# Characterization of the mutagenicity of selected nitrosamines using miniaturized Ames tests

Csaba Boglàri, Ph.D.

Scientific Director, Xenometrix AG

OpenTox Virtual Conference 2024

27.11.2024

Copyright: Xenometrix AG

# Objective

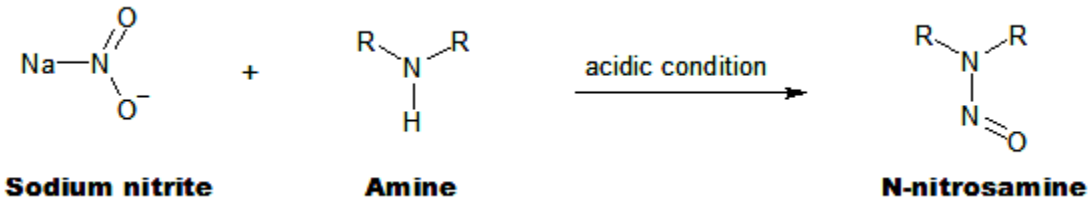
- Investigated chemical class: **Nitrosamines**
- Endpoint: **mutagenicity** – the capability of a substance to induce a genetic mutation in the DNA sequence
- Method: **Ames assay - bacterial reverse mutation test**
  - Low cost **in vitro** assay
  - **S.typhimurium** and **E.coli** Ames tester strains
  - **Point mutations** – changes in individual DNA bases – base pair substitution or frameshift mutations
  - The original test is performed in Petri dishes → **miniaturized versions** of the Ames test were applied: **MicroAmes6**, the 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.

Copyright: Xenometrix AG

# Nitrosamines - Background

- Formation of Nitrosamines [1]:




**Sodium nitrite** **Amine** **N-nitrosamine**

- Why is it a problem? [2]
  - Nitrosamines are generally considered as mutagenic impurities
  - Presence of nitrite and secondary amines → risk of nitrosamine formation
  - The above two are practically "omnipresent" → pharmaceuticals cannot be made "Nitrosamine-Free"
  - Acceptable limits are defined by authorities
  - Nitrosamine content has to be controlled according to ICH M7 guideline [3]

Literature source: [1] Shaikh et al., 2020 [2] Nudelman et al., 2023 [3] EMA/CHMP/ICH/83812/2013

Copyright: Xenometrix AG

# Nitrosamines | Acceptable intake

- If there is no sufficient carcinogenicity data → by default the acceptable intake is **18 ng / day** (pharmaceuticals) [4]
- Problems with the above threshold:
  - Technical difficulties to detect analytically [2]
  - 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 (e.g. HESI-GTTC) → **Ames protocol optimization**
  - Recommendations by authorities → **Enhanced Ames Test (EAT)**

Literature source: [2] Nudelman et al., 2023 [4] EMA/CHMP/428592/2019

Copyright: Xenometrix AG

# Enhanced Ames Test (EAT)

## for testing mutagenicity potential of Nitrosamines

- Recommendation by regulatory authorities (EMA [5], FDA [6]):

  - Tester strains: S. typhimurium TA98, TA100, TA1535, TA1537, and E. coli WP2 uvrA (pKM101) tester strains should be included.
  - 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 β-naphthoflavone induced hamster post-mitochondrial fractions (S9), i.e. inducers of cytochrome P450 enzymes

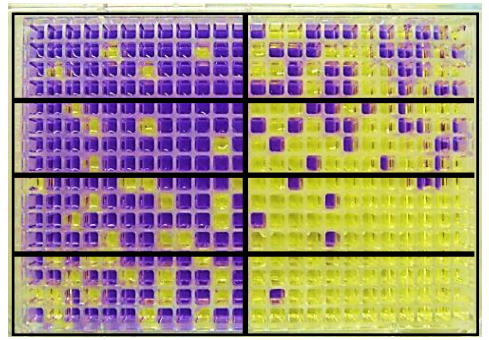
Literature source: [5] EMA/409815/2020 Rev.21 [6] Food & Drug Administration. (2023, August). Control of Nitrosamine Impurities in Human Drugs. Guidance for Industry

Copyright: Xenometrix AG

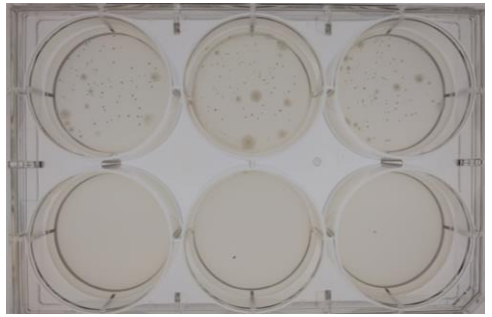
# Methods and Materials

- 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)**

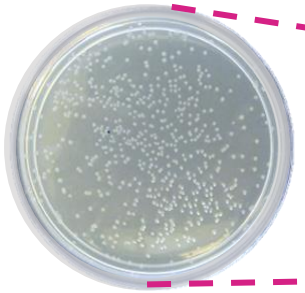


Literature source: [7] Thomas et al., 2024 [8] Dieckhoff et al., 2024 [9] Lijinsky et al., 1983

Copyright: Xenometrix AG

# MicroAmes6, a 6-well agar plate version

- **Petri dish-based assay**



- **MicroAmes6, 6-well agar plate format**

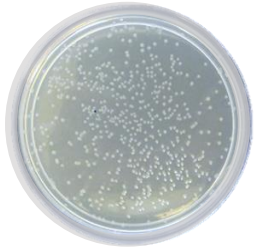
**Benefits of the miniaturized assays:**

- **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

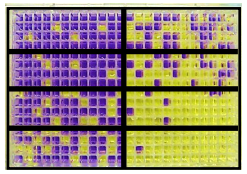
Copyright: Xenometrix AG

# Ames MPF™, the microplate fluctuation format

- **Petri dish-based assay**



- **Ames MPF™**



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

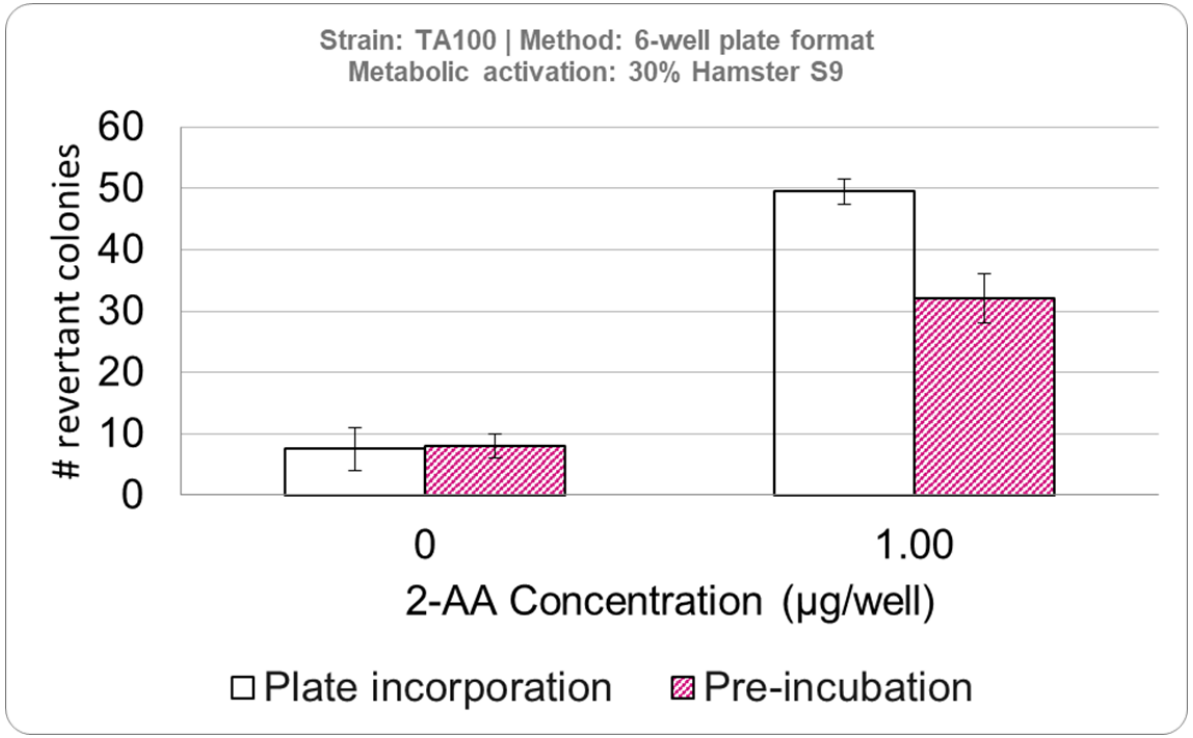
**Benefits of the miniaturized assays:**

- **Less hands-on time, more efficient** and **significantly easier handling** of the experiment with **8-channel dispenser**
- **Faster** and **easier** interpretation of results – **color change** facilitates the readout, **48 wells are checked for yellow wells**
- **Reduced handling** of material and plasticware - purchase, storage, disposal of **contaminated material**
- **Reduced volumes** for autoclave

Copyright: Xenometrix AG

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

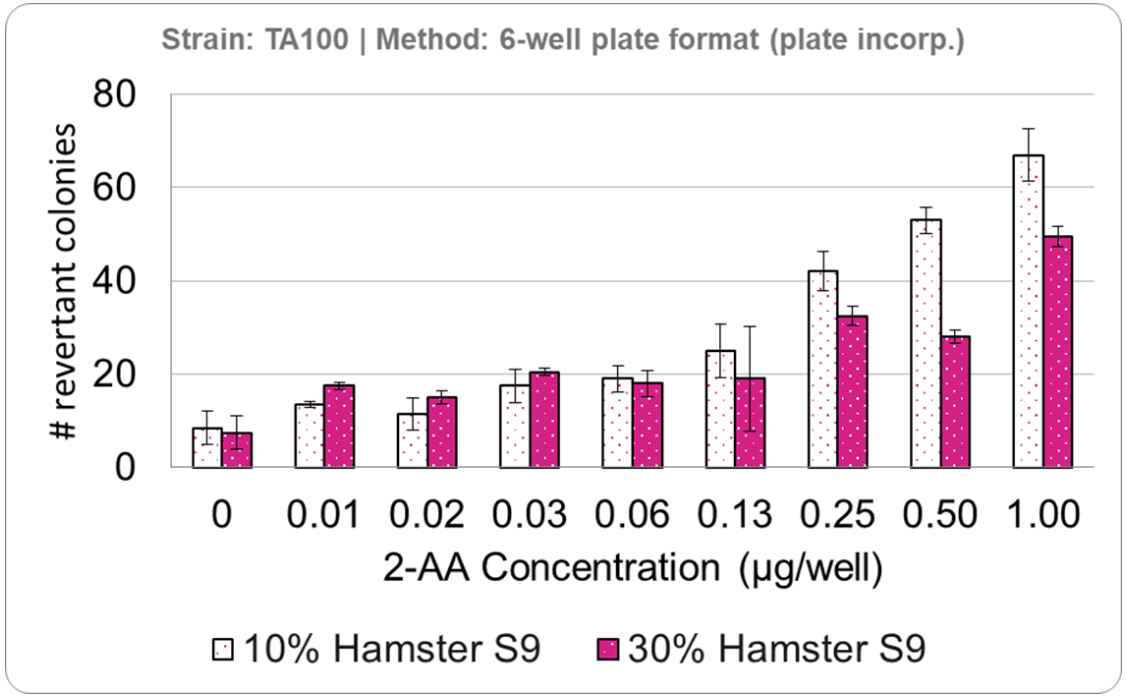
- The pre-incubation protocol is more suitable to test Nitrosamines compared to the plate incorporation protocol
- Ames MPF follows the pre-incubation principle by default → no adaptation of the original protocol was necessary
- MicroAmes6 plate incorporation protocol had to be adjusted → test in parallel 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%)
- 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** suggested by the comparable elicited fold induction towards 2-Aminoanthracene (2-AA) positive control.




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

# Volatility of Nitrosamines with small molecule weight

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 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].

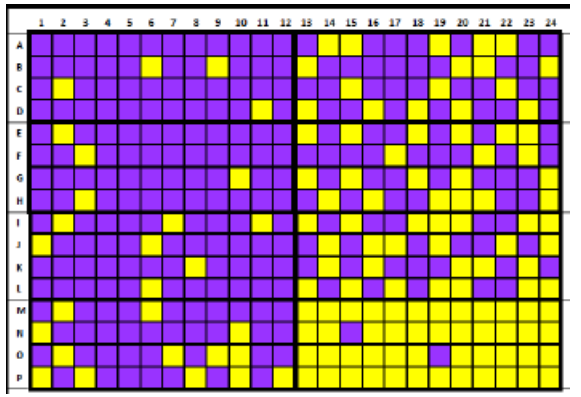
[1] Kim S, Chen J, Cheng T, et al. PubChem 2023 update. Nucleic Acids Res. 2023;51(D1):D1373–D1380. doi:10.1093/nar/gkac956
[2] Menezes, Helvécio & Amorim, Leiliane & Cardeal, Zenilda. (2013). Sampling and Analytical Methods for Determining VOC in Air by Biomonitoring Human Exposure. Critical Reviews in Environmental Science and Technology. 43. 10.1080/10643389.2011.604239.

Copyright: Xenometrix AG

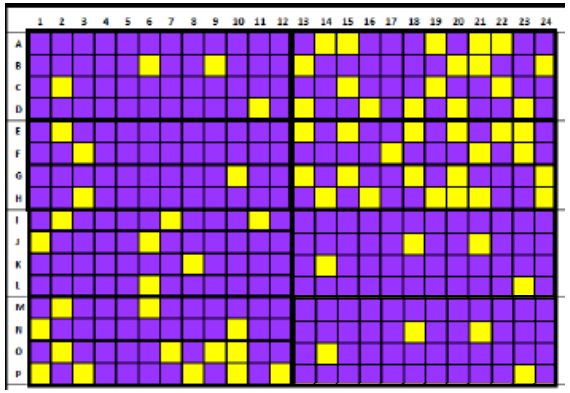
# Why is it important to address the volatility of test substances in the Ames test?

- 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

"Normal" plate in Ames MPF



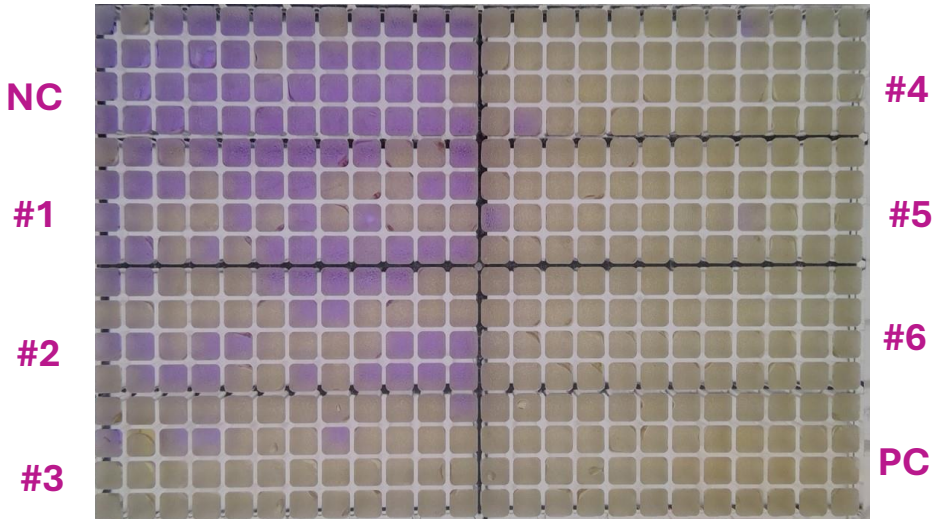
"Volatility" plate in Ames MPF



Copyright: Xenometrix AG

# Volatility of the test subtances - interference with the results

Non-volatile, strong positive compound



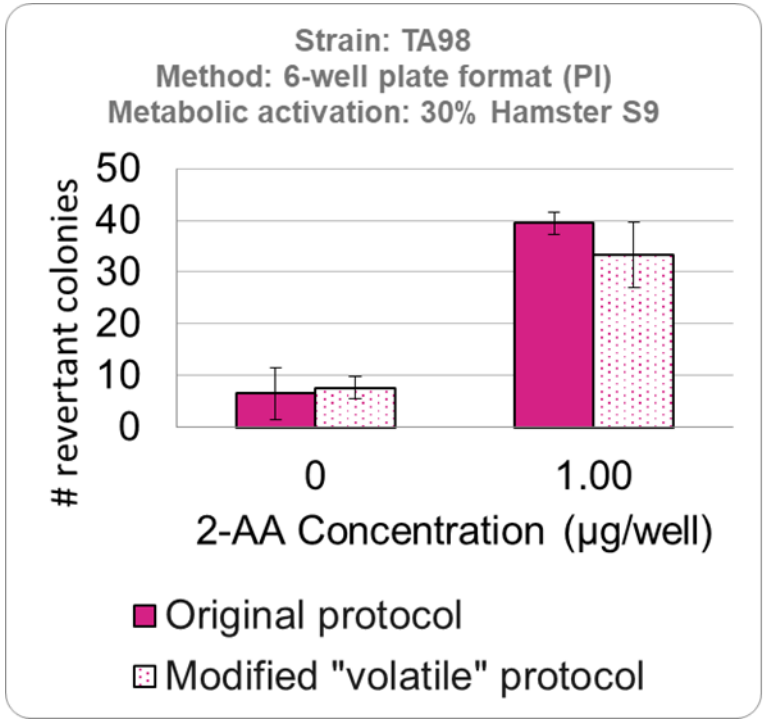

Volatile, strong positive compound

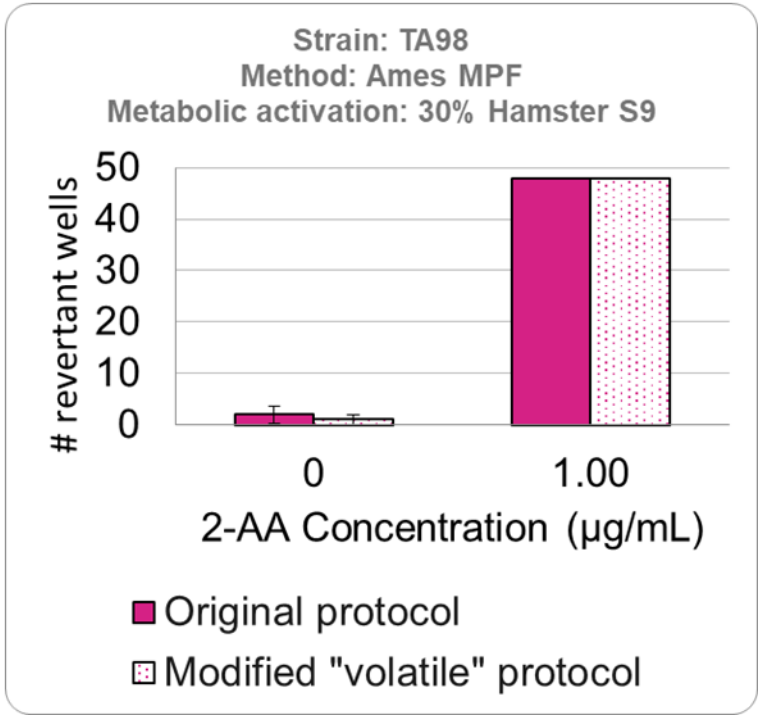

Comparison of the results of Ames MPF tests on the 384-well plates with two different test compounds. Left: non-volatile, strong positive compound. Right: volatile, strong positive compound. NC: Negative Control, #1 - #6: dilutions of the test compound, #1 is the lowest applied concentration of the compound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

Copyright: Xenometrix AG

# 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.**

Source of plate images: Boekel Scientific, ThermoFisher

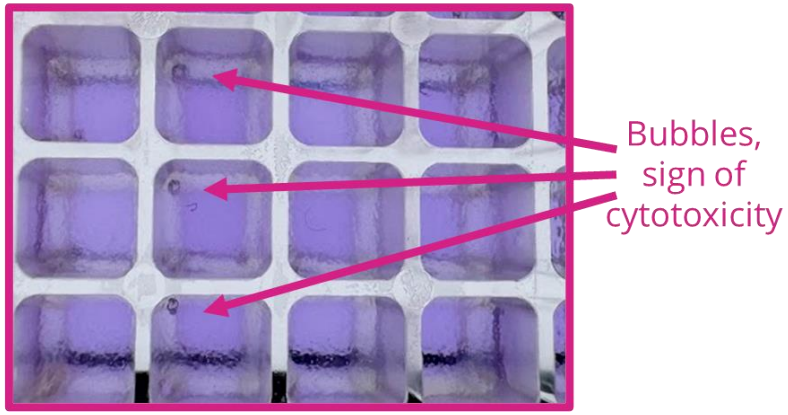
Copyright: Xenometrix AG

# Investigation of cytotoxicity

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

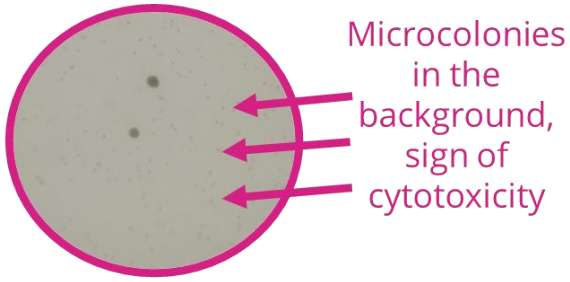
**Ames MPF: 384-well plate after 48 hours at 37°C**

Magnified image of the wells on the 384-well plate




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

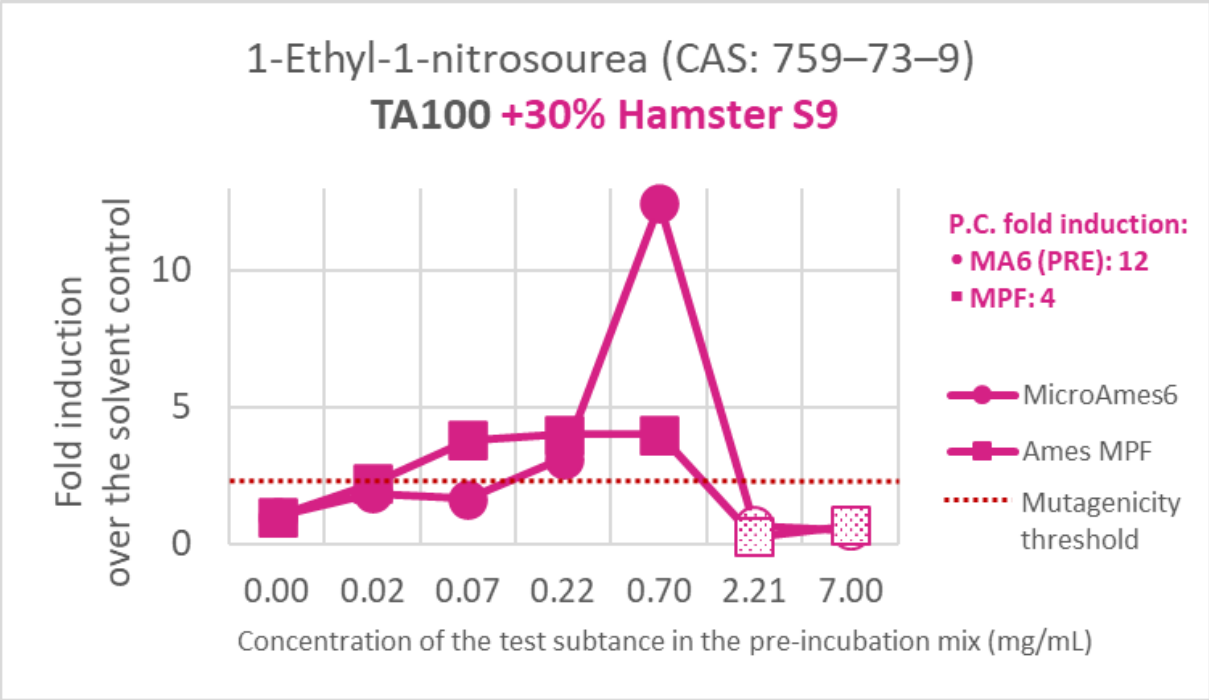
Magnified image of the well on the 6-well plate




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

Copyright: Xenometrix AG

# 1-Ethyl-1-nitrosourea
# Miniaturized Ames assay results

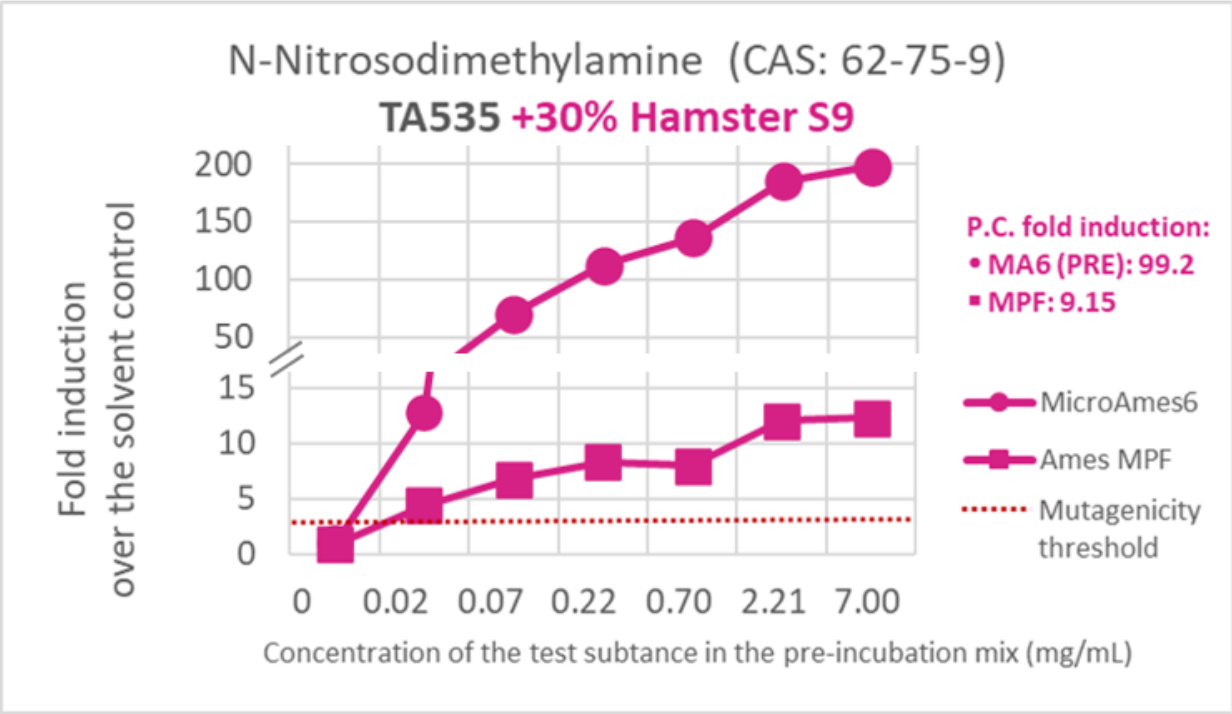



- Both miniaturized assays are **positive, in accodance with the Petri dish-based data from literature** [10]
- Normalized Lowest Effective Concentration of 1-Ethyl-1nitrosourea: **0.02 mg/mL Ames MPF** vs. **0.14 mg/mL Ames test in Petri Dishes**
- **Cytotoxicity observed** at concentrations > 0.7 ug/mL

Literature source: [10] NTP database

Copyright: Xenometrix AG

# N-Nitrosodimethylamine (NDMA) Miniaturized Ames assay results

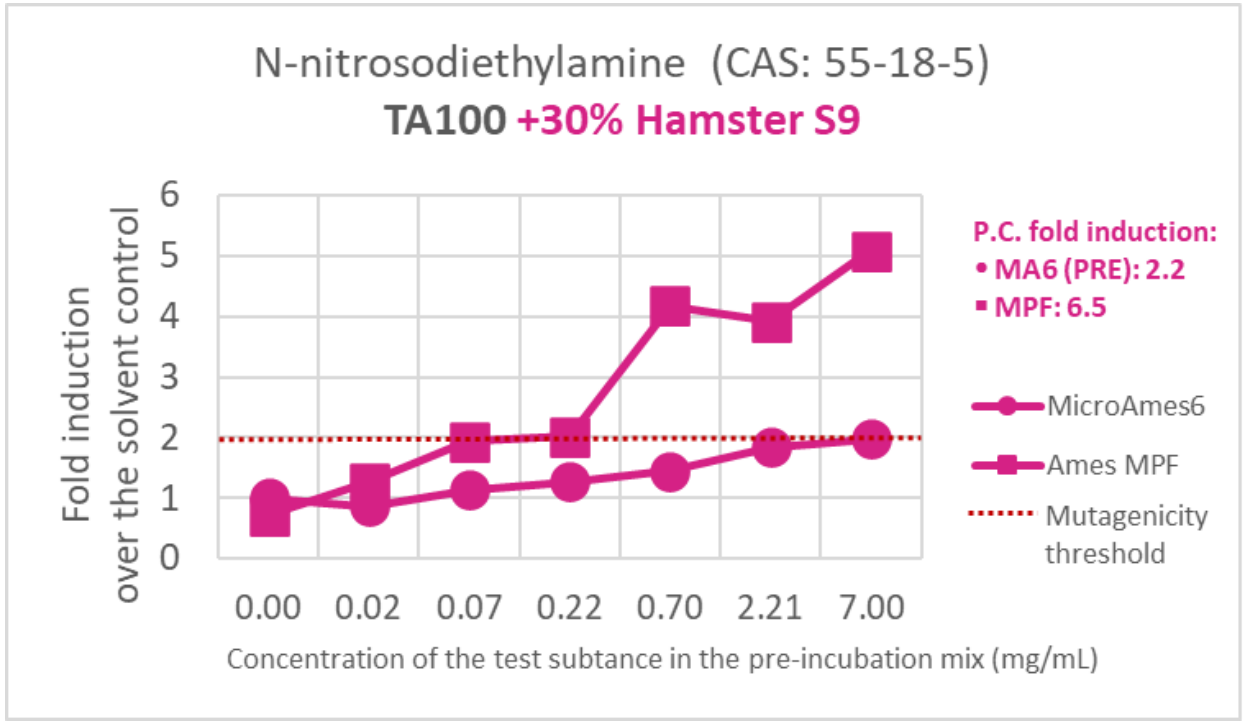



- Both miniaturized assays clearly positive, in accordance with Petri dish-based data
- Both miniaturized Ames assays positive at **0.02 mg/mL!** versus Petri dish-based assay positive at **0.7 mg/mL** NDMA-concentration [11]

Literature source: [11] Bringezu & Simon, 2022

Copyright: Xenometrix AG

# N-Nitrosodiethylamine (NDEA) Miniaturized Ames assay results

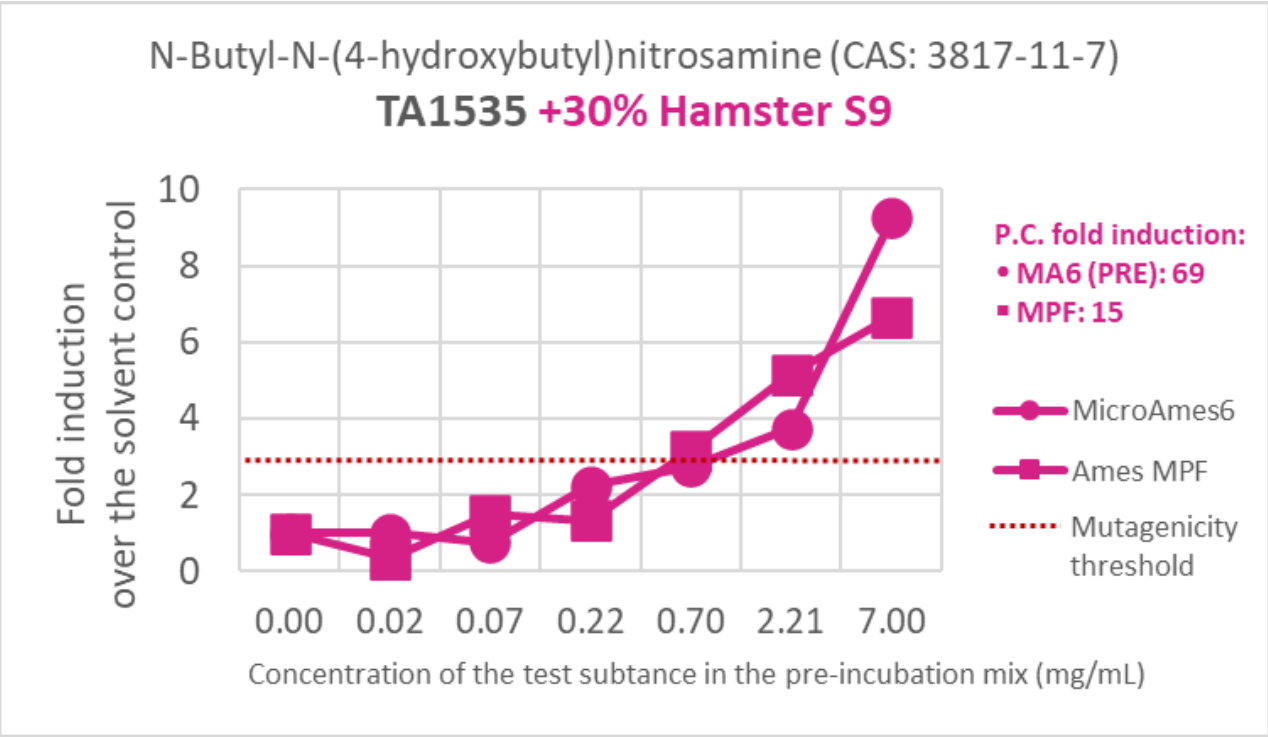



- Both miniaturized assays positive, in accordance with Petri dish-based data
- Normalized Lowest Effective Concentration for **Ames MPF is 0.22 mg/mL,** versus Petri dish-based assay: **1.4 mg/mL** NDMA [10]

Literature source: [10] NTP Database

Copyright: Xenometrix AG

# N-Butyl-N-(4-hydroxybutyl)nitrosamine Miniaturized Ames assay results




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

Literature source: [12] Nagao et al., 1977

Copyright: Xenometrix AG

# Summary of the results

| Summary Table | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Compound | CAS Nr. | Strain | Metabolic activation | MicroAmes6 | | Ames MPF | | Petri dish-based Agar Plate Test [10,11,12] | |
| | | | | Result | nLEC [µg/mL] | Result | nLEC [µg/mL] | Result | nLEC [µg/mL] |
| 1-Ethyl-1-nitrosourea | 759–73–9 | TA100 | 30% Hamster S9 | POS | 220 | POS | 20 | POS | 140 |
| N-Nitrosodimethylamine | 62-75-9 | TA1535 | 30% Hamster S9 | POS | 20 | POS | 20 | POS | 700 |
| N-nitrosodiethylamine | 55-18-5 | TA100 | 30% Hamster S9 | POS | 2214 | POS | 221 | POS | 1400 |
| N-Butyl-N-(4-hydroxybutyl)nitrosamine | 3817-11-7 | TA1535 | 30% Hamster S9 | POS | 2214 | POS | 700 | POS | 2436 |

The normalized LEC values (nLEC take into account the differences in the effective concentration during exposure in the pre-incubation mix between the liquid microplate fluctuation format (Ames MPF) and the pre-incubation agar-based Ames test systems (6-well agar plate format and the Ames test in Petri dishes); POS = positive.

Literature source: [10] NTP Database [11] Bringezu & Simon, 2022 [12] Nagao et al., 1977

Copyright: Xenometrix AG

# 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

Copyright: Xenometrix AG

XENOMETRIX
Swiss Commitment for Bioassays

**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**

**Visit our website for more data, materials and resources:**

**www.xenometrix.ch**

**Follow us on social media:**

**Posters presented on Nitrosamines at scientific meetings:**


# 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 (accessed 06.09.2023)
- [4] Questions and answers on “Information on nitrosamines formarketing authorisation holders”. EMA/CHMP/428592/2019 Rev. 3.European Medicines Agency, March 27, 2020. https://www.ema.europa.eu/en/documents/referral/nitrosamines-emea-h-a53-1490-questions-answers-information-nitrosamines-marketing-authorisation_en.pdf (accessed 09.06.2023)
- [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 (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.
- [8] Dieckhoff J, Bringezu F, Simon S. Metabolic activation of short-chain alkyl N-nitrosamines using Aroclor 1254 or phenobarbital/beta-naphthoflavone-induced rat or hamster S9 - A comparative analysis. Toxicol Rep. 2024 Jan 26;12:215-223. doi: 10.1016/j.toxrep.2024.01.012. PMID: 38322170; PMCID: PMC10844645.
- [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] Nationaly 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.
- [12] Nagao, Minako, Emako Suzuki, Kimie Yasuo, Takie Yahagi, Yuko Seino, Takashi Sugimura and Masashi Okada. “Mutagenicity of N-butyl-N-(4-hydroxybutyl)nitrosamine, a bladder carcinogen, and related compounds.” Cancer research 37 2 (1977): 399-407 .


Copyright: Xenometrix AG