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Optimized Miniaturized Ames Assays for the Evaluation of the Mutagenicity of Nitrosamines

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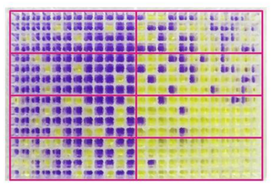
Introduction

N-nitrosamines are a class of chemical compounds recognized for their potent mutagenicity and carcinogenicity, arising as contaminants in various pharmaceutical preparations, food packaging material or in environmental samples. The testing of nitrosamines for mutagenic properties is critical for public health, necessitating an array of methodologies such as the Ames test, which has served as a benchmark for **mutagenicity assessment since decades**. Recent investigations have solidified the understanding of key parameters influencing **assay sensitivity**, specifically examining nitrosamines like N-nitrosodimethylamine (NDMA) and N-nitrosodiethylamine (NDEA). Enhanced sensitivities have been achieved through qualitative and quantitative analyses, pinpointing effective experimental conditions for accurate mutagenicity detection [1]. The formation of nitrosamines can occur during common processes, such as water purification, where secondary amines react under oxidative conditions, resulting in potent mutagenic by-products [2]. Considerable concerns have emerged surrounding the presence of these substances in pharmaceuticals, with contaminants potentially leading to critical safety issues. The regulatory landscape has been adapting in response; for instance, the FDA and EMA have heightened scrutiny of manufacturing processes to mitigate nitrosamine formation. The regulatory recommendation includes the application of the **Enhanced Ames Test (EAT)** principle with the recommendation to use higher S9 percentage – and to include **Hamster Liver S9** in addition to Rat Liver S9 [3,4].

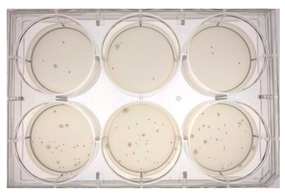
Methods: parallel running miniaturized Ames tests from the same overnight bacterial culture

Ames MPF™



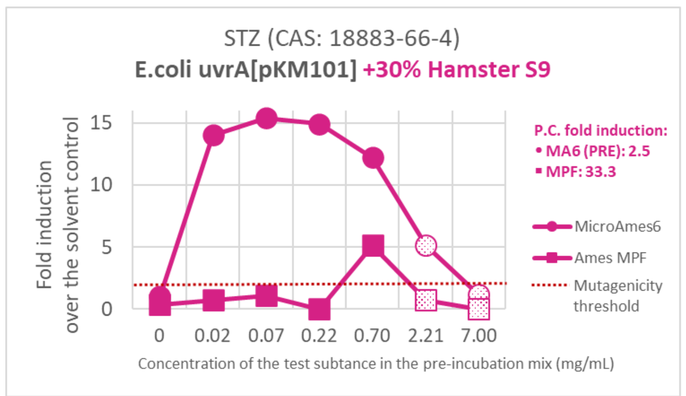
- Miniaturized Ames test in microplate fluctuation format
- Ames tester strains: TA100, TA1535, E.coli uvrA[pKM101]
- Water was used as solvent, unless solubility was an issue → DMSO
- Metabolic activation: 30% Hamster Liver S9 (in accordance with EAT)
- Input bacterial cell density: 10^8 cells per mL
- 25x concentrated stock solution of Nitrosamine test compounds
- Pre-incubation for 90 minutes – concentration was adjusted to have the same test compound concentration between the two miniaturized systems

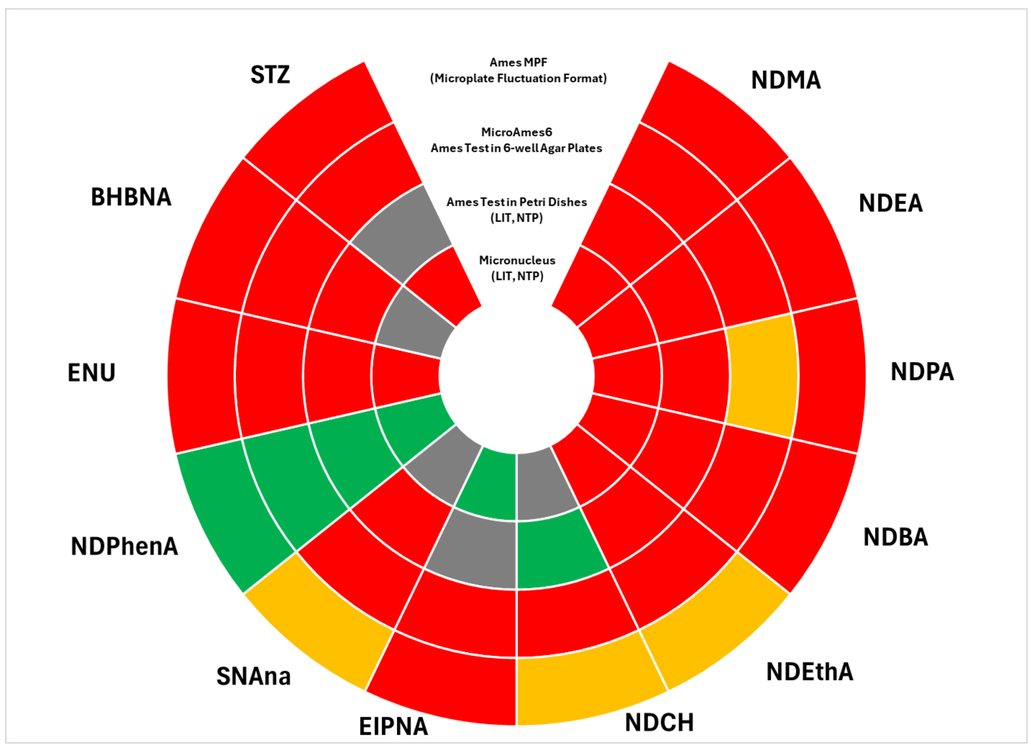
Pre-incubation MicroAmes6

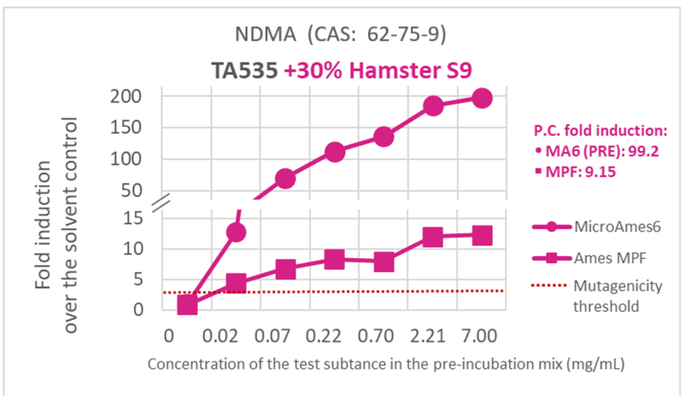


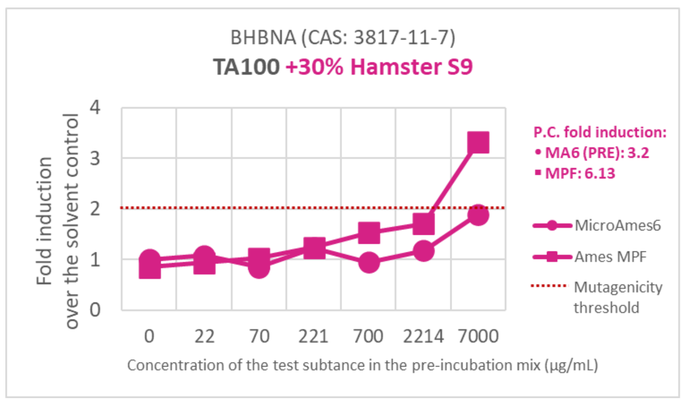
- Miniaturized Ames test in 6-well agar plate format with a pre-incubation step
- Ames tester strains: TA100, TA1535, E.coli uvrA[pKM101]
- Water was used as solvent, unless solubility was an issue → DMSO
- Metabolic activation: 30% Hamster Liver S9 (in accordance with EAT)
- Input bacterial cell density: 10^7 cells per mL
- 50x concentrated stock solution of Nitrosamine test compounds
- Pre-incubation for 30 minutes – concentration was adjusted to have the same test compound concentration between the two miniaturized systems

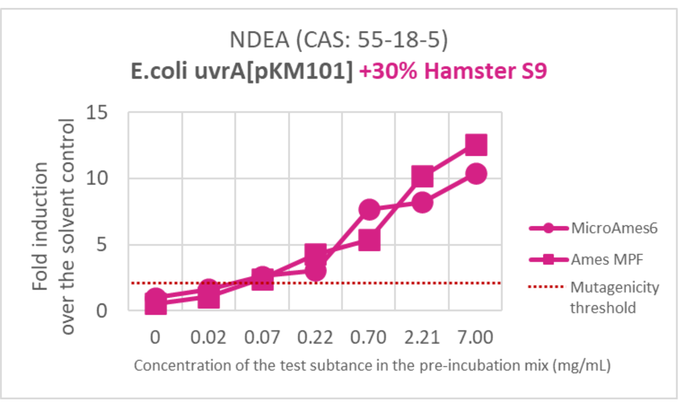
Results

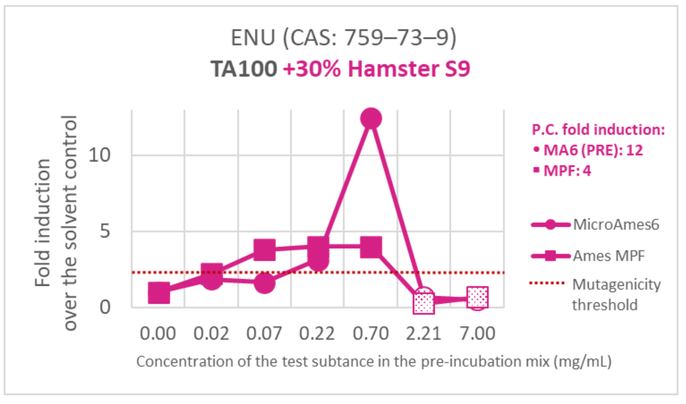


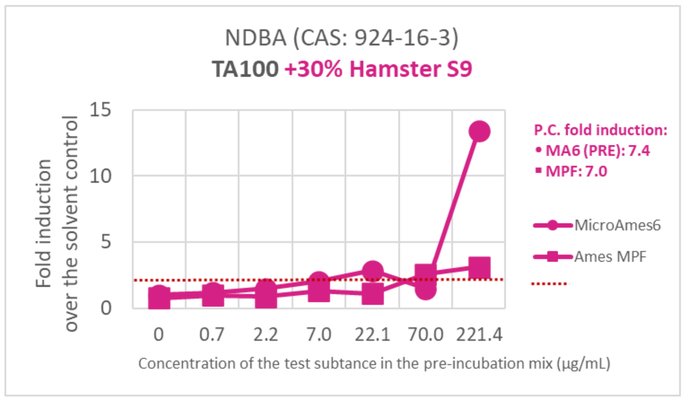


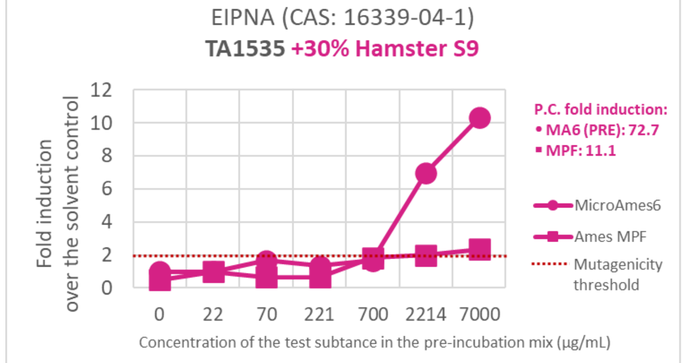


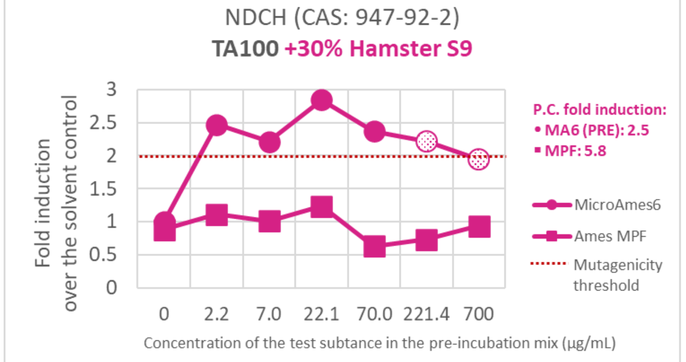












▲ Summary of the study and comparison of the miniaturized Ames test results with Petri dish-based Ames test data and micronucleus data from the NTP database [5] or scientific literature. For the miniaturized Ames assays we present cumulative test result, i.e. a compound with one positive result in one Ames tester strain is assessed as a positive compound. The compound is negative if it is tested negative with the miniaturized Ames assays in all three tester strains. The compound is equivocal if it is tested equivocal in the miniaturized Ames assays with at least one Ames tester strain, but all other strains are negative. For the Petri dish-based Ames test, the cumulative call was based on all available results published in the NTP database [5] or in the literature. In vitro or in vivo micronucleus test results for the corresponding Nitrosamines were collected from the NTP database [5]. Color codes: Red = positive, yellow = equivocal, green = negative, grey = not available.

◀ Selected experimental results from the study showing the performance of the miniaturized Ames assays ▶ All experiments were performed in the presence of 30% hamster liver S9. Test substance concentration in both miniaturized assay system correspond to the concentration of the test substance in the total volume during pre-incubation. The concentration of the Nitrosamine test substances were adjusted to have exactly the same effective concentration in the reaction mix during pre-incubation in both miniaturized Ames assays. Fold induction over the solvent control in the number of revertant wells or revertant colonies is presented as the function of the varying concentration of the Nitrosamine test substances, for Ames MPF and for MicroAmes6, respectively. Dashed red line is the threshold for positivity. Circles and squares with dotted pattern represent cytotoxic concentrations. Concurrent positive control fold induction values are indicated next to the graphs. MA6 = MicroAmes6, Ames assay in 6-well agar plate format; MPF: Ames test in microplate fluctuation format; PRE: pre-incubation protocol.

▼ Summary table of the 12 Nitrosamines with the abbreviations used, their corresponding CAS-number, molecule weight, boiling point and volatility properties. The volatility was estimated based on the molecule weight and the boiling point.

Test compound	Abbreviation	CAS	Molecule weight (g/mol)	Boiling point (°C)	Volatility
N-Nitrosodimethylamine	NDMA	62-75-9	74.08	151	Volatile
N-nitrosodiethylamine	NDEA	55-18-5	102.14	172	Volatile
N-Nitrosoethylisopropylamine	EIPNA	16339-04-1	116.16	185	Volatile
1-Ethyl-1-nitrosourea	ENU	759–73–9	117.11	182	Volatile
N-nitrosodipropylamine	NDPA	621-64-7	130.19	206	Volatile
N-Nitrosodiethanolamine	NDEthA	1116-54-7	134.13	114	Volatile
N-nitrosodibutylamine	NDBA	924-16-3	158.24	235	Volatile
N-Butyl-N-(4-hydroxybutyl)NA	BHBNA	3817-11-7	174.24	144	Volatile
(S)-N-Nitroso Anabasine	SNAna	1133-64-8	191.23	327	Semivolatile
N-Nitrosodiphenylamine	NDPhenA	86-30-6	198.22	101	Volatile
N-Nitrosodicyclohexylamine	NDCH	947-92-2	210.32	350	Semivolatile
Streptozocin	STREPZ	18883-66-4	265.22	408	Nonvolatile

Conclusion

Our data suggests that the miniaturized Ames assays are applicable to reliably assess the mutagenicity of Nitrosamines. Our optimized miniaturized Ames assays can be applied to test both volatile and non-volatile substances through the adjustment of the protocols. There is an **excellent concordance between the pre-incubation MicroAmes6 and the Petri dish-based Ames test data**: **out of the 8 Nitrosamines that are positive in the Petri dish-based Ames test, 7 Nitrosamines are also positive in the pre-incubation MicroAmes6.** We found **high agreement** between the Petri dish-based assay and the Ames MPF assay: **6 out of 8 compounds were positive in both the Petri dish-based assay and the Ames MPF.** The other two Nitrosamines published as positive in the Petri dish assay were equivocal in the Ames MPF assay. There is a good concordance between the two miniaturized Ames assays in predicting the mutagenicity of Nitrosamines, i.e. the **exact same assessment outcome** was gained with both miniaturized Ames assays for **8 out of 12 test substances**. N-Nitrosodicyclohexylamine tested with E.coli uvrA[pKM101], resulted in an equivocal result with MicroAmes6 versus a negative test result with Ames MPF. In the case of Streptozotocin MicroAmes6 was positive, Ames MPF gave an equivocal result with the E.coli uvrA[pKM101] tester strain. We conclude that the miniaturized Ames assays are an **environment- and resource-friendly alternative** to the Petri dish-based Agar plate test, while providing **high performance** and **excellent predictive power** for the **assessment of mutagenicity of Nitrosamines and other genotoxic impurities**. Significantly reduced hamster or rat liver S9 in line with 3R, reduced quantity of test compound as well as a significantly smaller volume of contaminated plastic waste are advanced skill sets for a sustainable environment.

References:
[1] 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.
[2] Breider F, von Gunten U. Quantification of Total N-Nitrosamine Concentrations in Aqueous Samples via UV-Photolysis and Chemiluminescence Detection of Nitric Oxide. Anal Chem. 2017 Feb 7;89(3):1574-1582. doi: 10.1021/acs.analchem.6b03595. Epub 2017 Jan 6. PMID: 27989108.
[3] Food & Drug Administration. (2023, August). Control of Nitrosamine Impurities in Human Drugs. Guidance for Industry
[4] European Medicines Agency. (2023, July). Questions and answers for marketing authorization holders/applicants on the CHMP Opinion for the Article 5(3) of Regulation (EC) No 726/2004 referral on nitrosamine impurities in human medicinal products
[5] National Toxicology Program (NTP) coordinated by United States Department of Health and Human Services

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.