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# Application of miniaturized Ames assays to assess mutagenicity of nitrosamine impurities

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## Introduction

Nitrosamines are a class of chemical compounds, many of which are potent carcinogens found in various industrial, agricultural, and consumer products, including tobacco smoke and processed meats. Their formation typically involves the reaction of nitrites and secondary amines. Due to their potential health risks, particularly their ability to induce genetic mutations and cancer, rigorous genotoxicity testing is essential. This testing includes in vitro assays like the Ames test to evaluate DNA damage and mutagenic potential. Understanding and mitigating nitrosamine exposure is crucial for public health safety and regulatory compliance.

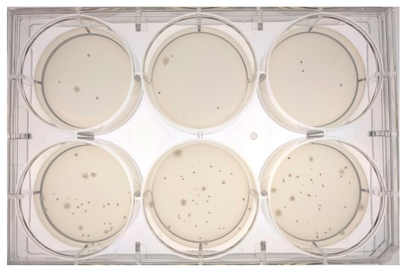
## Important highlights

- Mutagenicity of the Nitrosamine impurities can be evaluated using both agar-based and liquid miniaturized Ames test systems
- The miniaturized Ames assay in the agar-based 6-well plate format is compatible with the pre-incubation protocol and the increased S9 concentration in line with regulatory recommendation
- Volatility of short alkyl chain nitrosamines is addressed with modified protocols
- Miniaturized Ames assays can detect Nitrosamines at lower doses compared with the Petri dish-based traditional agar plate test

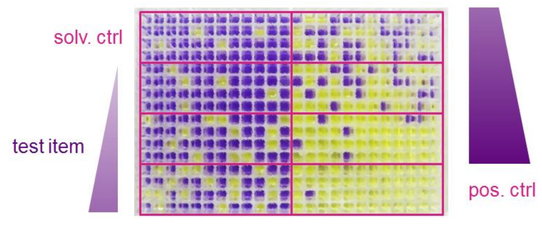
## Methods

We used overnight cultures of the Ames tester strains followed by 14 hours of incubation at 37°C shaking at 400 rpm. Following the **overnight incubation** the OD600 value was measured and the cell number was determined using a cell counting chamber. The overnight culture served as an input to the **miniaturized Ames assays**, either diluted or undiluted, for the 6-well plate format or the liquid microplate fluctuation format, respectively.

The **Xenometrix MicroAmes6** is an agar-based miniaturized Ames test in 6-well plate format. The test substance is considered positive if there is a 2-fold or higher fold induction over the negative control in the number of revertant colonies.

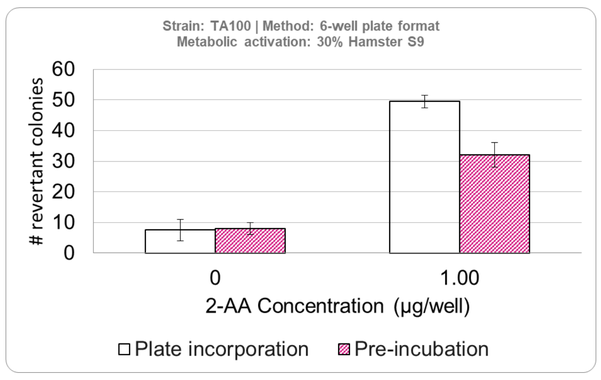



The **Xenometrix Ames MPF™** is a microplate fluctuation assay. The readout is color-based. If there is no reversion event, the well is purple, while the reversion is indicated by yellow coloration.



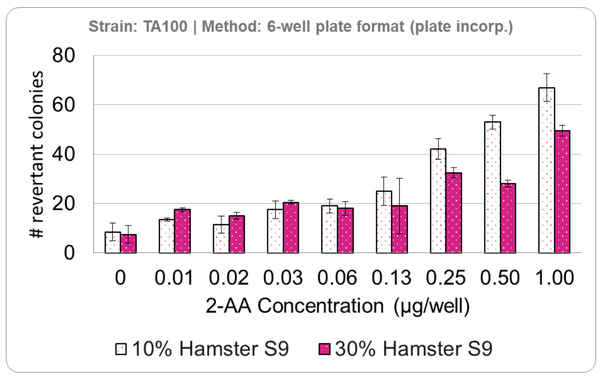

## Results

### Implementation of the pre-incubation protocol for the agar-based miniaturized Ames Test in 6-well plate format




The pre-incubation protocol is more suitable to test Nitrosamines compared to the plate incorporation protocol. Therefore, we decided to implement the pre-incubation protocol in a miniaturized Ames test in 6-well plate format. We found that the negative and positive control values were similar between the two protocols. The other miniaturized assay format, the Ames MPF follows the pre-incubation concept by default, thus no adaptation of the original protocol was necessary.

### Compatibility of the increased S9 concentration with the miniaturized Ames test in 6-well plate format




For the metabolic activation of substances in the miniaturized 6-well plate format generally 10% rat or hamster liver S9 is utilized. Regulatory recommendation includes the testing of nitrosamines at higher S9 concentration, i.e. 30% rat or hamster liver S9. For the Ames MPF assay 30% S9 is applied by default. The application of 30% hamster S9 is compatible with the miniaturized Ames assay in 6-well plate format suggested by the comparable elicited fold induction towards 2-Aminoanthracene (2-AA) positive control.

## Volatility of the short alkyl chain Nitrosamines

### Estimating the potential volatility of short alkyl chain Nitrosamines

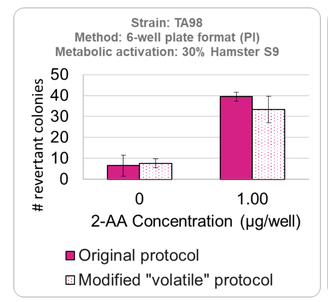
The boiling point (°C) of selected nitrosamines is used to evaluate the potential volatility of the compounds. The nitrosamines are presented in the increasing order of their molecule weight in the accompanying table. The boiling point-based estimation suggests that most of the short alkyl chain Nitrosamines are prone to volatility, being either volatile or semivolatile.

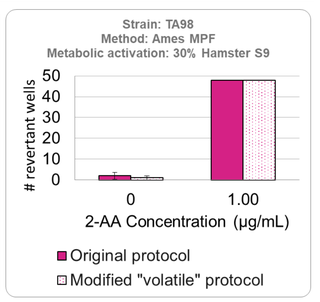
| Test compound | CAS | Molecule weight (g/mol) | Boiling point (°C) | Estimated volatility |
|---|---|---|---|---|
| N-Nitrosodimethylamine | 62-75-9 | 74.08 | 151 | Volatile |
| N-nitrosodiethylamine | 55-18-5 | 102.14 | 172 | Volatile |
| N-nitrosodipropylamine | 621-64-7 | 130.19 | 206 | Volatile |
| N-Nitrosodiethanolamine | 1116-54-7 | 134.13 | 114 | Volatile |
| N-nitrosodibutylamine | 924-16-3 | 158.24 | 235 | Volatile |
| N-Nitrosodiphenylamine | 86-30-6 | 198.22 | 101 | Volatile |
| N-Nitrosodicyclohexylamine | 947-92-2 | 210.32 | 350 | Semivolatile |

The following classification based on boiling point (BP) was applied: BP <100°C very volatile; 75°C<BP<250°C volatile; 250°C<BP<390°C semivolatile, 390°C<BP nonvolatile. Information about the physical-chemical properties was gained from [1]. The applied classification is based on [2].

### Why is it important to address the volatility of test substances in the Ames test?

- The effective concentration of the test substance might be lowered due to partial evaporation from the test system
- Concurrent negative and positive controls in the neighboring wells might be affected resulting in invalid results
- Occupational hazard: the operator of the assay can be exposed to volatile toxic substances

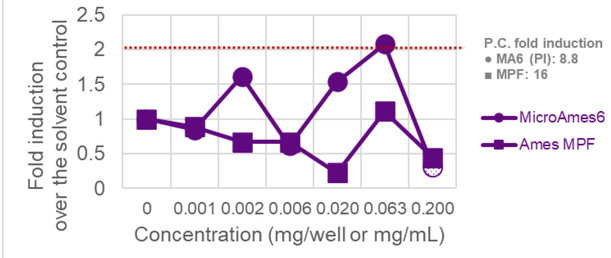


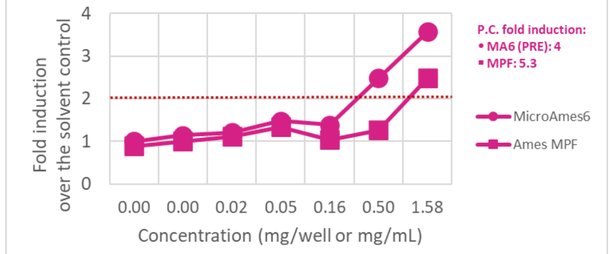

Graphs representing the comparison of the test performance with the original and the modified protocol, which is optimized for the testing of volatile substances. Both the 6-well plate format and the Ames MPF assays can be performed under the modified assay circumstances without affecting the negative or positive control performance.

## Performance of the miniaturized Ames assays in Nitrosamine testing

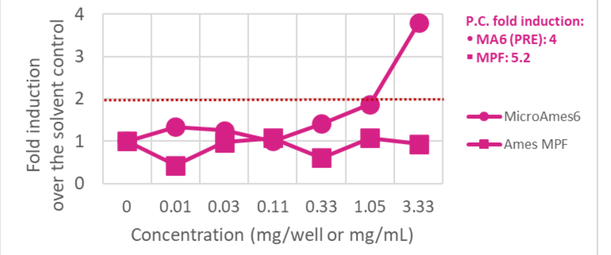
N-Nitrosodiphenylamine (CAS: 86-30-6)
**TA98 + 10% or 30% Rat Liver S9**



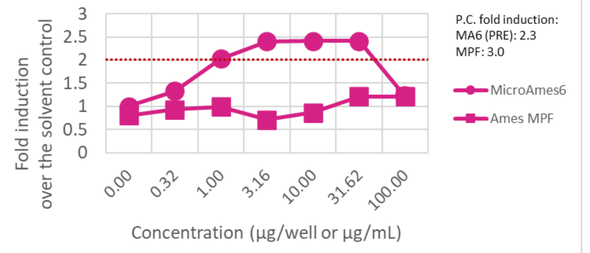

N-nitrosodiethylamine (CAS: 55-18-5)
**TA100 +30% Hamster S9**




N-Nitrosodiethanolamine (CAS: 1116-54-7)
**TA100 +30% Hamster S9**




N-Nitrosodimethylamine (CAS: 62-75-9)
**E.coli uvrA[pKM101] +30% Hamster Liver S9**




All experiments performed in the presence of rat or hamster liver S9 at the indicated concentrations. Fold induction over the solvent control in the number of revertants is presented as the function of the varying concentration of the Nitrosamine test substances. Circles represent the fold induction values of the MicroAmes6 experiment, while squares represent the Ames MPF fold induction values. Dashed red line is the threshold for positivity (2-fold induction). Concurrent positive control values are indicated next to the graphs. PI: plate incorporation protocol, PRE: pre-incubation protocol. The summary table shows the results of the miniaturized assays in comparison with Petri dish-based agar plate data from the NTP database or scientific literature. LEC = lowest effective concentration, which is the lowest concentration at which the fold induction over the solvent control exceeds the pre-defined threshold for mutagenicity (threshold is set to 2-fold); NEG = negative; POS = positive; EQ = equivocal; N.A. = not applicable. The LEC is provided as µg/well for MicroAmes6, µg/mL for Ames MPF and µg/plate for the Petri dish-based agar plate test. To ensure comparability between the different Ames test systems the concentration of the test compounds in the pre-incubation mix must be taken into consideration: The compound concentration is 1.6x higher (in µg/mL) in the pre-incubation mix of the Petri dish-based Agar plate test, and 7x higher (µg/mL) in the pre-incubation mix of MicroAmes6.

**Summary Table**

| Compound | CAS Nr. | Strain | Metabolic activation | MicroAmes6 | | Ames MPF | | Petri dish-based Agar Plate Test [NTP, Literature] | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Result | LEC [µg/well] | Result | LEC [µg/mL] | Result | LEC [µg/plate] |
| N-Nitrosodiphenylamine | 86-30-6 | TA98 | 10-30% Rat S9 | EQ | 63 | NEG | N.A. | NEG [3] | N.A. |
| N-nitrosodiethylamine | 55-18-5 | TA100 | 30% Hamster S9 | POS | 500 | POS | 1580 | POS [3] | 1000 |
| N-Nitrosodiethanolamine | 1116-54-7 | TA100 | 30% Hamster S9 | POS | 3333 | NEG | N.A. | POS [3] | 10000 |
| N-Nitrosodimethylamine | 62-75-9 | E.coli uvrA[pKM101] | 30% Hamster S9 | POS | 1 | NEG | N.A. | POS [4] | 150 |

## Conclusion

Our data suggests that the miniaturized Ames assays are applicable to reliably assess the mutagenicity of nitrosamines. The results obtained with the miniaturized Ames assays and the Petri dish-based Ames data are in good concordance for the tested nitrosamines. Further experiments are required with adjustments of compound concentration in the Ames MPF system. The miniaturized Ames tests can also be considered as resource-efficient assays that can be readily applied for the mutagenicity assessment of nitrosamines. Furthermore, the high-throughput testing provided by the miniaturized Ames assays can facilitate the generation of robust mutagenicity data on nitrosamine impurities, thus a better understanding of the concerns associated with it, and ultimately, the establishment of straightforward risk assessment strategies.

References:
[1] Kim S, Chen J, Cheng T, et al. PubChem 2023 update. Nucleic Acids Res. 2023;51(D1):D1373–D1380. doi:10.1093/nar/gkac956
[2] Menezes, Helvécio & Amorim, Leiliane & Cardeal, Zenilda. (2013). Sampling and Analytical Methods for Determining VOC in Air by Biomonitoring Human Exposure. Critical Reviews in Environmental Science and Technology. 43. 10.1080/10643389.2011.604239.
[3] National Toxicology Program (NTP) coordinated by United States Department of Health and Human Services
[4] Thomas DN, Wills JW, Tracey H, Baldwin SJ, Burman M, Williams AN, Harte DSG, Buckley RA, Lynch AM. Ames test study designs for nitrosamine mutagenicity testing: qualitative and quantitative analysis of key assay parameters. Mutagenesis. 2024 Mar 12;39(2):78-95. doi: 10.1093/mutage/gead033. PMID: 38112628; PMCID: PMC10928841.

The **Ames MPF** and **MicroAmes6** are proprietary miniaturised Ames assays developed by Xenometrix AG.
Check out our homepage **xenometrix.ch** for further information, and follow us on social media.