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Miniaturized Ames Tests for N-Nitrosamines: A Sensitive and Sustainable Genotoxicity Screening Approach

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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**. Increasing concern over nitrosamines has triggered international efforts to improve testing strategies for this class of compounds. Regulatory bodies such as the European Medicines Agency (EMA) and the U.S. Food and Drug Administration (FDA) recommend using Enhanced Ames Test conditions for evaluating the mutagenicity of N-nitrosamines. . 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 [1,2].

Highlights

- Miniaturized Ames assays are deployed under Enhanced Ames Test (EAT) conditions to assess Nitrosamine mutagenicity
- Genotoxic impurities are detected at low doses
- Optimized protocol for both volatile and non-volatile subtances
- Cytotoxicity assessment using XTT assay

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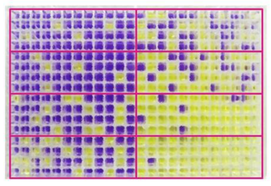
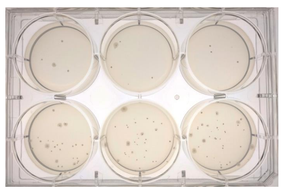
Methods

Ames MPF™



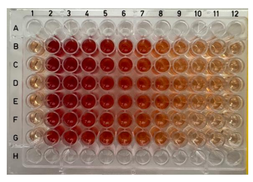
- Ames MPF is a miniaturized Ames test in liquid microplate fluctuation format.
- Pre-incubation for 90 minutes
- 30% Hamster S9 – in accordance with EAT
- Bacterial cell density: 10^8 cells per mL
- Compliant with ICH M7

Pre-incubation MicroAmes6



- MicroAmes6 is a miniaturized Ames test in 6-well agar plate format
- Pre-incubation for 30 minutes
- 30% Hamster S9 – in accordance with EAT
- Bacterial cell density: 10^7 cells per mL
- Compliant with ICH M7

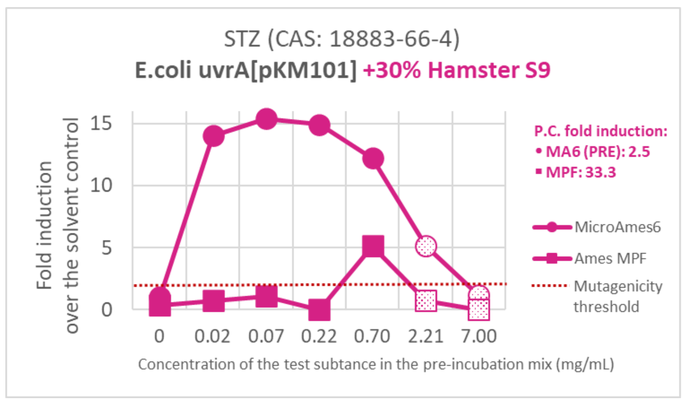
XTT assay

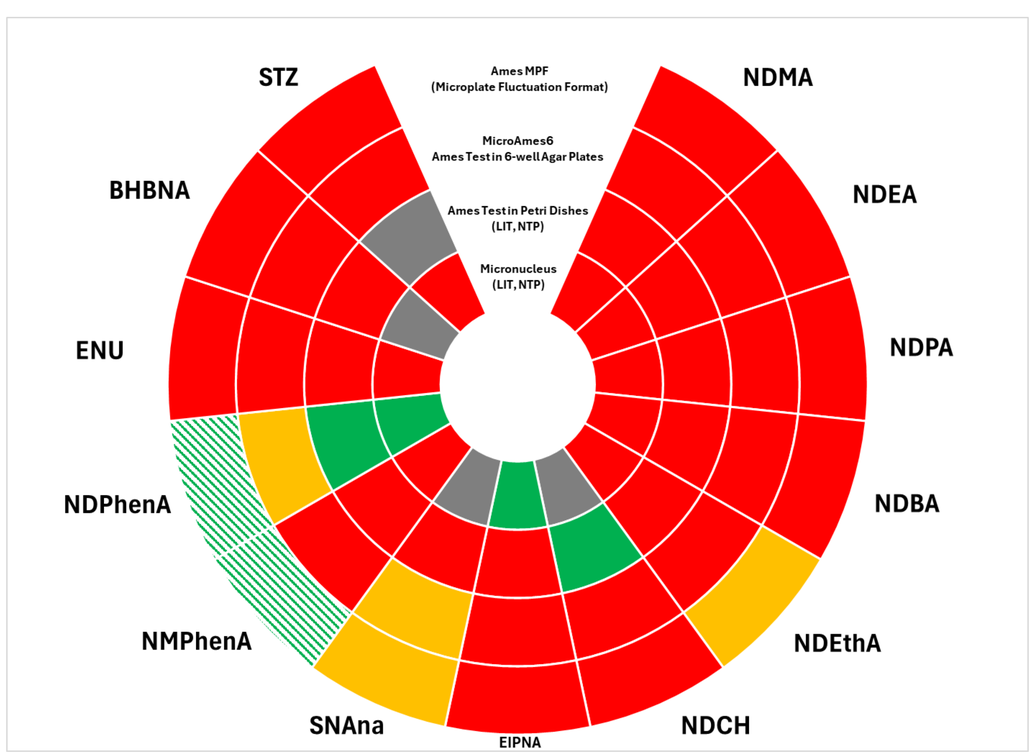


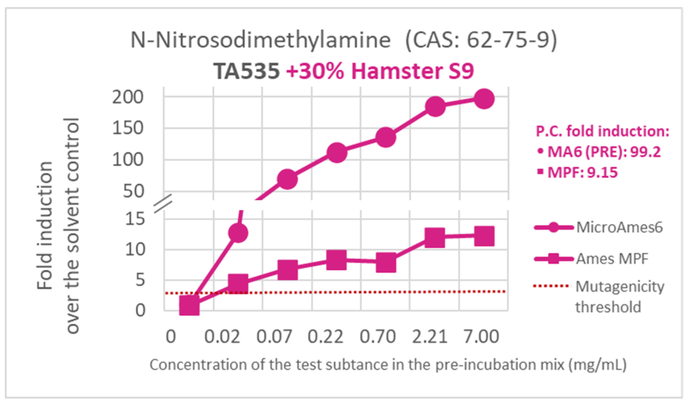
- XTT assay is a cell viability assay that can provide information on the cytotoxicity of test substances
- Cytotoxic effects are investigated 90 minutes exposure to the test substance – same pre-incubation mix as in Ames MPF

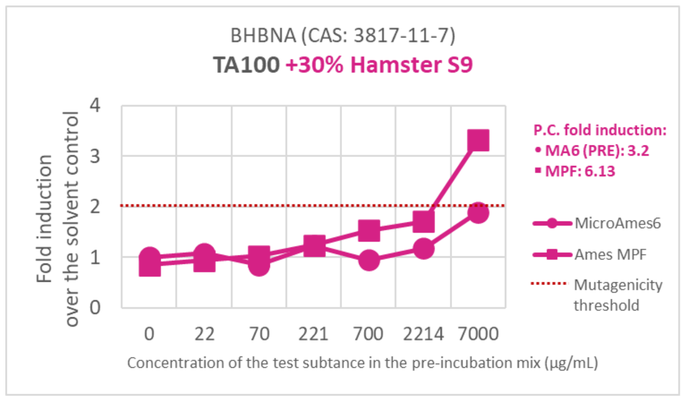
Results

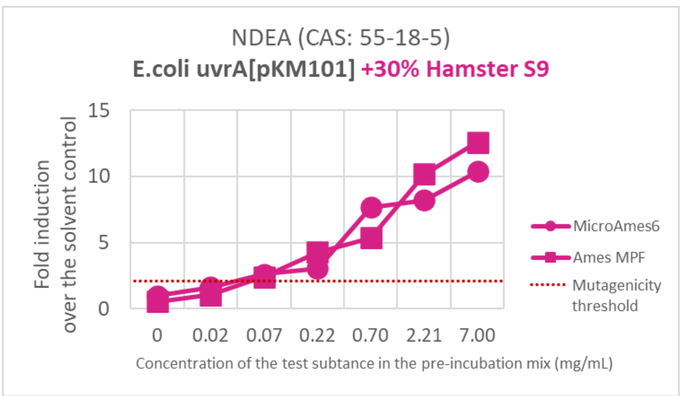
Mutagenicity Assessment of Nitrosamines using Miniaturized Ames Assays

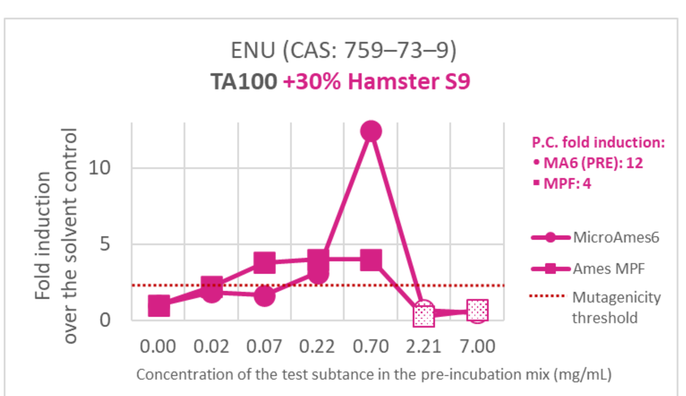






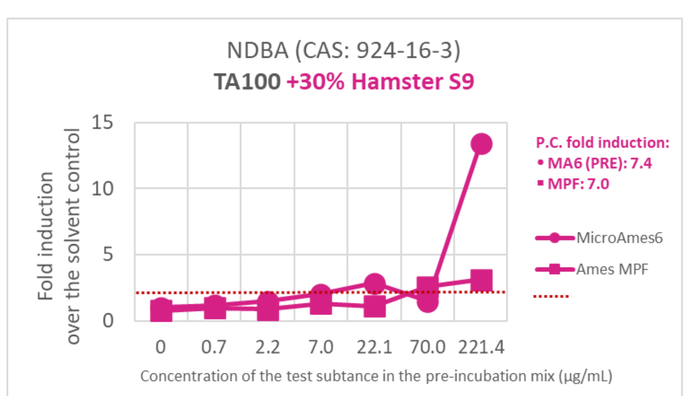






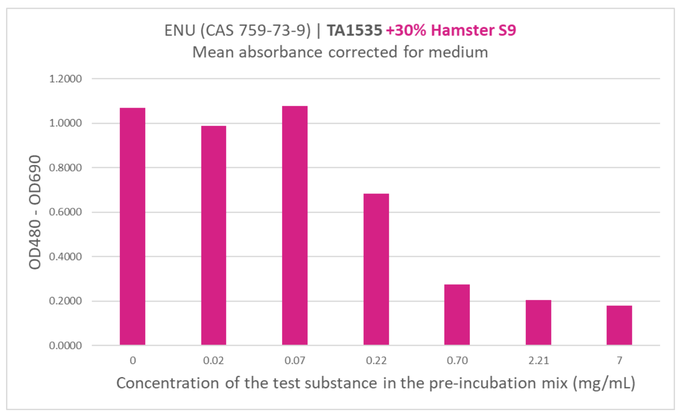
▲ Summary of the study and comparison of the miniaturized Ames test results with Petri dish-based Ames test data and micronucleus data from the HESI GTTC Nitrosamine study [3] or the NTP database [4]. For the miniaturized Ames assays we present cumulative test result, i.e. a compound with one positive 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 [4] or in the literature. In vitro or in vivo micronucleus test results for the corresponding Nitrosamines were collected from the NTP database [4]. Color codes: Red = positive, yellow = equivocal, green = negative, stripes = unclear result due to cytotoxicity and possible masking effect, grey = not available.

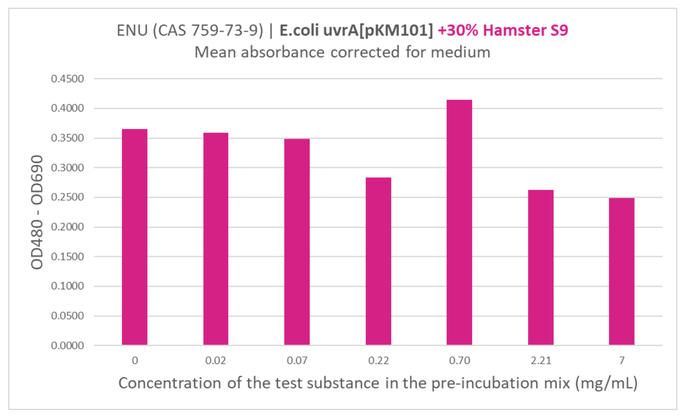
◀ Selected experimental results from the study showing the performance of the miniaturized Ames assays ▶ All experiments were performed in the presence of 30% hamster liver S9. Test substance concentration in both miniaturized assay system correspond to the concentration of the test substance in the 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.



Investigation of Nitrosamine Cytotoxicity using the XTT Assay

- 1-Ethyl-N-Nitrosourea (ENU, CAS 759-73-9) was exposed to Salmonella and E.coli tester strains
- Exposure to the test substance under the same conditions as during the pre-incubation in the Ames MPF (Enhanced Ames Test conditions)
- 10 µL Ames MPF pre-incubation mix is pipetted into the XTT reaction mix
- Absorbance is recorded at 480 nm (specific signal) and at 690 nm (background signal)
- Cytotoxic effect of 1-Ethyl-N-Nitrosurea is more pronounced in the TA1535 Salmonella strain than in the E.coli uvrA[pKM101]





Conclusion

The results indicate that miniaturized Ames assays are both efficient and reliable for evaluating the mutagenicity of N-nitrosamines. The adapted testing protocols enhanced sensitivity and provided the detection of mutagenic N-Nitrosamines at lower concentrations, and enabled more comprehensive assessments of potential cytotoxicity - validated by subsequent XTT testing. This study demonstrates the substantial potential of miniaturized Ames assays to improve genotoxicity testing. These formats not only maintain high sensitivity but also offer notable reductions in resource usage, including biological reagents and plastic materials.

References:
[1] Food & Drug Administration. (2023, August). Control of Nitrosamine Impurities in Human Drugs. Guidance for Industry
[2] 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
[3] Bercu J, Trejo-Martin A, Chen C, Schuler M, Cheung J, Cheairs T, Lynch AM, Thomas D, Czich A, Atrakchi A, McGovern TJ, Heflich RH, Vespa A, Froetschl R, Yang Y, Gandhi RD, Elloway J, Ziegler V, Hellmann A, Schaefer M, Tennant RE, Westerink W, Hoffmans R, Jolly R, Noteboom J, Gollapudi P, Sobol Z, McGettigan KK, Christensen JS, Simon S, Dieckhoff J, Zeller A, Marchand C, Waese K, Bishop ME, Leavitt P, Hargreaves V, Glick C, Liao Y, Elespuru R, Puglisi R. HESI GTTC ring trial: Concordance between Ames and rodent carcinogenicity outcomes for N-nitrosamines (NAs) with rat and hamster metabolic conditions. Regul Toxicol Pharmacol. 2025 Sep;161:105835. doi: 10.1016/j.yrtph.2025.105835. Epub 2025 Apr 29. PMID: 40311791
[4] National Toxicology Program (NTP) coordinated by United States Department of Health and Human Services