

# High-throughput mutagenicity testing of Nitrosamines using miniaturized enhanced Ames tests for chemical risk assessment



Dr. Csaba Boglári, Ph.D.  
Scientific Director, Xenometrix AG

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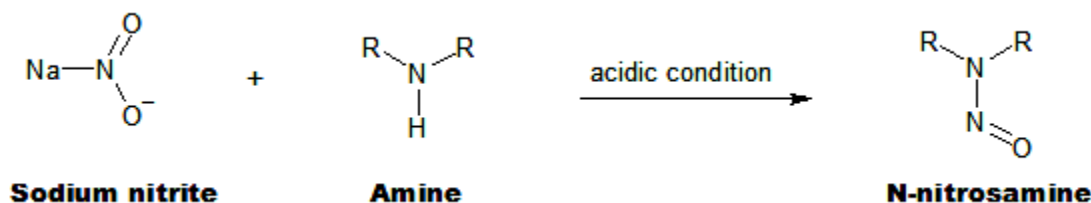
# Objective

- Investigated chemical class: **Nitrosamines**
- Endpoint: **mutagenicity**, base pair substitution or frameshift mutations
- Method: **Ames assay - bacterial reverse mutation test**
  - Low cost **in vitro** assay
  - **S.typhimurium** and **E.coli** Ames tester strains
  - The Ames test is performed in Petri dishes → **miniaturized versions** of the Ames test are applied in: **MicroAmes6**, 6-well agar plate format and the **Ames MPF™**, the microplate fluctuation format

Disclaimer | Xenometrix develops and manufactures ready-to-use assay kits that were applied in this project to generate data.

# Nitrosamines - Background

- Formation of Nitrosamines <sup>[1]</sup>:



- problem? <sup>[2]</sup>
  - Presence of nitrite and secondary amines → risk of nitrosamine formation
  - Sodium Nitrite & Amine Molecules are “omnipresent” → pharmaceuticals cannot be produced “Nitrosamine-Free”
  - Nitrosamines are considered as mutagenic impurities
  - Acceptable limits are defined by authorities
  - Nitrosamine content in pharmaceuticals has to be controlled according to ICH M7 guideline <sup>[3]</sup>

# Nitrosamines | Acceptable intake

- If there is no sufficient carcinogenicity data → by default the acceptable intake is **18 ng / day** (pharmaceuticals) <sup>[4]</sup>
- Problems with the above threshold:
  - Technical difficulties to detect analytically <sup>[2]</sup>
  - The threshold is not scientifically sound (Nitrosamines are also present in food → daily intake can easily exceed 200 ng)
- Goal: generate Ames, i.e. mutagenicity data for nitrosamines
  - International working groups → **Ames protocol optimization** <sup>[13]</sup>
  - Recommendations by regulatory authorities (EMA in Europe) → **Enhanced Ames Test (EAT)** <sup>[5] [6]</sup>

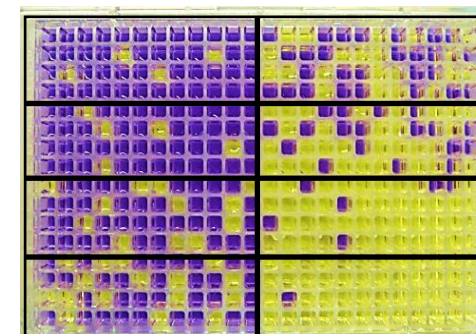
# Enhanced Ames Test (EAT) for testing mutagenicity potential of Nitrosamines

- Recommendation by regulatory authorities (EMA <sup>[5]</sup>, FDA <sup>[6]</sup>):
  - Tester strains: *S. typhimurium* TA98, TA100, TA1535, TA1537, and *E. coli* WP2 *uvrA* (pKM101) **tester strains** should be included – in compliance with **OECD TG 471** <sup>[16]</sup>
  - The **pre-incubation method** is recommended.
  - The recommended pre-incubation time: **30 minutes in the Petri dish-based Ames test**
  - FDA / EMA recommendations:
    1. Absence of a post-mitochondrial fraction (S9)
    2. Presence of 30% rat liver S9,
    3. Presence of **30% hamster liver S9**.
  - FDA / EMA recommendation: phenobarbital and  $\beta$ -naphthoflavone induced hamster post-mitochondrial fractions (S9), i.e. **inducers of cytochrome P450 enzymes**

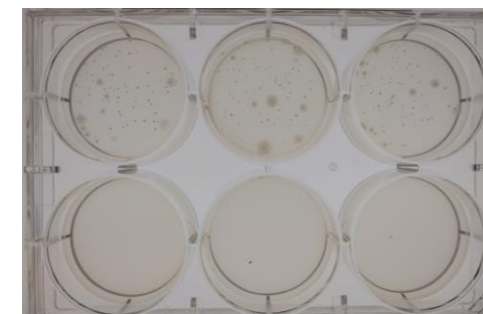
# Methods and Materials

- **Parallel running** miniaturized Ames assays from the **same overnight culture**:
  - Ames MPF™
  - Pre-incubation MicroAmes6
- Solvent:  $H_2O$  [7]
- Bacterial strains [7] [8]:
  - TA100
  - TA1535
  - E.coli WP2 uvrA[pKM101]
- Metabolic activation: **30% Hamster S9** [9]

**Ames MPF™**  
(microplate  
fluctuation  
format)

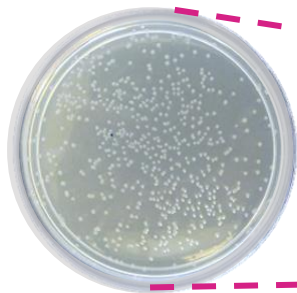


**Pre-  
incubation  
MicroAmes6**  
(6-well agar  
plate format)



# MicroAmes6, a 6-well agar plate version

- Petri dish-based assay



- MicroAmes6, 6-well agar plate format

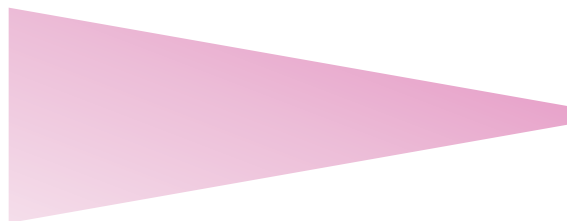
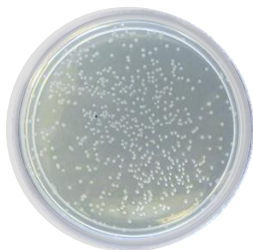


## Benefits of the miniaturized Ames assay in 6-well agar plate format:

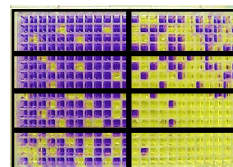
- **Efficient** and **significantly easier handling:**
- **Faster, easier** interpretation of results due to reduced number of colonies
- **Reduced** material, plasticware (purchase, storage, disposal)
- **Reduced volumes** for autoclave , incubators

# Ames MPF™, microplate fluctuation format

- Petri dish-based assay



- Ames MPF™



**Similar** method/technology to the classical Ames test, but in **miniaturized liquid microplate format**

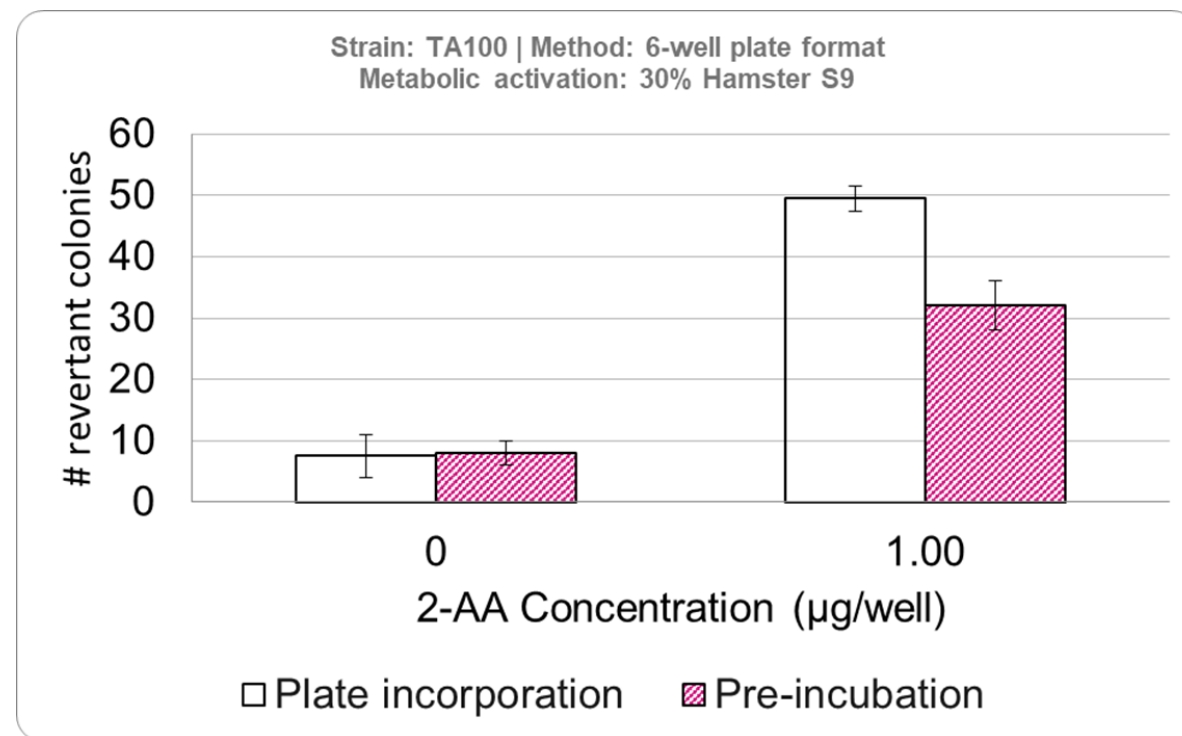
## Benefits of the miniaturized assay in liquid microplate format [14] [15]:

- **Less hands-on time, more efficient** and **significantly easier handling** of the experiment with **8-channel dispenser, automatable**
- **Faster** and **easier** interpretation of results – **color change** facilitates the readout
- **Reduced handling** of material and plasticware - purchase, storage, disposal of **contaminated material**
- **Reduced volumes** for autoclave and incubators



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

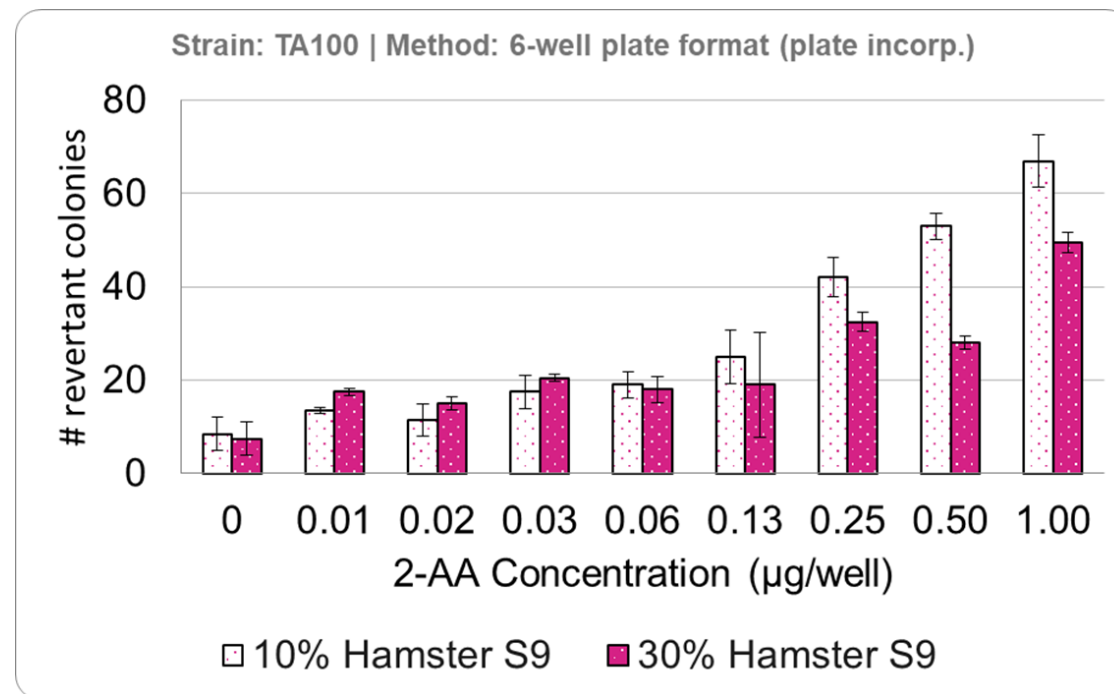
- The pre-incubation protocol is more suitable to test Nitrosamines compared to the plate incorporation protocol – experimental evidence, see literature [2]
- Ames MPF follows the pre-incubation principle by default
- MicroAmes6 plate incorporation protocol had to be adjusted → test in parallel plate incorporation vs. pre-incubation protocol
- The plate incorporation and the pre-incubation MicroAmes6 protocol gave comparable negative and positive control responses



2-AA = 2-Aminoanthracene: positive control chemical for TA100 strain with metabolic activation

# Comparison of different Hamster S9 concentrations in 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 (30%) [5] [6]
- For the Ames MPF assay 30% S9 is applied by default.
- The application of 30% hamster S9 is also **compatible with the miniaturized Ames assay in 6-well plate format**



2-AA = 2-Aminoanthracene: positive control chemical for TA100 strain with metabolic activation

[5] EMA/409815/2020 Rev.21 [6] Food & Drug Administration. (2023, August). Control of Nitrosamine Impurities in Human Drugs. Guidance for Industry

# Volatility of Nitrosamines with small molecule weight

- The nitrosamines are presented in the increasing order of their molecule weight
- Nitrosamines with **small molecule weight** 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].

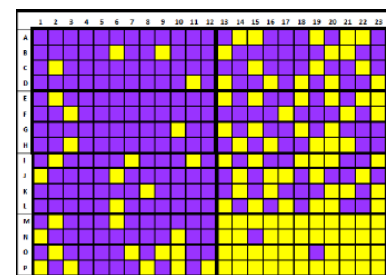
[1] Kim S, Chen J, Cheng T, et al. PubChem 2023 update. Nucleic Acids Res. 2023;51(D1):D1373–D1380. doi:10.1093/nar/gkac956

[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.

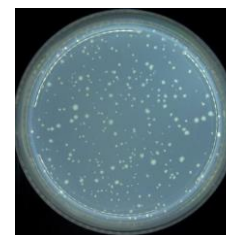
# Why is it important to address the volatility of test substances in the Ames test?

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

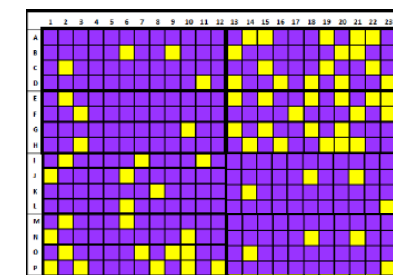
“Normal” plate



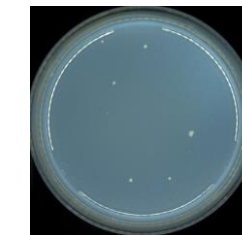
Positive Control plate next to the “Normal” Test Substance Plate



“Volatility” plate



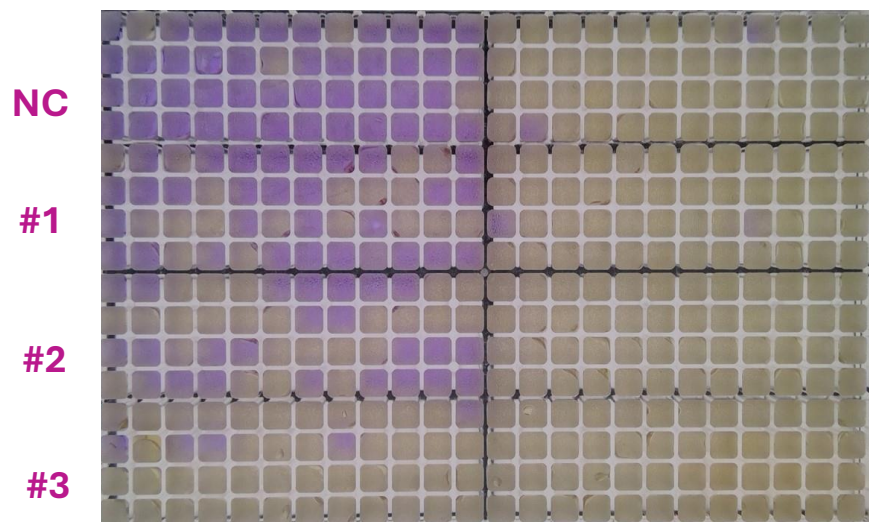
Positive Control plate next to the “Volatile & Cytotoxic” Test Substance Plate



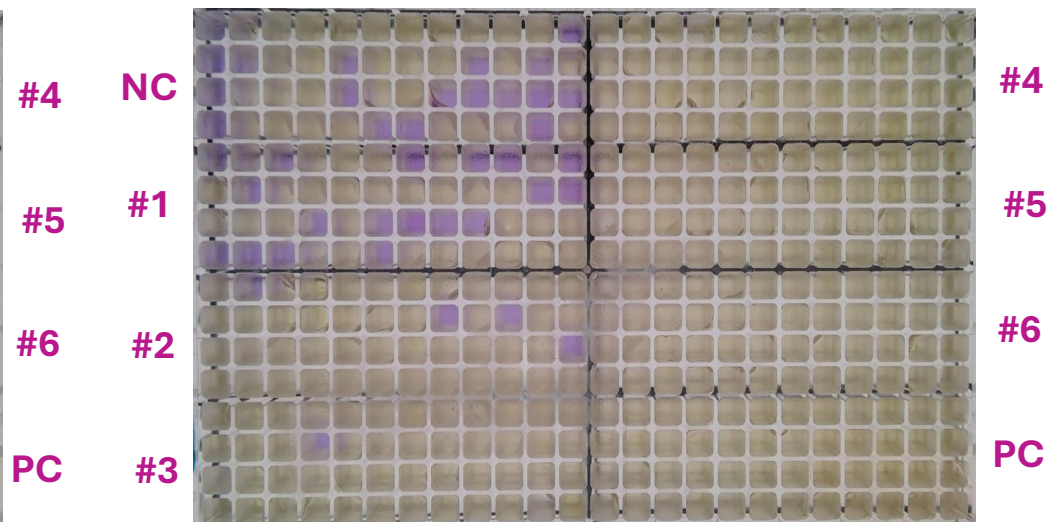
# Volatility of the test substances

- interference with the results
- clearly visible in Ames MPF

Non-volatile, strong positive compound



Volatile, strong positive compound

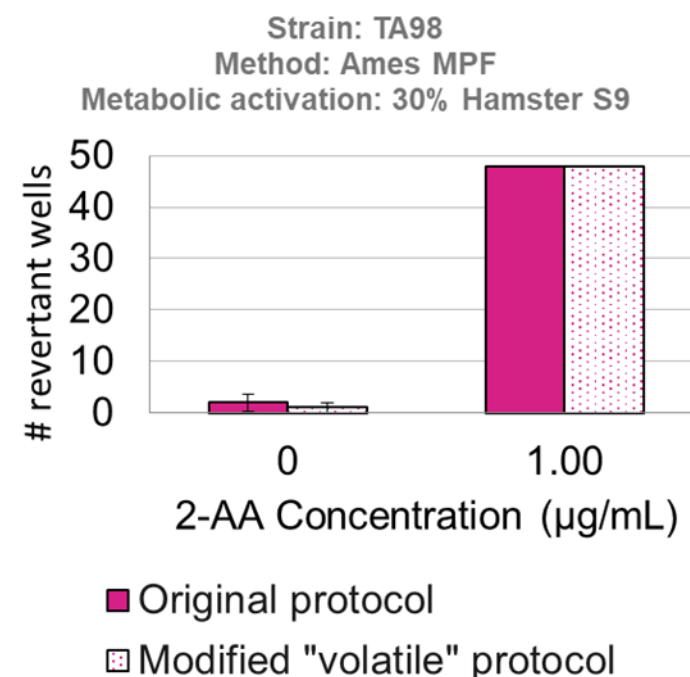
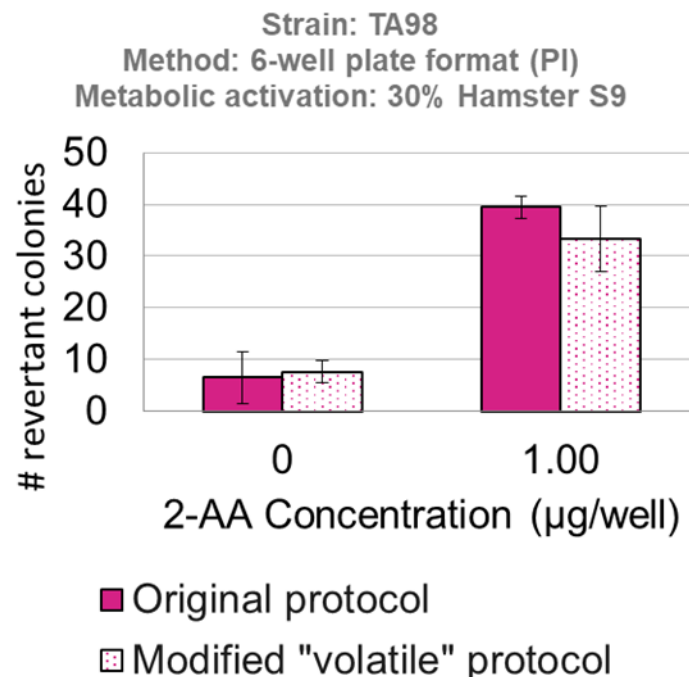


Comparison of the results of Ames MPF tests on the 384-well plates with two different test compounds. Left: non-volatile, strong positive compound. Right: volatile, strong positive compound. NC: Negative Control, #1 - #6: dilutions of the test compound, #1 is the lowest applied concentration of the compound in the test, #6 is the highest applied concentration of the compound in the test, PC: Positive Control.



# Verification if the foil interferes with the results of negative and positive control

■ Original protocol



▤ Modified "volatile" protocol



2-AA = 2-Aminoanthracene: positive control chemical for TA100 strain with metabolic activation

Graphs representing the comparison of the test performance with the original and the modified protocol (application of sealing foil on the plates), which is optimized for the testing of volatile substances.

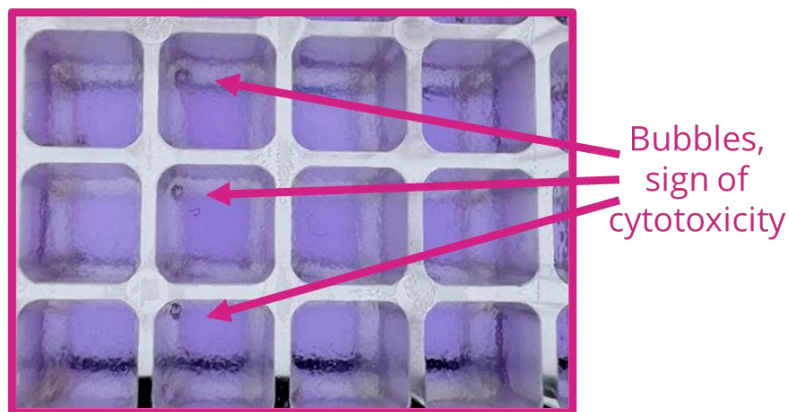
**Both the 6-well plate format and the Ames MPF assays can be performed under the modified assay circumstances without affecting the negative or positive control performance.**

# Investigation of cytotoxicity

- The assessment of **cytotoxicity** is essential in the Ames assay, as it can mask the mutagenicity of the tested sample, potentially causing **false negative results**.

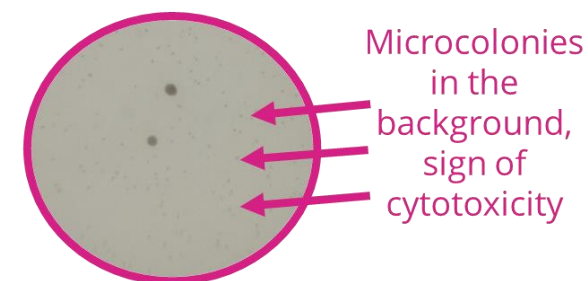
**Ames MPF: 384-well plate after 48 hours at 37°C**

Magnified image of the wells on the 384-well plate



**MicroAmes6: 6-well agar plates after 72 hours at 37°C**

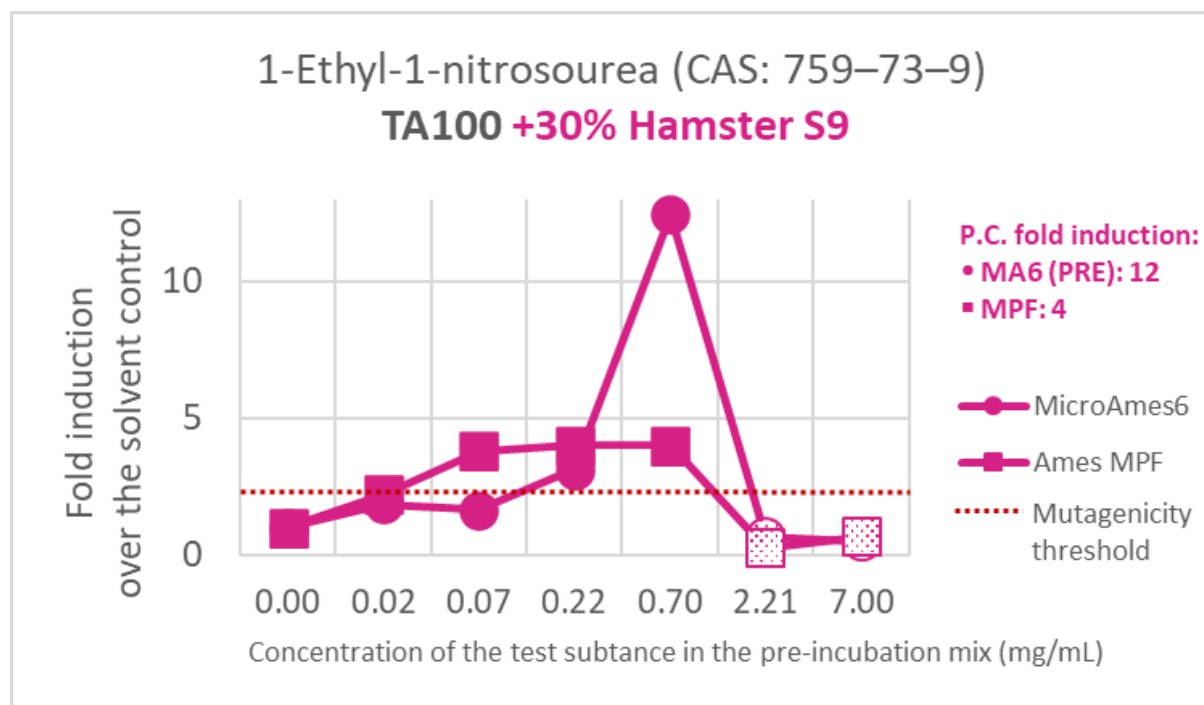
Magnified image of the well on the 6-well plate



- The above example of a cytotoxic Nitrosamine test substance provides an insight into how cytotoxicity can be investigated in the miniaturized Ames assays.

# 1-Ethyl-1-nitrosourea

## Miniaturized Ames assay results

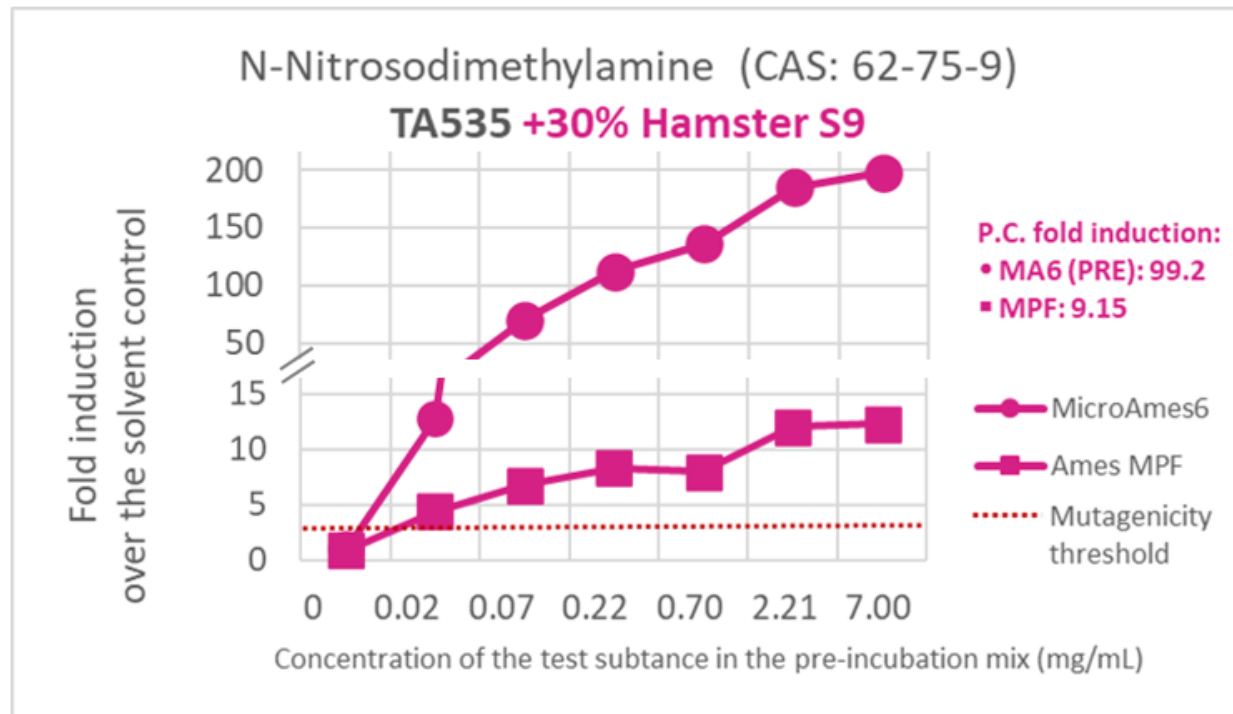


- Both miniaturized assays are **positive**, in accordance with the Petri dish-based data from literature <sup>[10]</sup>
- Normalized Lowest Effective Concentration of 1-Ethyl-1-nitrosourea: **0.02 mg/mL Ames MPF vs. 0.14 mg/mL Ames test in Petri Dishes**
- Cytotoxicity** observed at concentrations > 0.7 µg/mL



# N-Nitrosodimethylamine (NDMA)

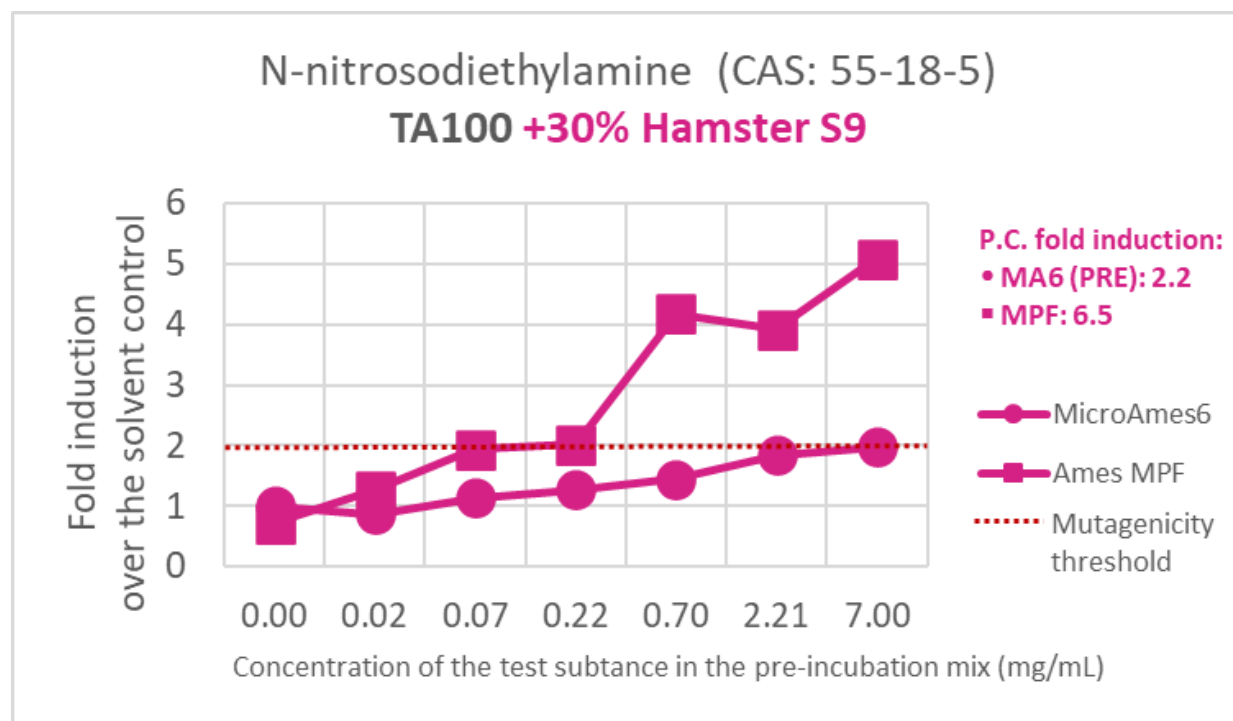
## Miniaturized Ames assay results



- Both miniaturized assays clearly positive, in accordance with Petri dish-based data
- Both miniaturized Ames assays positive at **0.02 mg/mL!** versus Petri dish-based assay positive at **0.7 mg/mL** NDMA-concentration <sup>[11]</sup>

# N-Nitrosodiethylamine (NDEA)

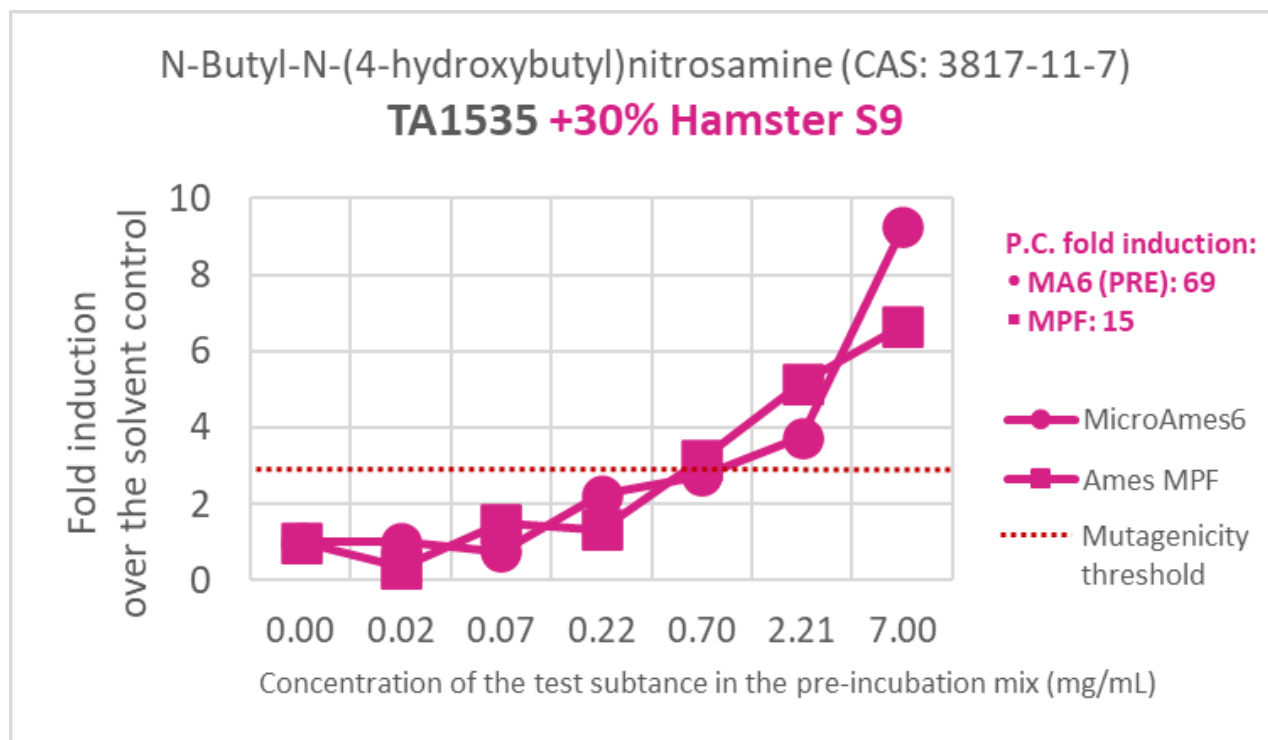
## Miniaturized Ames assay results



- Both miniaturized assays positive, in accordance with Petri dish-based data
- Normalized Lowest Effective Concentration for **Ames MPF is 0.22 mg/mL**, versus Petri dish-based assay: **1.4 mg/mL NDMA** <sup>[10]</sup>

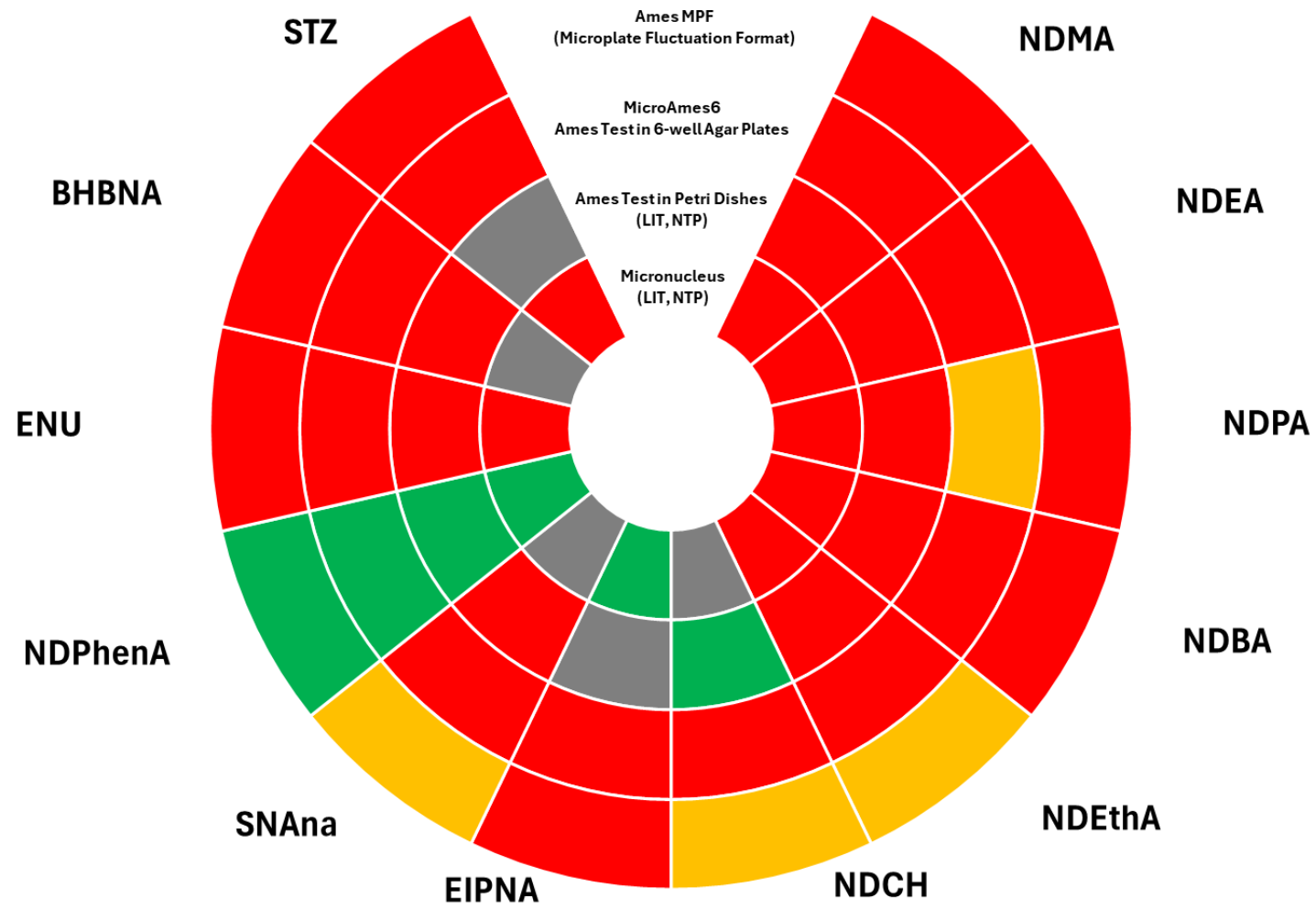
# N-Butyl-N-(4-hydroxybutyl)nitrosamine

## Miniaturized Ames assay results



- TA1535: both miniaturized assays: **positive results in accordance with the Petri dish assay** <sup>[12]</sup>
- **3x lower normalized Lowest Effective Concentration** achieved in Ames MPF compared to the Petri dish-based assay.

# Summary



Literature source: [10] NTP Database, 2022 [12] Nagao et al., 1977, [13] Bercu et al, 2025

# Conclusions

- Volatile and non-volatile Nitrosamines can be assessed using adjusted protocols
- Miniaturized Ames assays, the pre-incubation 6-well agar plate format and the microplate fluctuation format can be applied to assess the mutagenicity of Nitrosamine test substances
- Miniaturized Ames assays can detect mutagenic Nitrosamines at lower concentrations compared to the agar plate tests conducted on Petri dishes.
- Cytotoxicity can be assessed in both miniaturized Ames assays

# Literature references

- [1] Shaikh, Tabrez, Nitrosamine Impurities in Drug Substances and Drug Products (January 1, 2020). DOI: 10.5281/zenodo.3629095, Available at SSRN: <https://ssrn.com/abstract=3958595>
- [2] The Nitrosamine “Saga”: Lessons Learned from Five Years of Scrutiny Raphael Nudelman, Grace Kocks, Bruno Mouton, David J. Ponting, Joerg Schlingemann, Stephanie Simon, Graham F. Smith, Andrew Teasdale, and Anne-Laure Werner Organic Process Research & Development 2023 27 (10), 1719-1735 DOI: 10.1021/acs.oprd.3c00100
- [3] ICH guideline M7(R1) on assessment and control of DNA reactive(mutagenic) impurities in pharmaceuticals to limit potential carcinogenicrisk. EMA/CHMP/ICH/83812/2013. European Medicines Agency, August 25, 2015. [https://www.ema.europa.eu/en/documents/scientific-guideline/ich-guideline-m7r1-assessment-control-dna-reactive-mutagenic-impurities-pharmaceuticals-limit\\_en.pdf](https://www.ema.europa.eu/en/documents/scientific-guideline/ich-guideline-m7r1-assessment-control-dna-reactive-mutagenic-impurities-pharmaceuticals-limit_en.pdf) (accessed 06.09.2023)
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- [5] Questions and answers for marketing authorisation 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. [https://www.ema.europa.eu/en/documents/opinion-any-scientific-matter/nitrosamines-emea-h-a53-1490-questions-answers-marketing-authorisation-holders-applicants-chmp-opinion-article-53-regulation-ec-no-726-2004-referral-nitrosamine-impurities-human-medicinal-products\\_en.pdf](https://www.ema.europa.eu/en/documents/opinion-any-scientific-matter/nitrosamines-emea-h-a53-1490-questions-answers-marketing-authorisation-holders-applicants-chmp-opinion-article-53-regulation-ec-no-726-2004-referral-nitrosamine-impurities-human-medicinal-products_en.pdf) (accessed 22.11.2024)
- [6] Recommended Safety Testing Methods for Nitrosamine Impurities. Food & Drug Administration. <https://www.fda.gov/regulatory-information/search-fda-guidance-documents/cder-nitrosamine-impurity-acceptable-intake-limits#safety> (accessed 22.11.2024)
- [7] 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.
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- [9] Lijinsky W, Andrews AW. The superiority of hamster liver microsomal fraction for activating nitrosamines to mutagens in Salmonella typhimurium. Mutat Res. 1983 Oct;111(2):135-44. doi: 10.1016/0027-5107(83)90058-1. PMID: 6355832.
- [10] National Toxicology Program Database (link: <https://ntp.niehs.nih.gov/data>)
- [11] Bringezu & Simon, 2022 Bringezu F, Simon S. Salmonella typhimurium TA100 and TA1535 and E. coli WP2 uvrA are highly sensitive to detect the mutagenicity of short Alkyl-N-Nitrosamines in the Bacterial Reverse Mutation Test. Toxicol Rep. 2022 Feb 8;9:250-255. doi: 10.1016/j.toxrep.2022.02.005. PMID: 35198408; PMCID: PMC8850549.
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- [16] OECD (2020), Test No. 471: Bacterial Reverse Mutation Test, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, <https://doi.org/10.1787/9789264071247-en>.

If you have any further questions about testing the mutagenicity of Nitrosamines, please do not hesitate to reach out to me:

Csaba Boglári, Ph.D. [cbo@xenometrix.ch](mailto:cbo@xenometrix.ch)

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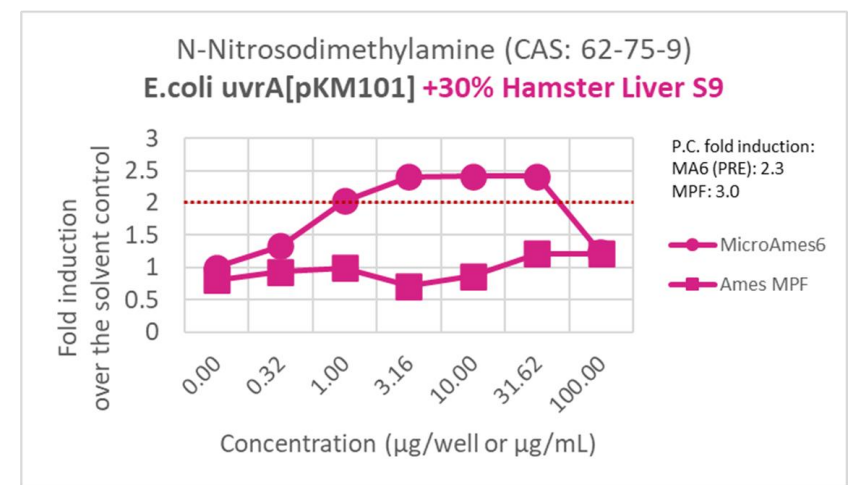
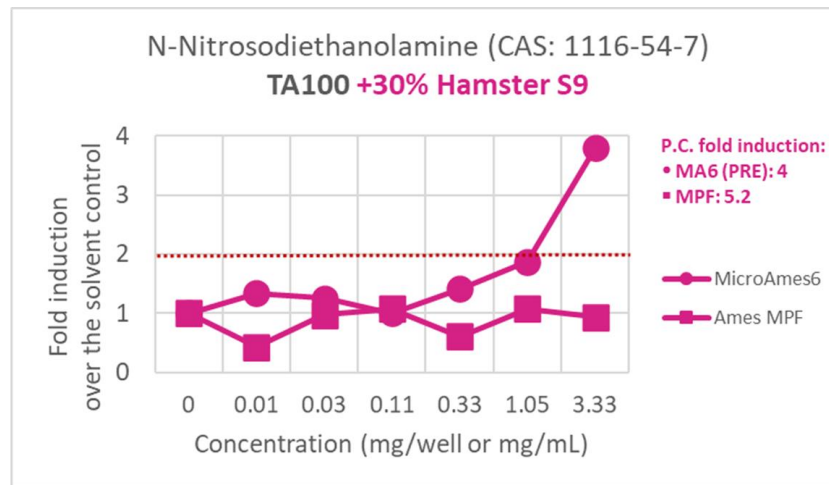
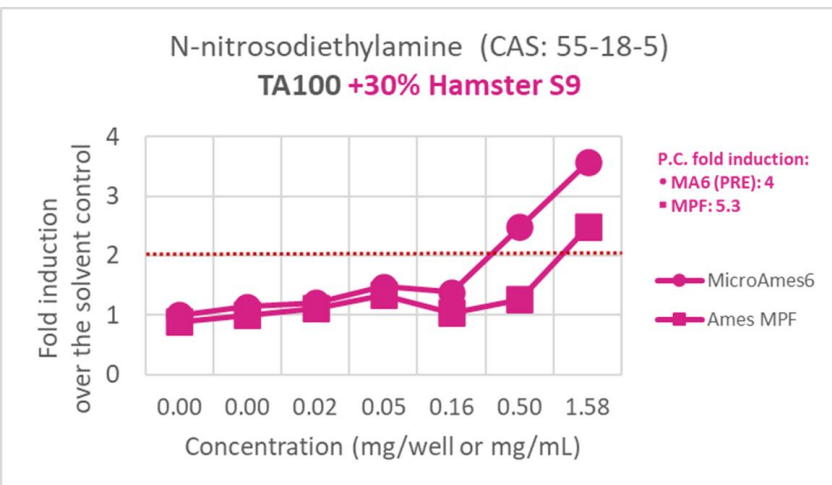
Posters presented on Nitrosamines at scientific meetings:



# Extra Slides



# Testing Nitrosamines with the miniaturized Ames tests



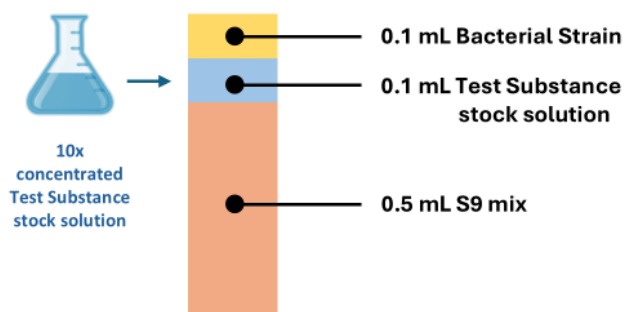
- Petri dish-based Ames test is positive for all 3 Nitrosamine compounds above (National Toxicology Program database)
- Pre-incubation MicroAmes6 provides positive results for all three selected Nitrosamines
- Ames MPF gave a positive result in the first case, but negative for the other two substances
- How can we explain these results?

[10] Source of Petri dish-based Ames assay data: National Toxicology Program Database (link: <https://ntp.niehs.nih.gov/data>)

# Comparison of the effective concentration of the compound between the different Ames assays

Focus is on the **exposure**, because that is when **mutations occur**!

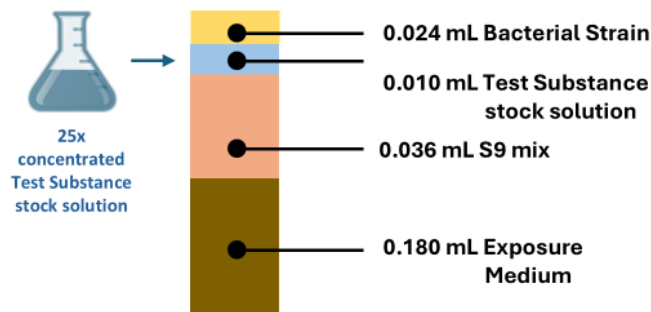
Pre-incubation Petri dish-based  
Traditional Ames test  
Top dose  
5000 µg / Plate



The effective concentration of the Test Substance  
in the total volume of the pre-incubation mix:

**7000 µg/mL**

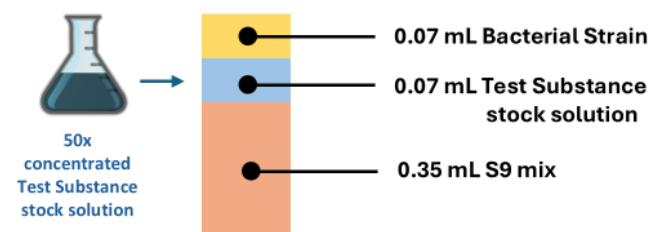
Ames MPF  
Microplate Fluctuation format  
Top dose  
5000 µg/ mL



The effective concentration of the Test Substance  
in the total volume during exposure:

**5000 µg/mL**

Pre-incubation MicroAmes6  
6-well plate format  
Top dose  
5000 µg / well

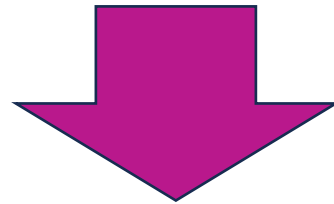


The effective concentration of the Test Substance  
in the total volume of the pre-incubation mix:

**35725 µg/mL**

# Comparison of the effective compound concentrations mg/plate versus mg/mL

- Compound concentrations in the pre-incubation mixes of the agar-based Ames systems are different as compared to Ames MPF during exposure



- Experiments with equal compound concentration in the Ames MPF system as compared to agar based assay for a precise comparison of the different Ames tests