

Microplate Agar (MPA) – A Promising Miniaturized Protocol for the Ames Test

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Statement of Purpose: During the development of new chemicals, biological and environmental biomaterials it is very important to test them to determine their possible hazardous effects. With this in mind, mutagenicity tests were developed in the 1970s and have evolved in subsequent years. Currently they are mandatory for all chemical, biomaterial and product registration by several health and environmental agencies around the world. Many tests are available, using cell free DNA to mammals. Among those assays, the Salmonella/microsome mutagenicity test, also called the Ames test, is the most popular and accepted assay throughout the world. This is an *in vitro* test, which uses bacteria and an exogenous metabolic activation system from mammals. The classical assay uses petri dishes and requires at least 500 mg to 1 g of test material. When testing new products, products under development, products for regulatory approval, leachates or extractable impurities, the quantity of test specimen is often the limiting factor.

To address this point, our research group recently developed a miniaturized version of the micro-suspension Salmonella/microsome assay (Kado et al, 1983) using 12-well microplates instead of conventional plates (Zwarg et al, 2018). Thirteen different compounds were tested in parallel using strains with low, medium and high revertant frequencies. The protocol, named the “Miniaturized Plate Assay” (MPA), provided equivalent sensitivity to the micro-suspension protocol, but it is less laborious and requires less test specimen and reagents, thus reducing the overall cost. This method can be applied to all the strains recommended by OECD and others, with specific diagnostic capabilities. This miniaturized protocol of the Ames test is now being discussed in a special OECD Standards Committee. We therefore expect a specific guideline on this method soon. As an example of how this MPA technique might be applied, the present work aims to verify the ability of this assay to assess the mutagenicity of 17 new chemical dyestuffs from the Max Weaver library recently donated to North Carolina State University (Kuenemann et al, 2017).

Methods: The MPA protocol was performed by adding to a tube, 50 µL of 5X concentrated cell suspension and 2 µL of the test specimen. Then 26 µL of this mixture was transferred to another tube containing 25 µL of sodium phosphate buffer, and 25 µL of S9 mix added to the first tube. After pre-incubation at 37°C for 90 min with shaking, 1 mL of molten agar was added to each tube, and 250 µL of the mixture was distributed in four microplate wells containing 2.8 mL of minimal agar. Colonies were counted by hand using a stereo-microscope after 66h of incubation at 37°C. Negative and positive controls were included. Toxicity was evaluated by careful observation

of the background of the microplates using a stereo-microscope. We used 6 strains capable of identifying different types of mutations and sensitive to different classes of compounds in the absence and presence of S9 mix containing rat liver S9 (5%) and cofactors. Single-dose experiments were performed, and the dyestuffs that were positive were re-tested in concentration response experiments for confirmation. One-way ANOVA followed by Tukey and linear regression were used to analyze the data. Results were expressed as positive and negative.

Results: From the 17 dyestuffs, 8 were mutagenic. The most sensitive strain was YG1041, followed by TA97a>TA102=TA100>TA98. YG7108 did not detect any mutagenic response. Negative and positive controls were reproducible and provided the expected variance coefficient. Less than 20 mg of test specimen was used for the completion of the MPA tests.

Strains/ Dyes	YG1041		TA98		TA97A		TA102		TA100		YG7108	
	Without S9	With S9	Without S9	With S9	Without S9	With S9	Without S9	With S9	Without S9	With S9	Without S9	With S9
1												
2												
3												
4												
5												
6												
7												
8	x	x	x	x	x	x					x	
9		x			x	x	x	x				
10	x	x										
11					x	x						
12	x	x										
13	x	x										
14												
15												
16										x	x	
17												
Legend	x											

Figure 1. Mutagenic response obtained for 17 dyestuffs tested with 6 strains with and without metabolic activation (S9).

Conclusions: We concluded that the MPA technique was successful in identifying mutagenicity of new compounds using less than 20 mg of test specimen and 6 different strains with and without metabolic activation. Its application saved time, money and material, in comparison to the standard Ames test. We believe that this MPA approach can be used for the testing of new biomaterials, including their precursors, leachables and extractables, and so provide early identification of their potential mutagenicity.

References:

Kado et al. Mutation Research 1983;121(1):15-32
Kuenemann et al. Chemical Science 2017;8:4334-4339
Zwarg et al. Environmental & Molecular Mutagenesis 2018;59(6):488-50.

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