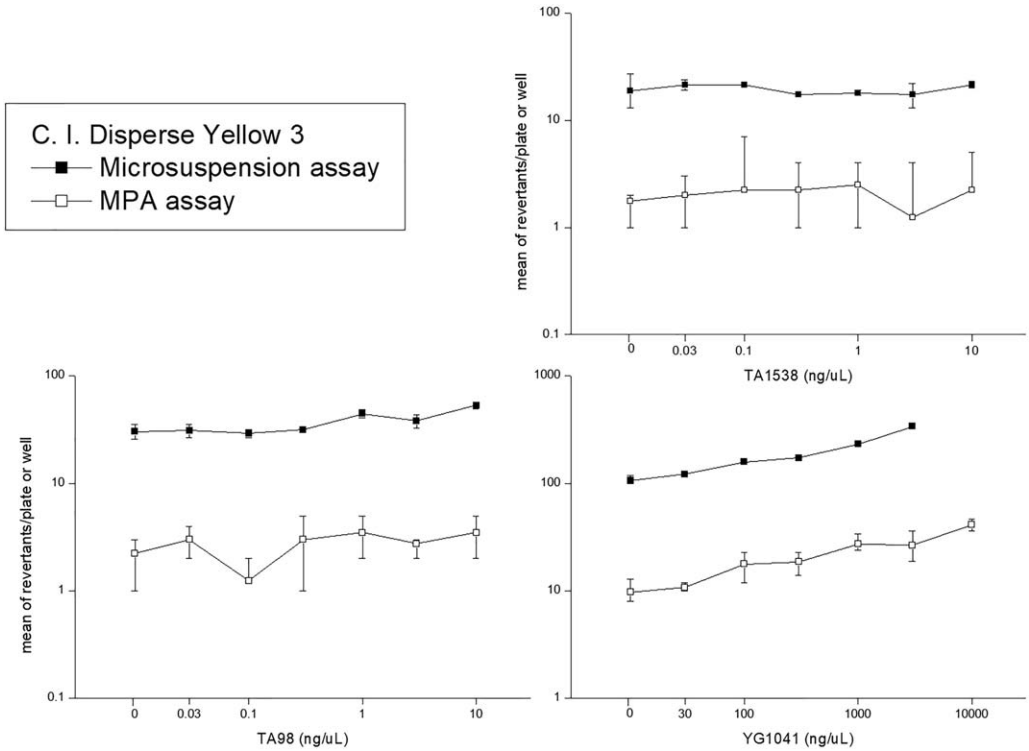

Fig. 15. Response curve, in log-scale, for C.I. Disperse Yellow 3 using strains TA1538, TA98, and YG1041 with S9 in the microsuspension and MPA protocols.

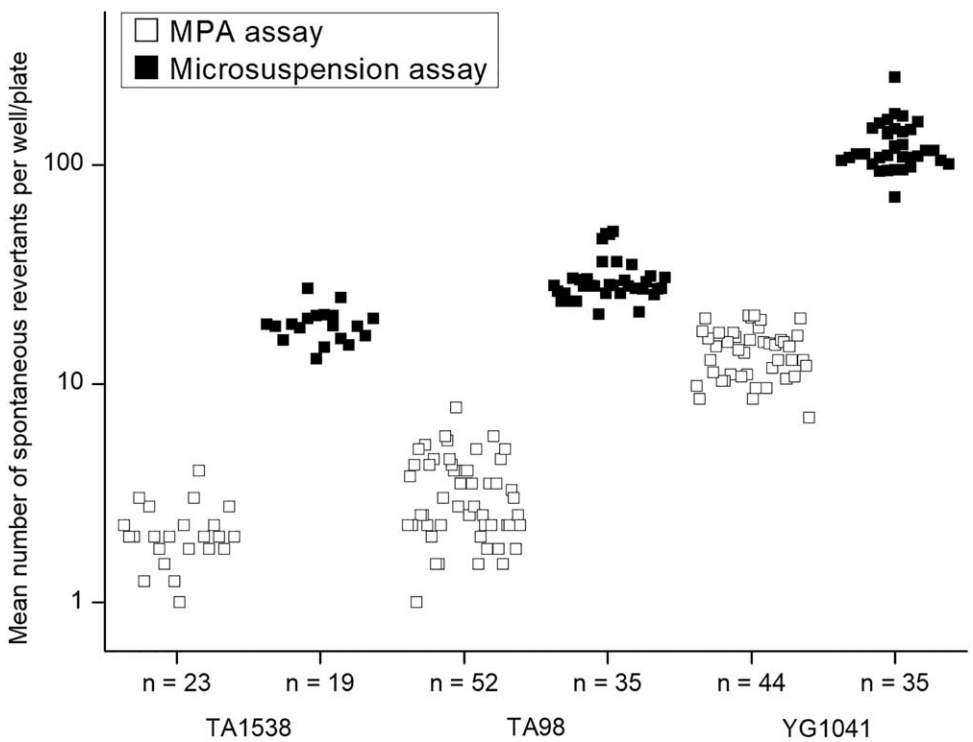

Fig. 16. Mean of revertants per plate/well for the negative controls obtained for the study.

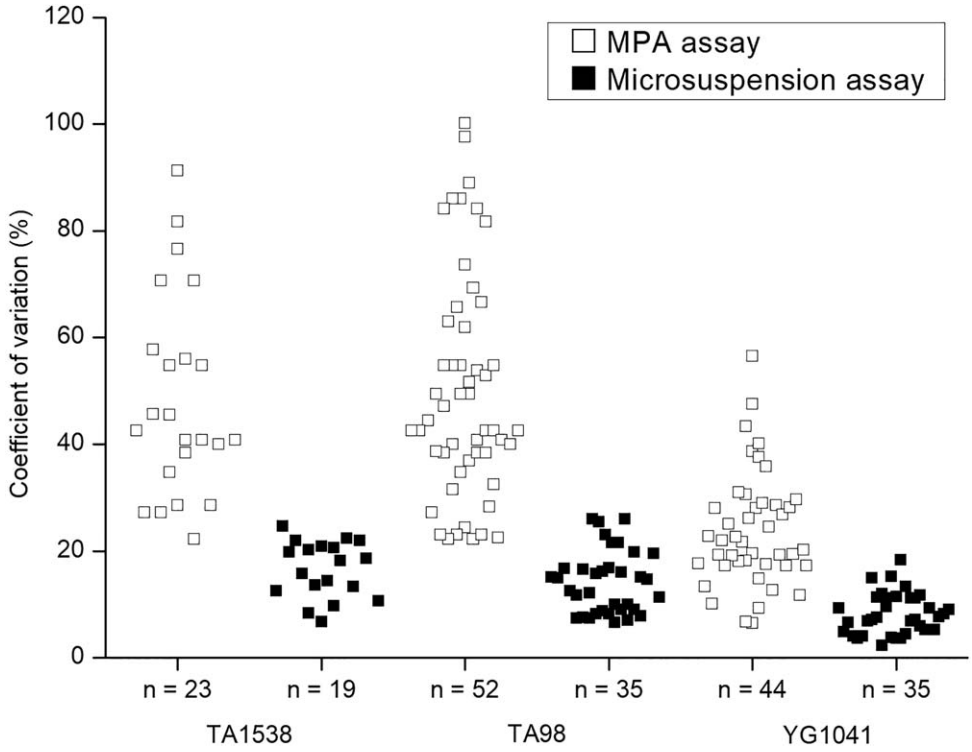

Fig. 17. Coefficients of variation for the negative controls observed for each strain in microsuspension and MPA protocols.

strains with low and medium spontaneous mutant frequencies are used. This remains to be verified, but we assume that the limitation of MPA may be compensated by the reduction of sample quantity and costs provided by the miniaturization procedure. Therefore MPA can be considered a suitable tool especially with strains with higher spontaneous mutant frequencies, e.g. TA100, TA97a, TA102, TA104, and YG1041.

CONCLUSIONS

This is the first study to examine a miniaturized version of the microsuspension Salmonella/microsome assay using agar microplates, and demonstrates that the modified method exhibits a similar sensitivity to the microsuspension assay. The method requires less sample and less reagents in comparison to the microsuspension protocol, thereby reducing overall costs. It can be particularly suitable for environmental studies such as EDA or monitoring programs.

AUTHORS' CONTRIBUTION

Concept of the idea: Umbuzeiro and Brack.

Development of experiments: Zwarg, Morales and Maselli.

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