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...
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

<table>
<thead>
<tr>
<th>Compounds</th>
<th>CAS</th>
<th>Supplier</th>
<th>Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>-S9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1,6-dinitropyrene</td>
<td>42397-64-8</td>
<td>Sigma-Aldrich Co.</td>
<td>98%</td>
</tr>
<tr>
<td>3,6-dinitrobenzo(a)pyrene</td>
<td>847862-64-0</td>
<td>NARD Chemical Ltd.</td>
<td>99%</td>
</tr>
<tr>
<td>1-nitropyrene</td>
<td>5522-43-0</td>
<td>Sigma-Aldrich Co.</td>
<td>99%</td>
</tr>
<tr>
<td>4-nitropyrene</td>
<td>57835-02-4</td>
<td>Tokyo Chemical Industry</td>
<td>97%</td>
</tr>
<tr>
<td>3-nitrofluoranthenene</td>
<td>892-21-7</td>
<td>Santa Cruz Biotechnology</td>
<td>94%</td>
</tr>
<tr>
<td>4-nitroquinoline 1-oxide</td>
<td>56-57-5</td>
<td>Sigma-Aldrich Co.</td>
<td>98%</td>
</tr>
<tr>
<td>+S9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-aminoanthracene</td>
<td>613-13-8</td>
<td>Aldrich Chem Co.</td>
<td>96%</td>
</tr>
<tr>
<td>Benzo(a)pyrene</td>
<td>50-32-8</td>
<td>Aldrich Chem Co.</td>
<td>97%</td>
</tr>
<tr>
<td>C.I. Disperse Blue 373</td>
<td>51868-46-3</td>
<td>Shanghai Orgchem Co. Ltd.</td>
<td>95%</td>
</tr>
<tr>
<td>C.I. Disperse Orange 30</td>
<td>12223-23-3</td>
<td>Shanghai Orgchem Co. Ltd.</td>
<td>95%</td>
</tr>
<tr>
<td>C.I. Disperse Orange 37</td>
<td>13301-61-6</td>
<td>Sigma-Aldrich Co.</td>
<td>96%</td>
</tr>
<tr>
<td>C.I. Disperse Violet 93</td>
<td>52697-38-8</td>
<td>Shanghai Orgchem Co. Ltd.</td>
<td>95%</td>
</tr>
<tr>
<td>C.I. Disperse Yellow 3</td>
<td>2832-40-8</td>
<td>Sigma-Aldrich Co.</td>
<td>96%</td>
</tr>
</tbody>
</table>
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 Proudlow 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., 1991a, 1991b]. Myers et al. [1987] when analyzing an 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.

1. Add 2 μL sample and 50 μL cell suspension
2. Transfer 26 μL
3. Add 25 μL phosphate buffer
4. Add 25 μL S9 mix
5. Pre-incubation at 37 °C for 90 min with shaking at 180 rpm
6. Add 1 mL molten agar
7. Distribute 250 μL in four wells

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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

![Fig. 2. Picture showing the revertant colonies in the 12-well microplates after 66 hr incubation.](image-url)

**TABLE II.** Minimal Effect Concentration (MEC₂) in ng/µL and MEC₂ Ratio (Microsuspension/MPA) for the Tested Compounds in the Absence of Metabolic Activation (−S9) for MPA and Microsuspension Assay

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Strains/Protocols</th>
<th>TA1538</th>
<th>Microsuspension</th>
<th>Ratio</th>
<th>TA98</th>
<th>Microsuspension</th>
<th>Ratio</th>
<th>YG1041</th>
<th>Microsuspension</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,6-dinitropyrene</td>
<td>MPA</td>
<td>0.002</td>
<td>0.005</td>
<td>2.5</td>
<td>0.001</td>
<td>0.001</td>
<td>1.0</td>
<td>0.00003</td>
<td>0.00005</td>
<td>1.7</td>
</tr>
<tr>
<td>3,6-dinitrobenz(a)pyrene</td>
<td>Microsuspension</td>
<td>0.008</td>
<td>0.007</td>
<td>0.9</td>
<td>0.002</td>
<td>0.004</td>
<td>2.0</td>
<td>0.00005</td>
<td>0.0001</td>
<td>2.0</td>
</tr>
<tr>
<td>1-nitropyrene</td>
<td>MPA</td>
<td>0.02</td>
<td>0.01</td>
<td>0.5</td>
<td>0.001</td>
<td>0.002</td>
<td>2.0</td>
<td>0.00002</td>
<td>0.0001</td>
<td>0.5</td>
</tr>
<tr>
<td>4-nitropyrene</td>
<td>Microsuspension</td>
<td>0.005</td>
<td>0.002</td>
<td>0.4</td>
<td>0.004</td>
<td>0.002</td>
<td>0.5</td>
<td>0.0001</td>
<td>0.00009</td>
<td>0.9</td>
</tr>
<tr>
<td>3-nitrofluoranthene</td>
<td>MPA</td>
<td>0.009</td>
<td>0.002</td>
<td>0.2</td>
<td>0.001</td>
<td>0.001</td>
<td>1.0</td>
<td>0.00001</td>
<td>0.00004</td>
<td>4.0</td>
</tr>
<tr>
<td>4-nitroquinoline 1-oxide</td>
<td>Microsuspension</td>
<td>0.003</td>
<td>0.003</td>
<td>1.0</td>
<td>0.003</td>
<td>0.003</td>
<td>1.0</td>
<td>0.007</td>
<td>0.006</td>
<td>0.9</td>
</tr>
</tbody>
</table>

**TABLE III.** Minimal Effect Concentration (MEC₂) in ng/µL and MEC₂ Ratio (Microsuspension/MPA) for the Tested Compounds in the Presence of Metabolic Activation (+S9) for MPA and Microsuspension Assay

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Strains/Protocols</th>
<th>TA1538</th>
<th>Microsuspension</th>
<th>Ratio</th>
<th>TA98</th>
<th>Microsuspension</th>
<th>Ratio</th>
<th>YG1041</th>
<th>Microsuspension</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-aminoanthracene</td>
<td>MPA</td>
<td>0.4</td>
<td>0.4</td>
<td>1.0</td>
<td>0.9</td>
<td>1.2</td>
<td>1.3</td>
<td>0.1</td>
<td>0.3</td>
<td>3.0</td>
</tr>
<tr>
<td>Benzo(a)pyrene</td>
<td>Microsuspension</td>
<td>0.9</td>
<td>1.2</td>
<td>1.3</td>
<td>3.0</td>
<td>4.0</td>
<td>1.3</td>
<td>0.5</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>C.I. Disperse Blue 373</td>
<td>Negative</td>
<td>0.2</td>
<td>0.16</td>
<td>0.8</td>
<td>1.00</td>
<td>1.100</td>
<td>1.0</td>
<td>0.16</td>
<td>0.12</td>
<td>0.8</td>
</tr>
<tr>
<td>C.I. Disperse Orange 30</td>
<td>Negative</td>
<td>1.100</td>
<td>1.100</td>
<td>1.0</td>
<td>1.0</td>
<td>1.7</td>
<td>1.7</td>
<td>1.50</td>
<td>1.80</td>
<td>1.2</td>
</tr>
<tr>
<td>C.I. Disperse Orange 37</td>
<td>Negative</td>
<td>1.0</td>
<td>1.7</td>
<td>1.7</td>
<td>0.7</td>
<td>0.2</td>
<td>0.3</td>
<td>1.7</td>
<td>2.0</td>
<td>0.7</td>
</tr>
<tr>
<td>C.I. Disperse Violet 93</td>
<td>Negative</td>
<td>0.4</td>
<td>1.0</td>
<td>2.5</td>
<td>0.4</td>
<td>1.0</td>
<td>2.5</td>
<td>0.7</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>C.I. Disperse Yellow 3</td>
<td>Negative</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>620</td>
<td>460</td>
<td>0.7</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

*Not possible to calculate MEC₂ 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.
3-Nitrofluoranthene
- Microsuspension assay
- MPA assay

4-Nitroquinoline 1-oxide
- Microsuspension assay
- MPA assay

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.
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.
Data analysis and Interpretation of results: Zwarg, Morales, Maselli, Brack, Umbuzeiro.
Writing the manuscript: Zwarg, Morales, Umbuzeiro.

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