XENOMETRIX

Ames II

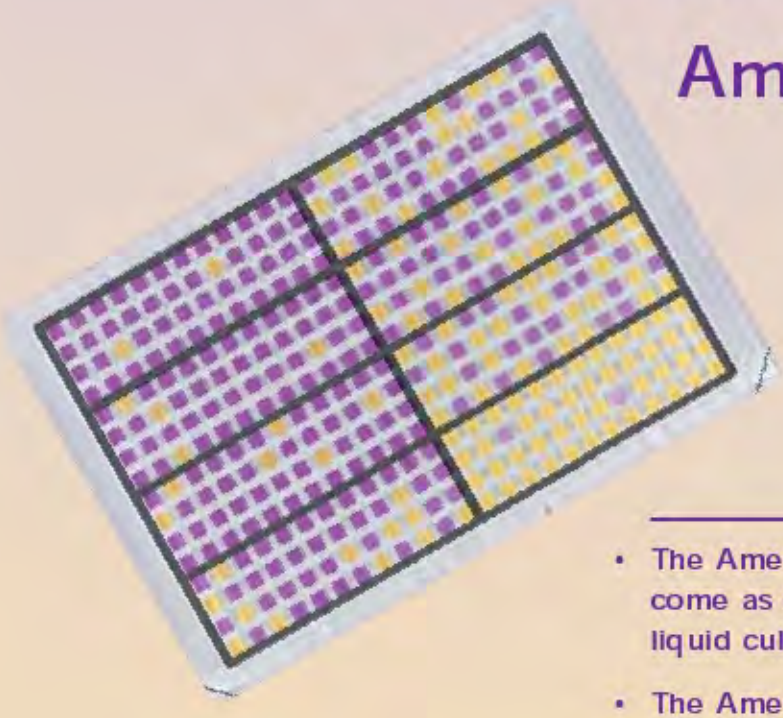
Ames MPF™ 98/100

Ames MPF™ 1535

Ames MPF™ 1537

Ames MPF™ E.Coli

Ames MPF™ PENTA I



- The Ames MPF and Ames II Fluctuation Assays come as complete ready to use kits with strains, liquid culture media, positive control and S9
- The Ames MPF and Ames II Fluctuation Assays can be used to detect genotoxic activity in chemicals, cosmetics, pharmaceuticals, foodstuffs, water, air, soils or sediments.
- Quality controlled S. typhimurium and E.Coli strains (genotyped and phenotyped)
- 3 times less compound required
- 5 times less operator intervention
- 3 fold less contaminated waste
- Less consumption of rat liver S9
- 10 times lower cost per analysis
- OECD Guideline 471 and FDA compliant
- Colorimetric determination to reduce reporting errors

Form AA09
01-2012

Ames MPF™ Mutagenicity Assay

Technical Documentation

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PROUDLY DISTRIBUTED BY ANIARA

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1. Introduction

The traditional Ames plate incorporation test (ref.1) is one of the most commonly performed genotoxicity assays in the world, forming an important component of many regulatory submissions. However, with the increasing number of chemicals flowing through the drug development process, and the increasing demand for early indications of mutation and potential carcinogenesis, the number of Ames screening assays required is growing year by year. The traditional full-format Ames test cannot currently serve this market, since it requires too much chemical, labor and time to serve as a screening tool.

2. Principle of the Ames MPF™ Mutagenicity Assay

The traditional Ames plate incorporation test is the most generally used and validated bacterial reverse-mutation test. The test employs several mutant strains of *Salmonella typhimurium*, carrying mutations in the operon coding for histidine biosynthesis. Similarly, E. coli strains can be used to test certain classes of chemicals, such as some oxidizing mutagens, cross-linking agents and hydrazines. The E. coli strains have mutations in the tryptophan biosynthesis operon. When such biosynthesis-deficient bacteria are exposed to mutagenic agents, under certain conditions reverse mutation from histidine or tryptophan auxotrophy to prototrophy occurs.

The increasing number of compounds to be screened and the fact, that new compounds are produced only in very small scale at this stage, were among the reasons to develop an alternative screening test to the standard Ames test.

The Ames MPF™ Mutagenicity Assay corresponds to the Ames Fluctuation Assay and is based on the same principle as the traditional test, but it sets a new standard for this type of testing, offering several advantages over the traditional Ames test. The Ames Fluctuation Assay is cited in the guidelines of OECD and FDA.

The Ames MPF™ Fluctuation Assay, available from Aniara, is a liquid microplate modification of the Ames test which offers a higher speed format, colorimetry, easy handling and the possibility of automated plating and plate reading. The assay is fast and efficient, consumes a lower amount of test chemical, and shows good correlation with the traditional assay. Due to the possibility of automation, hundreds of substances can be run within a month. The excellent correlation of the liquid microplate version with the traditional, agar-based Ames test has been shown in several publications.

3. Strains used in the Ames MPF™ Mutagenicity Assay

The four *Salmonella typhimurium* strains provided in the Ames MPF™ test kit are TA98, TA100; TA1535, and TA1537. The TA98 and TA1537 strains are used for the detection of frameshift mutations, and TA100 and TA1535 for base pair substitutions (ref.1). TA98 and TA100 strains are the two most often used strains in the plate incorporation assay for screening purposes (reduced Ames test), and they have the largest data sets (refs. 2 and 3). TA1535 and TA1537 are used to identify certain chemical classes of mutagens which are not detected by TA98 and TA 100 (refs. 9 and 10).
The E.coli strains available are wp2 *uvrA* and wp2 [pKM101]. They can be used individually or combined ("E.coli Combo") as described in the Instructions for Use.

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4. Ames MPF™ Mutagenicity Assay Description

Bacteria are exposed to 6 concentrations of a test agent, as well as a positive and a negative control, for 90 minutes in medium containing sufficient histidine (or tryptophan for E.coli) to support approximately two cell divisions. After 90 minutes, the exposure cultures are diluted in pH indicator medium lacking histidine (or tryptophan), and aliquoted into 48 wells of a 384-well plate. Within two days, cells which have undergone the reversion to prototrophy will grow into colonies. Metabolism by the bacterial colonies lowers the pH of the medium, changing the color of the medium in that well from purple to yellow. This color change can be detected visually or by microplate reader. The number of wells containing revertant colonies are counted for each dose and compared to a zero dose (solvent) control. It is recommended to test each dose in triplicate.

A dose-related increase in the number of revertant colonies upon exposure to a test chemical relative to the zero-dose controls indicates that the chemical is mutagenic in the Ames MPF™ Fluctuation Assay.

Genotypes of the Ames MPF™ *Salmonella typhimurium* and *E.coli* strains

Strain	Mutation	Type	Target	Cell Wall	Repair	pKM101
S.typhimurium						
TA98	*hisD3052*	Frameshifts	GCGCGCGC	*rfa*	*uvrB*	yes
TA100	*hisG46*	Base-pair subst.	GGG	*rfa*	*uvrB*	yes
TA1535	*hisG46*	Base-pair subst.	GGG	*rfa*	*uvrB*	no
TA1537	*hisC3076*	Frameshifts	+1 frameshift (near C-C-C run)	*rfa*	*uvrB*	no
E.coli wp2						
uvrA	*trpE65*	Base-pair subst.	A:T	-	*uvrA*	no
[pKM101]	*trpE65*	Base-pair subst.	A:T	-	-	yes

rfa: This mutation leads to a defective lipopolysaccharide (LPS) layer that coats the cell surface, making the bacteria more permeable to bulky chemicals.

uvrB/uvrA: The uvrB/uvrA deletion mutation eliminates the accurate excision repair mechanism, thereby allowing more DNA lesions to be repaired by error-prone DNA repair mechanisms.

pKM101: This R factor plasmid enhances chemical and UV-induced mutagenesis via an error-prone recombinational DNA repair pathway. The plasmid also confers ampicillin resistance.

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5. AmesMPF™ Mutagenicity Assay Test Kits

The Ames MPF™ kits are available in the following combinations for the testing of 1 or 10 compounds:

- Kits with single strains: TA98, TA100, TA1535, TA1537, E.coli uvrA, E.coli [pKM101]
- E.coli Combo with E. coli uvrA and [pKM101]
- 2-strain kit: TA98 and TA100 (also as AQUA kit for testing of water samples)
- 4-strain kit: TA98, TA100, TA1535, TA1537
- Penta I kit: TA98, TA100, TA1535, TA1537; E.coli Combo

1 Sample Kits

The 1 Sample Kits contain all the bacteria and media ready-to-use, and step-by-step Instructions for Use. It allows to test 1 sample in triplicate, 6 concentrations, positive and negative controls, with and without metabolic activation (S9) or 3 samples without replicate, 6 concentrations, positive and negative controls, in the absence and presence of metabolic activation.

10 Sample Kits

These kits allow testing of at least 10 compounds in triplicate, 6 concentrations, positive and negative controls, in the absence and presence of metabolic activation.

S9 and positive controls

Kits are available with and without S9 and strain-specific positive control chemicals.

Design your own kit

All components necessary for the assay are also available separately.

2 shipping versions

All kits are available either with bacteria in a liquid medium which are shipped on dry ice or with bacteria in a semi-solid medium which are shipped at ambient temperature. This version saves shipping costs and is less susceptible to shipping delays, but the bacteria need to be re-suspended in media before the start of the overnight culture. Otherwise the kits are identical and give comparable results.

Ames MPF 98/100 AQUA

We also offer a kit with TA98 and TA100 for the testing of aqueous samples. By using a 10-fold concentrated Exposure Medium the aqueous sample is only weakly diluted in the exposure step to 74% rather than the normal 25-fold dilution in the standard kit. The AQUA format and media can also be used with the other Ames *S. typhimurium* strains. The Ames MPF 98/100 AQUA kit is available for the testing of 1 or 5 samples.

All Ames MPF kits are available with strains in semi-solid medium which are shipped at room temperature, but - like the strains in liquid medium - must be stored immediately at -70℃ upon arrival.

Please contact Aniara for further information if you have a -20℃ freezer only.

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6. Automation of the Ames MPF™ Mutagenicity Assay

The Ames MPF™ Fluctuation Assay can be automated. The technically identical Ames II Mutagenicity Assay (also available from Aniara) has been validated in an automated robotic system and proved to allow fast, accurate and reliable toxicological screening (refs. 4, 5, 11). The same conclusions apply to the Ames MPF™ Assay.

7. Validation and Comparative Studies

The performance of the Ames II assay which uses the same principles and procedures as the Ames MPF™ assay has been evaluated before and was shown to be an effective and reliable screening method to identify mutagens, with excellent concordance to published results using the traditional Ames assay. (ref. 7, 11, 12).

An internal validation study published as poster at the EEMS congress in Prague in 2006 compared the performance of the TA100 MPF™ with the TAMix strains of the Ames II assay, and with published results of TA100 in the plate incorporation assay (ref. 6). This study demonstrated excellent correlation between TA100 and TAMix in the liquid microplate format with published data of TA100 in the plate incorporation assay.

A further study published as poster at the SOT congress in Charlotte in 2007 (Ref. 8) compared 24 reference compounds using TA98, TA100, TA1535 and TA1537 in the Ames MPF™ format with published data using the plate incorporation assay and found again excellent concordance between the two assay formats.

8. Advantages of the Ames MPF™ Mutagenicity Assay

- 10 times lower cost per analysis
- 3 fold less contaminated waste
- 3 times less compound required
- 5 times less operator intervention
- Ready-to-use reagents and quality controlled bacterial strains
- No autoclaving of media or sterility testing required
- Colorimetric determination to reduce reporting errors
- OECD Guideline 471 compliant

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9. Xenometrix Services

1) Training Programs and pre- and post sale support:
   Training on how to perform the Ames MPF™ Fluctuation Assay in our or your facility; support of the setup in your own laboratory. Free support with experimental set-up and data evaluation by phone or Email by our experienced staff.

2) Client Research Laboratory:
   Xenometrix has, at its Allschwil facility, a fully staffed and equipped laboratory for the purpose of performing an optimal Ames MPF™ Fluctuation Assay and reporting work for customers.
   Xenometrix offers to perform assays, interpret data and produce detailed reports.
   The Client Research Laboratory staff is trained in all aspects of the Ames MPF™ Fluctuation Assay, making for a very efficient and cost-effective process. Depending upon answers to a client's questionnaire, detailed reports are generally completed within 7 days. Data can also be communicated immediately upon assay completion (3 days).

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10. Literature

Ref. 1 D.M.Maron and B.N. Ames; Revised methods for the Salmonella Mutagenicity test; Mutation Research 113 (1983), 173-215

Ref. 2 T. Kubo, K. Urano and H. Utsumo; Mutagenicity characteristics of 255 environmental chemicals; J. Health Science 48 (6), (2002), 545-554

Ref. 3 A. Hakura et. Al.: Salmonella/human S9 mutagenicity test: a collaborative study with 58 compounds; Mutagenesis 20 (3) (2005), 217-228

Ref. 4 K Braun; Automation of the Ames II™ Assay: High Through-put screening of mutagenic substances; Aventis Pharma Deutschland GmbH, DI & A, Lead Optimization, Drug Safety Evaluation, MipTec ICAR (2001).

Ref. 5 M Crook; Automation of the Ames II™ toxicology test; MipTec-ICAR (2000) P07.

Ref. 6 S. Flückiger-Isler and M. Kamber; The Ames MPF™ 98/100 Assay: Novel mutagenicity testing in liquid microplate format using S. typhimurium TA98 and TA100; EEMS Prague (2006)

Ref. 7 S Flückiger-Isler, M Baumeister, K Braun, V Gervais, N Hasler-Nguyen, R Reimann, J van Gompel, H-G Wunderlich, G Engelhardt; Assessment of the performance of the Ames II™ assay: A collaborative study with 19 coded compounds; Mutation Res (2004) 558, 181-197.

Ref. 8 S. Flückiger-Isler and M. Kamber; Novel Mutagenicity Testing in Liquid Microplate Format using *S. typhimurium* TA98, TA100, TA1535 and TA1537. SOT Charlotte (2007)

Ref. 9 L.R. Ferguson and W.A. Denny; Frameshift mutagenesis by acridines and other reversibly-binding DNA ligands. Mutagenesis 5, 529-540 (1990).

Ref 10 M.J. Prival and E. Zeiger; Chemicals mutagenic in *Salmonella typhimurium* strain TA1535 but not in TA100. Mutat. Res. 412, 251-260 (1998).

Ref 11 M. Kamber, S. Flückiger-Isler, G. Engelhardt, R, Jaeckh and E. Zeiger; Comparison of the Ames II and traditional Ames test responses with respect to mutagenicity, strain specificities, need for metabolism and correlation with rodent carcinogenicity. Mutagenesis 24, 359 - 366 (2009).

Ref 12 GdA Umbuzeiro, C.M. Rech, S. Correia, A.M. Bergamasco, G.H.L. Cardenette, s. Flückiger, and M. Kamber; Comparison of the Salmonella/microsome microsuspension assay with the new microplate fluctuation (MPF) protocol for testing the mutagenicity of environmental samples. Environ Mol Mutagen 51, 31-38 (2010).

Ref 13 F. Atienzar; Evaluation of a Battery of early Genotoxicity Assays to Predict Regulatory Testing. ADMET meeting, Brussels 22-23 Jan 2009

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Product	Art. No.	Content
AMES MPF 98 TEST KITS		
Ames MPF TA98 - 1 Sample Kit (Semi-Solid)	AA01-110	1 sample kit
Ames MPF TA98 - 1 Sample Kit - Rat Liver S9 - Pos.Control (Semi-Solid)	AA01-110-S1-P	1 Sample Kit
Ames MPF TA98 - 1 Sample Kit - Rat Liver S9 - Pos.Control S2 (Semi-Solid)	AA01-110-S2-P	1 sample kit
Ames MPF TA98 - 10 Sample Kit (Semi-Solid)	AA10-110	10 samples kit
Ames MPF TA98 - 10 Sample Kit - Rat Liver S9 - Pos.Control (Semi-Solid)	AA10-110-S1-P	10 Samples Kit
Ames MPF TA98 - 10 Sample Kit - Rat Liver S9 - Pos.Control S2 (Semi-Solid)	AA10-110-S2-P	10 samples kit
Ames MPF TA98 - 1 Sample Kit (Liquid/Frozen)	AB01-110	1 sample kit
Ames MPF TA98 - 1 Sample Kit - Rat Liver S9 - Pos.Control (Liquid/Frozen)	AB01-110-S1-P	1 Sample Kit
Ames MPF TA98 - 1 Sample Kit - Rat Liver S9 - Pos.Control S2 (Liquid/Frozen)	AB01-110-S2-P	1 sample kit
Ames MPF TA98 - 10 Sample Kit (Liquid/Frozen)	AB10-110	10 samples kit
Ames MPF TA98 - 10 Sample Kit - Rat Liver S9 - Pos.Control (Liquid/Frozen)	AB10-110-S1-P	10 Samples Kit
Ames MPF TA98 - 10 Sample Kit - Rat Liver S9 - Pos.Control S2 (Liquid/Frozen)	AB10-110-S2-P	10 samples kit
AMES MPF 100 TEST KITS		
Ames MPF TA100 - 1 Sample Kit (Semi-Solid)	AA01-111	1 sample kit
Ames MPF TA100 - 1 Sample Kit - Rat Liver S9 - Pos.Control (Semi-Solid)	AA01-111-S1-P	1 Sample Kit
Ames MPF TA100 - 1 Sample Kit - Rat Liver S9 - Pos.Control S2 (Semi-Solid)	AA01-111-S2-P	1 sample kit
Ames MPF TA100 - 10 Sample Kit (Semi-Solid)	AA10-111	10 samples kit
Ames MPF TA100 - 10 Sample Kit - Rat Liver S9 - Pos.Control (Semi-Solid)	AA10-111-S1-P	10 Samples Kit
Ames MPF TA100 - 10 Sample Kit - Rat Liver S9 - Pos.Control S2 (Semi-Solid)	AA10-111-S2-P	10 samples kit
Ames MPF TA100 - 1 Sample Kit (Liquid/Frozen)	AB01-111	1 sample kit
Ames MPF TA100 - 1 Sample Kit - Rat Liver S9 - Pos.Control (Liquid/Frozen)	AB01-111-S1-P	1 Sample Kit
Ames MPF TA100 - 1 Sample Kit - Rat Liver S9 - Pos.Control S2 (Liquid/Frozen)	AB01-111-S2-P	1 sample kit
Ames MPF TA100 - 10 Sample Kit (Liquid/Frozen)	AB10-111	10 samples kit
Ames MPF TA100 - 10 Sample Kit - Rat Liver S9 - Pos.Control (Liquid/Frozen)	AB10-111-S1-P	10 Samples Kit
Ames MPF TA100 - 10 Sample Kit - Rat Liver S9 - Pos.Control S2 (Liquid/Frozen)	AB10-111-S2-P	10 samples kit
AMES MPF 1535 TEST KITS		
Ames MPF TA1535 - 1 Sample Kit (Semi-Solid)	AA01-112	1 sample kit
Ames MPF TA1535 - 1 Sample Kit - Rat Liver S9 - Pos.Control (Semi-Solid)	AA01-112-S1-P	1 Sample Kit
Ames MPF TA1535 - 1 Sample Kit - Rat Liver S9 - Pos.Control S2 (Semi-Solid)	AA01-112-S2-P	1 sample kit

Product	Art. No.	Content
Ames MPF TA1535 - 10 Sample Kit (Semi-Solid)	AA10-112	10 samples kit
Ames MPF TA1535 - 10 Sample Kit - Rat Liver S9 - Pos.Control (Semi-Solid)	AA10-112-S1-P	10 Samples Kit
Ames MPF TA1535 - 10 Sample Kit - Rat Liver S9 - Pos.Control S2 (Semi-Solid)	AA10-112-S2-P	10 samples kit
Ames MPF TA1535 - 1 Sample Kit (Liquid/Frozen)	AB01-112	1 Sample Kit
Ames MPF TA1535 - 1 Sample Kit - Rat Liver S9 - Pos.Control (Liquid/Frozen)	AB01-112-S1-P	1 Sample Kit
Ames MPF TA1535 - 1 Sample Kit - Rat Liver S9 - Pos.Control S2 (Liquid/Frozen)	AB01-112-S2-P	1 sample kit
Ames MPF TA1535 - 10 Sample Kit (Liquid/Frozen)	AB10-112	10 Samples Kit
Ames MPF TA1535 - 10 Sample Kit - Rat Liver S9 - Pos.Control (Liquid/Frozen)	AB10-112-S1-P	10 Sample Kit
Ames MPF TA1535 - 10 Sample Kit - Rat Liver S9 - Pos.Control S2 (Liquid/Frozen)	AB10-112-S2-P	10 samples kit
AMES MPF 1537 TEST KITS		
Ames MPF TA1537 - 1 Sample Kit (Semi-Solid)	AA01-113	1 sample kit
Ames MPF TA1537 - 1 Sample Kit - Rat Liver S9 - Pos.Control (Semi-Solid)	AA01-113-S1-P	1 Sample Kit
Ames MPF TA1537 - 1 Sample Kit - Rat Liver S9 - Pos.Control S2 (Semi-Solid)	AA01-113-S2-P	1 sample kit
Ames MPF TA1537 - 10 Sample Kit (Semi-Solid)	AA10-113	10 samples kit
Ames MPF TA1537 - 10 Sample Kit - Rat Liver S9 - Pos.Control (Semi-Solid)	AA10-113-S1-P	10 Samples Kit
Ames MPF TA1537 - 10 Sample Kit - Rat Liver S9 - Pos.Control S2 (Semi-Solid)	AA10-113-S2-P	10 samples kit
Ames MPF TA1537 - 1 Sample Kit (Liquid/Frozen)	AB01-113	1 Sample Kit
Ames MPF TA1537 - 1 Sample Kit - Rat Liver S9 - Pos.Control (Liquid/Frozen)	AB01-113-S1-P	1 Sample Kit
Ames MPF TA1537 - 1 Sample Kit - Rat Liver S9 - Pos.Control S2 (Liquid/Frozen)	AB01-113-S2-P	1 sample kit
Ames MPF TA1537 - 10 Sample Kit (Liquid/Frozen)	AB10-113	10 Samples Kit
Ames MPF TA1537 - 10 Sample Kit - Rat Liver S9 - Pos.Control (Liquid/Frozen)	AB10-113-S1-P	10 Samples Kit
Ames MPF TA1537 - 10 Sample Kit - Rat Liver S9 - Pos.Control S2 (Liquid/Frozen)	AB10-113-S2-P	10 samples kit
AMES MPF 98-100 TEST KITS		
Ames MPF TA98-100 - 1 Sample Kit (Semi-Solid)	AA01-210	1 sample kit
Ames MPF TA98-100 - 1 Sample Kit - Rat Liver S9 - Pos.Control (Semi-Solid)	AA01-210-S1-P	1 Sample Kit
Ames MPF TA98-100 - 1 Sample Kit - Rat Liver S9 - Pos.Control S2 (Semi-Solid)	AA01-210-S2-P	1 sample kit
Ames MPF TA98-100 - 10 Sample Kit (Semi-Solid)	AA10-210	10 samples kit
Ames MPF TA98-100 - 10 Sample Kit - Rat Liver S9 - Pos.Control (Semi-Solid)	AA10-210-S1-P	10 Samples Kit
Ames MPF TA98-100 - 10 Sample Kit - Rat Liver S9 - Pos.Control S2 (Semi-Solid)	AA10-210-S2-P	10 samples kit
Ames MPF TA98-100 - 1 Sample Kit (Liquid/Frozen)	AB01-210	1 Sample Kit

Product	Art. No.	Content
Ames MPF TA98-100 - 1 Sample Kit - Rat Liver S9 - Pos.Control (Liquid/Frozen)	AB01-210-S1-P	1 Sample Kit
Ames MPF TA98-100 - 1 Sample Kit - Rat Liver S9 - Pos.Control S2 (Liquid/Frozen)	AB01-210-S2-P	1 sample kit
Ames MPF TA98-100 - 10 Sample Kit (Liquid/Frozen)	AB10-210	10 Samples Kit
Ames MPF TA98-100 - 10 Sample Kit - Rat Liver S9 - Pos.Control (Liquid/Frozen)	AB10-210-S1-P	10 Samples Kit
Ames MPF TA98-100 - 10 Sample Kit - Rat Liver S9 - Pos.Control S2 (Liquid/Frozen)	AB10-210-S2-P	10 samples kit
Ames MPF TA98-100 Plasticware for 1 Sample Kit	APPW-0610	1 kit
AMES MPF 98-100-1535-1537 COMBINATION TEST KITS		
Ames MPF TA98-100-35-37 (Combination) - 1 Sample Kit (Semi-Solid)	AA01-410	1 sample kit
Ames MPF TA98-100-1535-1537 - 1 Sample Kit - S9 - Pos.Control (Semi-Solid)	AA01-410-S1-P	1 Sample Kit
Ames MPF TA98-100-1535-1537 - 1 Sample Kit - S9 - Pos.Control S2 (Semi-Solid)	AA01-410-S2-P	1 sample kit
Ames MPF TA98-100-35-37 (Combination) - 10 Sample Kit (Semi-Solid)	AA10-410	10 samples kit
Ames MPF TA98-100-1535-1537 - 10 Sample Kit - S9 - Pos.Control (Semi-Solid)	AA10-410-S1-P	10 samples kit
Ames MPF TA98-100-1535-1537 - 10 Sample Kit - S9 - Pos.Control S2 (Semi-Solid)	AA10-410-S2-P	10 samples kit
Ames MPF TA98-100-35-37 (Combination) - 1 Sample Kit (Liquid/Frozen)	AB01-410	1 sample kit
Ames MPF TA98-100-1535-1537 - 1 Sample Kit - S9 - Pos.Control (Liquid/Frozen)	AB01-410-S1-P	1 Sample Kit
Ames MPF TA98-100-1535-1537 - 1 Sample Kit - S9 - Pos.Control S2 (Liquid/Frozen)	AB01-410-S2-P	1 sample kit
Ames MPF TA98-100-35-37 (Combination) - 10 Sample Kit (Liquid/Frozen)	AB10-410	10 samples kit
Ames MPF TA98-100-1535-1537 - 10 Sample Kit -S9 - Pos.Control (Liquid/Frozen)	AB10-410-S1-P	10 Samples Kit
Ames MPF TA98-100-1535-1537 - 10 Sample Kit - S9 - Pos.Control S2 (Liquid/Frozen)	AB10-410-S2-P	10 samples kit
AMES MPF PENTA I TEST KITS		
Ames MPF PENTA I - 1 Sample Kit (Semi-Solid)	AC01-512	1 Sample Kit
Ames MPF PENTA I - 1 Sample Kit - Rat Liver S9 - Pos.Contr. (Semi-Solid)	AC01-512-S1-P	1 Sample Kit
Ames MPF PENTA I - 1 Sample Kit - Rat Liver S9 - Pos.Contr. S2 (Semi-Solid)	AC01-512-S2-P	1 sample kit
Ames MPF PENTA I - 10 Sample Kit (Semi-Solid)	AC10-512	10 Samples Kit
Ames MPF PENTA I - 10 Sample Kit - Rat Liver S9 - Pos.Contr. (Semi-Solid)	AC10-512-S1-P	10 Samples Kit
Ames MPF PENTA I - 10 Sample Kit - Rat Liver S9 - Pos.Contr. S2 (Semi-Solid)	AC10-512-S2-P	10 samples kit
Ames MPF PENTA I - 1 Sample Kit (Liquid/Frozen)	AD01-512	1 Sample Kit
Ames MPF PENTA I - 1 Sample Kit - Rat Liver S9 - Pos.Contr. (Liquid/Frozen)	AD01-512-S1-P	1 Sample Kit
Ames MPF PENTA I - 1 Sample Kit - Rat Liver S9 - Pos.Contr. S2 (Liquid/Frozen)	AD01-512-S2-P	1 sample kit
Ames MPF PENTA I - 10 Sample Kit (Liquid/Frozen)	AD10-512	10 Samples Kit

Product	Art. No.	Content
Ames MPF PENTA I - 10 Sample Kit - Rat Liver S9 - Pos.Contr. (Liquid/Frozen)	AD10-512-S1-P	10 Samples Kit
Ames MPF PENTA I - 10 Samples Kit - Rat Liver S9 - Pos.Contr. Kit S2 (Liquid/Frozen)	AD10-512-S2-P	10 samples kit
AMES MPF E.COLI pKM TEST KITS		
Ames MPF E.Coli pKM - 1 Sample Kit (Semi-Solid)	AC01-116	1 Sample Kit
Ames MPF E.Coli pKM - 1 Sample Kit - Rat Liver S9 - Pos.Contr. (Semi-Solid)	AC01-116-S1-P	1 Sample Kit
Ames MPF E.Coli pKM - 1 Sample Kit - Rat Liver S9 - Pos.Contr. S2 (Semi-Solid)	AC01-116-S2-P	1 sample kit
Ames MPF E.Coli pKM - 10 Sample Kit (Semi-Solid)	AC10-116	10 Samples Kit
Ames MPF E.Coli pKM - 10 Sample Kit - Rat Liver S9 - Pos.Contr. (Semi-Solid)	AC10-116-S1-P	10 Samples Kit
Ames MPF E.Coli pKM - 10 Sample Kit - Rat Liver S9 - Pos.Contr. S2 (Semi-Solid)	AC10-116-S2-P	10 samples kit
Ames MPF E.Coli pKM - 1 Sample Kit (Liquid/Frozen)	AD01-116	1 Sample Kit
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Ames MPF E.Coli pKM - 1 Sample Kit - Rat Liver S9 - Pos.Contr. S2 (Liquid/Frozen)	AD01-116-S2-P	1 sample kit
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Ames MPF E.Coli pKM - 10 Sample Kit - Rat Liver S9 - Pos.Contr. (Liquid/Frozen)	AD10-116-S1-P	10 Samples Kit
Ames MPF E.Coli pKM - 10 Sample Kit - Rat Liver S9 - Pos.Contr. S2(Liquid/Frozen)	AD10-116-S2-P	10 samples kit
AMES MPF E.COLI uvrA TEST KITS		
Ames MPF E.Coli uvrA - 1 Sample Kit (Semi-Solid)	AC01-115	1 Sample Kit
Ames MPF E.Coli uvrA - 1 Sample Kit - Rat Liver S9 - Pos.Contr. (Semi-Solid)	AC01-115-S1-P	1 Sample Kit
Ames MPF E.Coli uvrA - 1 Sample Kit - Rat Liver S9 - Pos.Contr. S2 (Semi-Solid)	AC01-115-S2-P	1 sample kit
Ames MPF E.Coli uvrA - 10 Sample Kit (Semi-Solid)	AC10-115	10 Samples Kit
Ames MPF E.Coli uvrA - 10 Sample Kit - Rat Liver S9 - Pos.Contr. (Semi-Solid)	AC10-115-S1-P	10 Samples Kit
Ames MPF E.Coli uvrA - 10 Sample Kit - Rat Liver S9 - Pos.Contr. S2 (Semi-Solid)	AC10-115-S2-P	10 samples kit
Ames MPF E.Coli uvrA - 1 Sample Kit (Liquid/Frozen)	AD01-115	1 Sample Kit
Ames MPF E.Coli uvrA - 1 Sample Kit - Rat Liver S9 - Pos.Contr. (Liquid/Frozen)	AD01-115-S1-P	1 Sample Kit
Ames MPF E.Coli uvrA - 1 Sample Kit - Rat Liver S9 - Pos.Contr. S2 (Liquid/Frozen)	AD01-115-S2-P	1 sample kit
Ames MPF E.Coli uvrA - 10 Sample Kit (Liquid/Frozen)	AD10-115	10 Samples Kit
Ames MPF E.Coli uvrA - 10 Sample Kit - Rat Liver S9 - Pos.Contr. (Liquid/Frozen)	AD10-115-S1-P	10 Samples Kit
Ames MPF E.Coli uvrA - 10 Sample Kit - Rat Liver S9 - Pos.Contr. S2 (Liquid/Frozen)	AD10-115-S2-P	10 samples kit
AMES MPF E.COLI COMBO TEST KITS		

Product	Art. No.	Content
Ames MPF E.Coli Combo - 1 Sample Kit (Semi-Solid)	AC01-117	1 Sample Kit
Ames MPF E.Coli Combo - 1 Sample Kit - Rat Liver S9 - Pos.Contr. (Semi-Solid)	AC01-117-S1-P	1 Sample Kit
Ames MPF E.Coli Combo - 1 Sample Kit - Rat Liver S9 - Pos.Contr. S2 (Semi-Solid)	AC01-117-S2-P	1 sample kit
Ames MPF E.Coli Combo - 10 Sample Kit (Semi-Solid)	AC10-117	10 Samples Kit
Ames MPF E.Coli Combo - 10 Sample Kit - Rat Liver S9 - Pos.Contr. (Semi-Solid)	AC10-117-S1-P	10 Samples Kit
Ames MPF E.Coli Combo - 10 Sample Kit - Rat Liver S9 - Pos.Contr. S2 (Semi-Solid)	AC10-117-S2-P	10 samples kit
Ames MPF E.Coli Combo - 1 Sample Kit (Liquid/Frozen)	AD01-117	1 Sample Kit
Ames MPF E.Coli Combo - 1 Sample Kit - Rat Liver S9 - Pos.Contr. (Liquid/Frozen)	AD01-117-S1-P	1 Sample Kit
Ames MPF E.Coli Combo - 1 Sample Kit - Rat Liver S9 - Pos.Contr. S2 (Liquid/Frozen)	AD01-117-S2-P	1 sample kit
Ames MPF E.Coli Combo - 10 Sample Kit (Liquid/Frozen)	AD10-117	10 Samples Kit
Ames MPF E.Coli Combo - 10 Sample Kit - Rat Liver S9 - Pos.Contr. (Liquid/Frozen)	AD10-117-S1-P	10 Samples Kit
Ames MPF E.Coli Combo - 10 Sample Kit - Rat Liver S9 - Pos.Contr. S2 (Liquid/Frozen)	AD10-117-S2-P	10 samples kit
AMES UmuC EASY AQ and UmuC EASY CS TEST KITS		
Ames MPF U-muC Easy AQ (Aqueous Samples) 1-Day Microplate Format Genotoxicity Assay	AF06-118	6 samples kit (2x96 wells)
Ames MPF UmuC Easy CS (Concentrated Samples) 1-Day Microplate Format Genotoxicity Assay	AG06-118	6 samples kit (2x96 wells)
AMES MPF 98-100 AQUA TEST KITS		
Ames MPF Aqua 98-100 - 48 Measuring Points	AJ01-210	1 sample kit
Ames MPF Aqua 98-100 - 48 Measuring Points with Lyophilized Rat Liver S9 and Positive Controls	AJ01-210-S1-P	1 sample kit
Ames MPF Aqua 98-100 - 240 Measuring Points	AJ05-210	5 samples kit
Ames MPF Aqua 98-100 - 240 Measuring Points with Lyophilized Rat Liver S9 and Positive Controls	AJ05-210-S1-P	5 sample kit
PETRIFILM AQUA PRODUCTS		
Petrifilm Aqua Yeast Molds (AQYM)	A3M-6408	2x50 tests
Petrifilm Aqua Yeast Molds (AQYM)	A3M-6413	1000 tests
Petrifilm Aqua Enterobacteriaceae (AQEB)	A3M-6418	2x25 tests
Petrifilm Aqua Enterobacteriaceae (AQEB)	A3M-6428	1000 tests
Petrifilm Aqua Heterotrophic (ACHC)	A3M-6450	2x50 tests
Petrifilm Aqua Heterotrophic (ACHC)	A3M-6452	1000 tests
Petrifilm Aqua Coliform Plate (AQCC)	A3M-6457	2x25 tests
Petrifilm Aqua Coliform Plate (AQCC)	A3M-6458	1000 tests

Product	Art. No.	Content
AMES MPF YG TEST KITS		
Ames MPF YG Test Kit - 1 Sample	AK01-000	1 sample kit
Ames MPF YG Test Kit - 6 Samples	AK06-000	6 samples kit
AMES RAT LIVER S9		
Ames MPF S9 CoFactor Kit	APCO-0800	20 ml
Aroclor Induced Lyophilized Microsomal Rat Liver S9	APRS-AC00	0.4 ml
Ames Rat Liver S9 (Lyophilized) , 1254 Aroclor induced 1 ml	APRS-AC01	1 ml
Ames Rat Liver S9 (Lyophilized), 1254 Aroclor induced 2 ml	APRS-AC02	2 ml
Phenobarbital/Beta-Naphtoflavone Induced Lyophilized Rat Liver S9	APRS-PB00	0.4 ml
Ames Rat Liver S9 (Lyophilized), Phenobarbital 5/6 Benzofl. induced 1 ml	APRS-PB01	1 ml
Ames Rat Liver S9 (Lyophilized), Phenobarbital 5/6 Benzofl. induced 2 ml	APRS-PB02	2 ml
AMES MPF MEDIA (LIQUID)		
Ames MPF 50 Exposure Medium (RT) (Liquid/Frozen)	APMM-EM02	50 ml
Ames MPF TA25 - 10X Exposure Medium (RT) (Liquid/Frozen)	APMM-EM03	25 ml
Ames MPF, Ames II & E.coli 50 ml Growth Medium (RT) (Liquid/Frozen)	APMM-GM00	50 ml
Ames MPF 550 Indicator Medium (RT) (Liquid/Frozen)	APMM-IM10	550 ml
Ames MPF TA100 Indicator Medium (RT) (Liquid/Frozen)	APMM-IM11	100 ml
AMES MPF E.COLI MEDIA (LIQUID)		
Ames MPF Exposure Medium (RT) - 50 ml - E.Coli (Liquid/Frozen)	APME-EM22	50 ml
Ames MPF, Ames II & E.coli 50 ml Growth Medium (RT) (Liquid/Frozen)	APMM-GM00	50 ml
Ames MPF Reversion Indicator Medium (RT) - 550 ml - E.Coli (Liquid/Frozen)	APME-IM31	550 ml
Ames MPF Reversion Indicator Medium (RT) - 100 ml - E.Coli (Liquid/Frozen)	APME-IM32	100 ml
AMES MPF - S. typhimurium STRAINS (LIQUID)		
Ames TA100 S. typhimurium Strain (Liquid/Frozen)	APLI-0111	50 µl
Ames TA1535 S. typhimurium Strain (Liquid/Frozen)	APLI-0112	50 µl
Ames TA1537 S. typhimurium Strain (Liquid/Frozen)	APLI-0113	50 µl
Ames Liquid EC uvrA S. typhimurium Strain (Liquid/Frozen)	APLI-0115	50 µl

Product	Art. No.	Content
Ames Liquid EC pKM101 S. typhimurium Strain (Liquid/Frozen)	APLI-0116	50 µl
AMES MPF - S. typhimurium STRAINS (SEMISOLID)		
Ames TA98 S. typhimurium Strain (Semi-Solid)	APSS-0110	50 µl
Ames TA100 S. typhimurium Strains (Semi-Solid)	APSS-0111	50 µl
Ames TA1535 S. typhimurium Strain (Semi-Solid)	APSS-0112	50 µl
Ames TA1535 S. typhimurium Strain (Semi-Solid)	APSS-0113	50 µl
Ames Semisolid EC uvraA S. typhimurium Strains (Semi-Solid)	APSS-0115	50 µl
Ames Semisolid EC pKM101 S. typhimurium Strains (Semi-Solid)	APSS-0116	50 µl
Ames MPF Semisolid Strain (TA1535 (psK1002))	APSS-0118	50 µl
POSITIVE CONTROLS		
Ames 2-Aminoanthracene Positive Control	APPC-AA01	100 µg
Ames N4-Aminocytidine Positive Control	APPC-AC04	10 mg
Ames 9-Aminoacridine Positive Control	APPC-AR05	1000 µg
Ames 2-Nitrofluorene Positive Control	APPC-NF00	20 µg
Ames 4-Nitroquinolone-N-Oxide Positive Control (50 micrograms)	APPC-NQ02	50 µg
Ames 4-Nitroquinolone-N-Oxide Positive Control (500 micrograms)	APPC-NQ03	500 µg
PIPETTORS		
Repeating 8 Channel-Pipettor, (25-1250 microliter)	A1160-1250	25-1250 microliter
Tips Ovation (5x192 stk.) (1400 microliter)	A4060-3132	1400 microliter
Transferpette S TreffLab 8 Channel, 5-50 Microliter	A96-10688-06-1	5 to 50 microliter
Transferpette S TreffLab 8 Channel, 20-200 Microliter	A96-10688-09-1	20 to 200 microliter
Tips For Transferpette, 960 Stk., 200 Microliter (Yellow)	AR-2130602	200 microliter
PLASTICWARE		
Ames MPF 1 Strain Kit Plasticware	APPW-0611	1 kit
Sterile 24-Well Exposure Plates (126 Plates)	APPW-0725	126 plates
Sterile 384-Well Plates (32 plates)	APPW-0924	32 plates
Sterile Culture Tubes With Fliter Cap (50 ml, 9x20 Tubes)	APPW-1018	9x20 tubes
Sterile Reagent Reservoirs (32x5)	APPW-1132	32x5 reservoirs

Product	Art. No.	Content
Sterile 96-Well Chemical Plate (100 Plates)	APPW-8100	160 Plates
ANALYTICAL SERVICE		
Ames MPF Ames II Analytical Service	ASSA-0710	1 compound

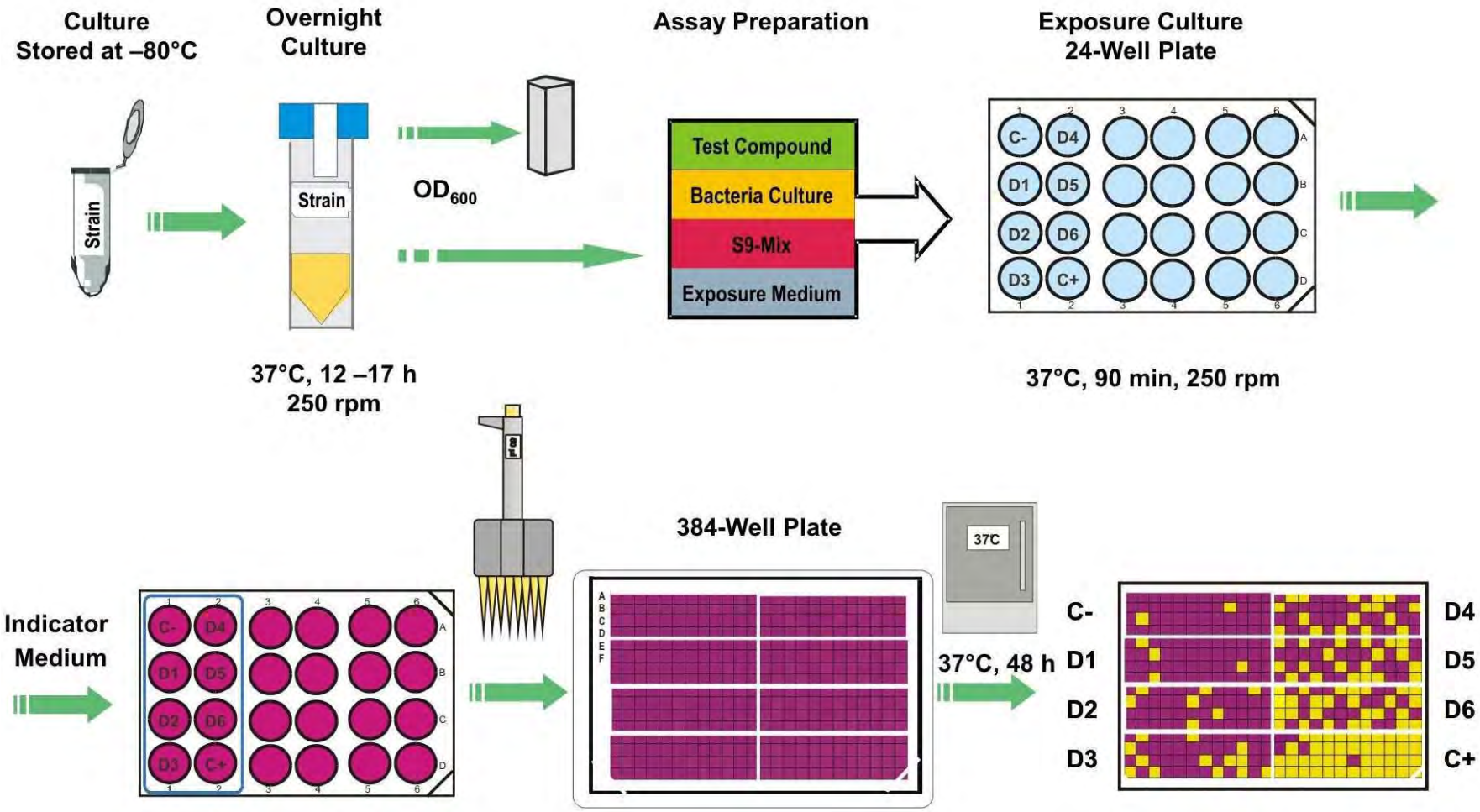
Product	Product Size	Art. No.
PAC Acid Phosphatase (310)	300 tests with 4 microplates, 8 reservoirs	AKPA96-310
SRB Sulforhodamine B (1200)	1200 tests without microplates	AKSR96-1200
SRB Sulforhodamine B (1210)	1200 tests with 16 microplates, 56 reservoirs	AKSR96-1210
SRB Sulforhodamine B (300)	300 tests without microplates	AKSR96-300
SRB Sulforhodamine B (310)	300 tests with 4 microplates, 14 reservoirs	AKSR96-310
SRB Sulforhodamine B (9600)	9600 tests without microplates (2 g)	AKSR96-9600
XTT Tetrazolium Hydroxide (1200)	1200 tests without microplates	AKXT96-1200
XTT Tetrazolium Hydroxide (1210)	1200 tests with 16 microplates, 32 reservoir	AKXT96-1210
XTT Tetrazolium hydroxide (2400)	2400 tests without microplates	AKXT96-2400
XTT Tetrazolium Hydroxide (300)	300 tests without microplates	AKXT96-300
XTT Tetrazolium Hydroxide (310)	300 tests with 4 microplates, 8 reservoirs	AKXT96-310
XTT Tetrazolium Hydroxide (9600)	9600 tests without microplates (500 mg)	AKXT96-9600
Two Parameter		
LDHE-XTT (1200)	2 x 1200 tests without microplates	AKLEX96-1200
LDHE-XTT (1210)	2 x 1200 tests with 32 microplates, 40 reservoirs	AKLEX96-1210
LDHE-XTT (300)	2 x 300 tests without microplates	AKLEX96-300
LDHE-XTT (310)	2 x 300 tests with 8 microplates, 10 reservoirs	AKLEX96-310
NR - CVDE (1200)	2 x 1200 tests without microplates	AKRCV96-1200
NR - CVDE (1210)	2 x 1200 tests with 16 microplates, 72 reservoirs	AKRCV96-1210
NR - CVDE (300)	2 x 300 tests without microplates	AKRCV96-300
NR - CVDE (310)	2 x 300 tests with 4 microplates, 18 reservoirs	AKRCV96-310
NR - SRB (1200)	2 x 1200 tests without microplates	AKRSR96-1200
NR - SRB (1210)	2 x 1200 tests with 16 microplates, 88 reservoirs	AKRSR96-1210
NR - SRB (300)	2 x 300 tests without microplates	AKRSR96-300
NR - SRB (310)	2 x 300 tests with 4 microplates, 22 reservoirs	AKRSR96-310
SRB - CVDE (1200)	2 x 1200 tests without microplates	AKSRCV96-1200
SRB - CVDE (1210)	2 x 1200 tests with 16 microplates, 88 reservoirs	AKSRCV96-1210
SRB - CVDE (300)	2 x 300 tests without microplates	AKSRCV96-300
SRB - CVDE (310)	2 x 300 tests with 4 microplates, 22 reservoirs	AKSRCV96-310
XTT - CVDE (300)	2 x 300 tests without microplates	AKXCV96-300
XTT - CVDE (1200)	2 x 1200 tests without microplates	AKXCV96-1200

Product	Product Size	Art. No.
XTT - CVDE (1210)	2 x 1200 tests with 16 microplates, 64 reservoirs	AKXCV96-1210
XTT - CVDE (310)	2 x 300 tests with 4 microplates, 16 reservoirs	AKXCV96-310
XTT - PAC (1200)	2 x 1200 tests without microplates	AKXPAC96-1200
XTT - PAC (1210)	2 x 1200 tests with 16 microplates, 56 reservoirs	AKXPAC96-1210
XTT - PAC (300)	2 x 300 tests without microplates	AKXPAC96-300
XTT - PAC (310)	2 x 300 tests with 4 microplates, 14 reservoirs	AKXPAC96-310
XTT - SRB (1200)	2 x 1200 tests without microplates	AKXSR96-1200
XTT - SRB (1210)	2 x 1200 tests with 16 microplates, 80 reservoirs	AKXSR96-1210
XTT - SRB (300)	2 x 300 tests without microplates	AKXSR96-300
XTT - SRB (310)	2 x 300 tests with 4 microplates, 20 reservoirs	AKXSR96-310
XTT-NR (1200)	2 x 1200 tests without microplates	AKXN96-1200
XTT-NR (1210)	2 x 1200 tests with 16 microplates, 48 reservoirs	AKXN96-1210
XTT-NR (300)	2 x 300 tests without microplates	AKXN96-300
XTT-NR (310)	2 x 300 tests with 4 microplates, 9 reservoirs	AKXN96-310
Three Parameter		
GLU-XTT-CVDE (1200)	1200 tests without microplates	AKGXCV96-1200
GLU-XTT-CVDE (1210)	1200 tests with 32 microplates, 80 reservoirs	AKGXCV96-1210
GLU-XTT-CVDE (300)	300 tests without microplates	AKGXCV96-300
GLU-XTT-CVDE (310)	300 tests with 8 microplates, 20 reservoirs	AKGXCV96-310
LDHe-XTT-NR (1200)	3 x 1200 tests without microplates	AKLEXR96-1200
LDHe-XTT-NR (1210)	3 x 1200 tests with 32 microplates, 72 reservoirs	AKLEXR96-1210
LDHe-XTT-NR (300)	3 x 300 tests without microplates	AKLEXR96-300
LDHe-XTT-NR (310)	3 x 300 tests with 8 microplates, 18 reservoirs	AKLEXR96-310
LDHe-XTT-SRB (1200)	3 x 1200 tests without microplates	AKLEXSR96-1200
LDHe-XTT-SRB (1210)	3 x 1200 tests with 32 microplates, 88 reservoirs	AKLEXSR96-1210
LDHe-XTT-SRB (300)	3 x 300 tests without microplates	AKLEXSR96-300
LDHe-XTT-SRB (310)	3 x 300 tests with 8 microplates, 22 reservoirs	AKLEXSR96-310
XTT-NR-CVDE (1200)	3 x 1200 tests without microplates	AKXTRCV96-1200
XTT-NR-CVDE (1210)	3 x 1200 tests with 16 microplates, 96 reservoirs	AKXTRCV96-1210
XTT-NR-CVDE (2400)	2400 tests without microplates	AKXTRCV96-2400
XTT-NR-CVDE (300)	3 x 300 tests without microplates	AKXTRCV96-300

Product	Product Size	Art. No.
XTT-NR-CVDE (310)	3x300 tests with 4 microplates, 24 reservoirs	AKXTRCV96-310
XTT-NR-SRB (1200)	3 x 1200 tests without microplates	AKXTRS96-1200
XTT-NR-SRB (1210)	3 x 1200 tests with 32 microplates, 112 reservoirs	AKXTRS96-1210
XTT-NR-SRB (300)	3 x 300 tests without microplates	AKXTRS96-300
XTT-NR-SRB (310)	3 x 300 tests with 8 microplates, 28 reservoirs	AKXTRS96-310
XTT-SRB-CVDE (1200)	3 x 1200 tests without microplates	AKXTSCV96-1200
XTT-SRB-CVDE (1210)	3 x 1200 tests with 32 microplates, 112 reservoirs	AKXTSCV96-1210
XTT-SRB-CVDE (300)	3 x 300 tests without microplates	AKXTSCV96-300
XTT-SRB-CVDE (310)	3 x 300 tests with 8 microplates, 28 reservoirs	AKXTSCV96-310
Four Parameter		
LDHE-GLU-XTT-PAC (1200)	4 x 1200 tests without microplates	AKLGXP96-1200
LDHE-GLU-XTT-PAC (1210)	4 x 1200 tests with 48 microplates, 80 reservoirs	AKLGXP96-1210
LDHE-GLU-XTT-PAC (300)	4 x 300 tests without microplates	AKLGXP96-300
LDHE-GLU-XTT-PAC (310)	4 x 300 tests with 12 microplates, 20 reservoirs	AKLGXP96-310
LDHE-GLU-XTT-SRB (1200)	4 x 1200 tests without microplates	AKLGXS96-1200
LDHE-GLU-XTT-SRB (1210)	4 x 1200 tests with 48 microplates, 104 reservoirs	AKLGXS96-1210
LDHE-GLU-XTT-SRB (300)	4 x 300 tests without microplates	AKLGXS96-300
LDHE-GLU-XTT-SRB (310)	4 x 300 tests with 12 microplates, 26 reservoirs	AKLGXS96-310
LDHE-XTT-NR-SRB (1200)	4 x 1200 tests without microplates	APANI96-1200
LDHE-XTT-NR-SRB (1210)	4 x 1200 tests with 48 microplates, 120 reservoirs	APANI96-1210
LDHE-XTT-NR-SRB (300)	4 x 300 tests without microplates	APANI96-300
LDHE-XTT-NR-SRB (310)	4 x 300 tests with 12 microplates, 30 reservoirs	APANI96-310

Appendix I

Short Ames MPF™ Assay Description



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Advantages

- 10 times lower cost per analysis
- 3 fold less contaminated waste
- 3 times less compound required
- 5 times less operator intervention
- Ready-to-use reagents and quality controlled bacterial strains
- No autoclaving of media or sterility testing required
- Colorimetric determination to reduce reporting errors
- OECD Guideline 471 compliant

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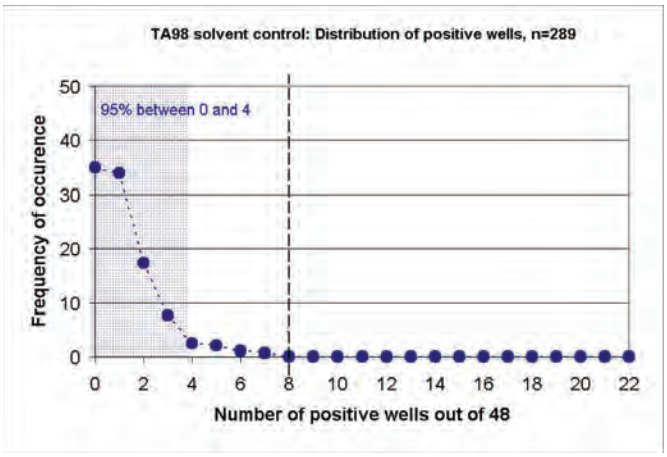
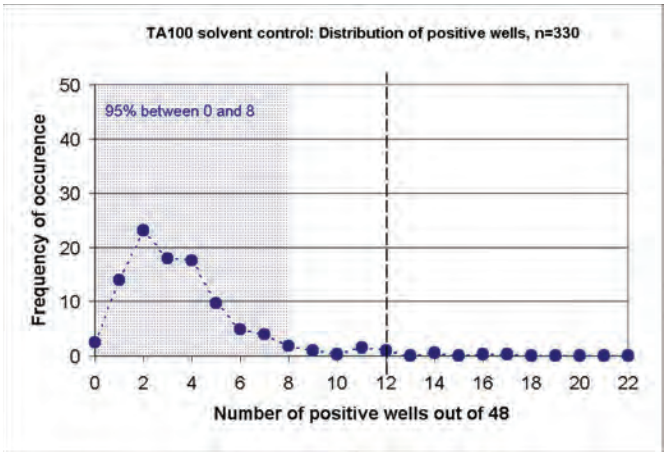
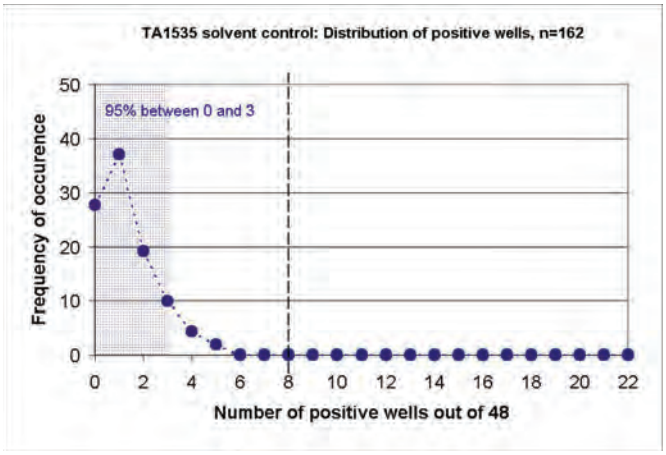
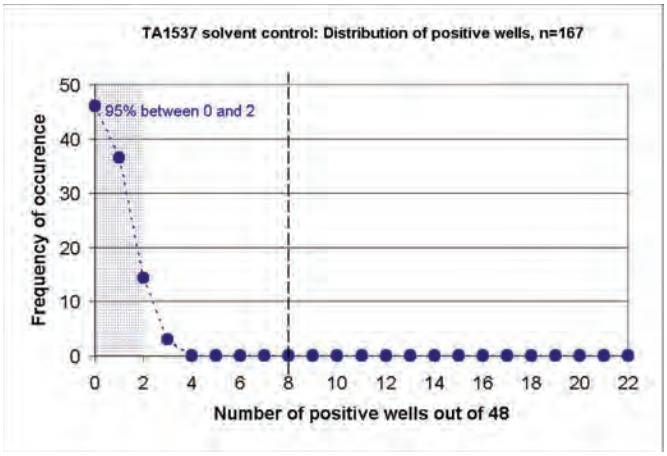
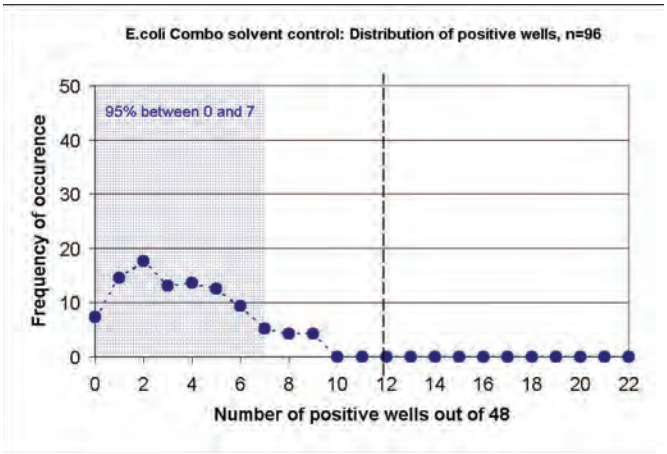
Historic solvent control values (DMSO) for the PENTA I strains











Appendix II: Selected References

From Genes to Molecular Epidemiology

European Environmental Mutagen Society
36th Annual Meeting

July 2 - 6, 2006

Prague Congress Centre,
Prague, Czech Republic

P-171

THE AMES MPF 98/100 ASSAY: NOVEL MUTAGENICITY TESTING IN LIQUID MICROPLATE FORMAT USING S. TYPHIMURIUM TA98 AND TA100

Sini Flückiger-Isler

Markus Kamber

Xenometrix, Allschwil, Switzerland

Genetic toxicity testing has moved towards the earlier stages of drug discovery in order to identify genotoxic liabilities of new compounds in the pipeline. Scaled-down versions of the original Ames plate incorporation test using the *S. typhimurium* strains TA98 (frameshifts) and TA100 (base-pairs) are often used for this purpose. Because in early development many compounds are available in very small quantities, a liquid microplate version with these strains was developed to decrease compound consumption and to increase the through-put of the assay.

TA98 is already successfully used in the Ames II assay, in combination with TAMix, a mixture of strains to detect base-pair mutations. TA100 with its high spontaneous reversion rate was as yet not suitable for the microplate format with its 48-well limit.

We were able to decrease the spontaneous reversion rate of TA100 to a level low enough to be used in the microplate format without loss of sensitivity. The mutagenic response to 13 reference compounds, examined in TA100 and in the Ames II TAMix cultures, resulted in comparable results: Seven compounds were stronger mutagenic in TA100 than in TAMix, and both strains showed similar responses with one compound. One compound had a weaker effect in TA100. As expected, one compound was detected by TAMix only and three chemicals showed no mutagenic activity in both strains.

The new Ames MPF 98/100 test by Xenometrix using a liquid format and 384-well microplates offers a time and cost-effective pre-regulatory alternative to the plate incorporation method. As both assays use the same *Salmonella* strains, TA98 and TA100, results can be compared with existing data sets. The new test kit including ready-to-use media and bacteria enables rapid screening of a large number of compounds and consumes six times less test substances and consumables than the plate incorporation method, and reduces hands-on time.

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THE AMES MPF™ PENTA I ASSAY:

Mutagenicity Testing in Liquid Microplate Format Using OECD Guideline 471 Compliant Strains *S. typhimurium* TA98, TA100, TA1535, TA1537 and *E.coli* WP2 *uvrA* plus *E.coli* WP2 [pKM101]

Sini Flückiger-Isler and Markus Kamber
Xenometrix, Gewerbestrasse 25, CH - 4123 Allschwil, Switzerland

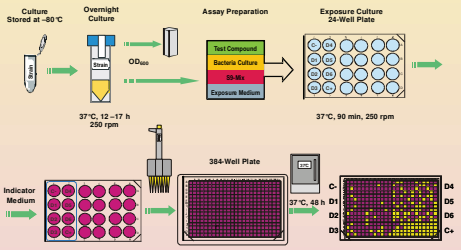
Introduction:

The necessity of testing compounds for genotoxic liabilities is constantly increasing. In drug discovery, genotoxic substances should be removed from further development as early as possible, often at stages where very limited quantities are available. But also the testing of environmental samples, or new regulatory requirements (REACH) for re-testing of existing chemicals increase the need for higher throughput mutagenicity assays. We have earlier introduced the liquid Ames II and Ames MPF (microplate format) assays, which have the advantage of requiring less test compound, consumables and hands-on-time. We are now able to offer in this format all strains required by the OECD guideline 471 for Testing of Chemicals.
The complete bacterial reverse mutation test includes at least five tester strains. *S. typhimurium* TA98, TA100, TA1535 and TA1537 are already successfully used in the microplate format. These 4 tester strains have GC base pairs at the primary reversion site and may therefore not detect certain classes of chemicals. A tester strain with an AT base pair at its primary reversion site was until now not available in the microplate format.
The mutagenic response to 13 reference compounds, including streptonigrin, mitomycin C, aldehydes oxidizing agents and hydrazines, was examined in *E.coli* WP2 *uvrA* and *E.coli* WP2 [pKM101]. The two strains had different sensitivities towards different mutagens. When combined during exposure as "*E.coli* Combo", it was always the more sensitive strain that dominated the response. When compared with published plate incorporation data the results were found to be identical.
The new Ames MPF *E.Coli* Combo assay was combined with the Ames MPF 98/100/1535/1537 assay to create the Ames MPF PENTA I test which meets the strain requirements of the OECD guideline 471. The new Ames MPF PENTA I assay kit is based on the fluctuation method using a preincubation procedure of 90 minutes. The use of a liquid format and 384-well microplates offers a time- and cost effective alternative to the plate incorporation test. As both formats use the same tester strains, results can be compared with existing data sets. The new assay kit includes ready-to-use media and quality controlled bacteria, and allows for high throughput testing.

Test method:

The Ames MPF™ assays are performed in 384-well plates with the histidine auxotroph *Salmonella typhimurium* tester strains TA98, TA100, TA1535 and TA1537, and the tryptophan auxotroph *E.coli* tester strains WP2uvrA plus WP2[pKM101]. After overnight growth, exposure with test chemicals is performed in 24-well plates (6 concentrations in triplicate, together with solvent and positive controls) in the absence and presence of S9 mix. After treatment, a specially formulated medium containing a pH indicator and lacking the required amino acid is added. Each well of the 24-well plate is distributed into 48 wells of a 384 well-plate and incubated for two days to allow revertant bacteria to grow. Mutagenicity is measured by a color change from purple to yellow (pH drop due to bacterial metabolism).

The experiments presented here were done with 3-6 concentrations.



Results:

Table 1: Reference compounds as detected by the Ames MPF™ E.coli *uvrA*, Ames MPF™ E.coli pKM101 and Ames MPF™ E.coli Combo assay. Comparison with published results in the Ames plate incorporation assay.

Compound	Ames MPF S9	uvrA	[pKM101]	Combo	Ames Plate Incorporation uvrA	[pKM101]	uvrA[pKM101]	TA102
Cumene hydroperoxide	-	+	+	+		+	+	
Formaldehyde	-	-	+	+		-/+	+	
Mitomycin C	-	-	+	+		+	-	
Streptonigrin	-	+	+	+		+	+	
Danthron	+	-	-	-		-	-	
Glutaraldehyde	-	-	+	+		+	+	
N4-aminocytidine	-	+	+	+	+			
Hydrazine sulfate	-	+	+	+				+
4-nitroquinoline-N-oxide	-	+	+	+		+	+	
Methyl methanesulfonate	-	+	+	+		+	+	
	+	+	+	+		+	+	
2-aminoanthracene	+	+	+	+		+	+	
2-nitrofluorene	-			-		-	-	
9-aminoacridine	-			-	-			

Figure 1:

Ames MPF™ *E.coli* strains WP2 uvrA and WP2[pKM101] exposed individually or combined ("Ames MPF Combo") to 11 mutagens

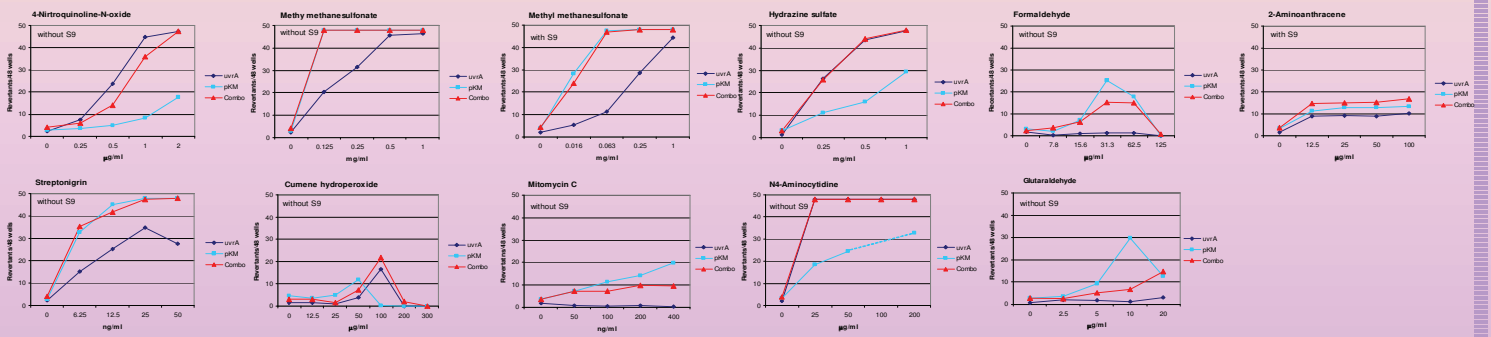
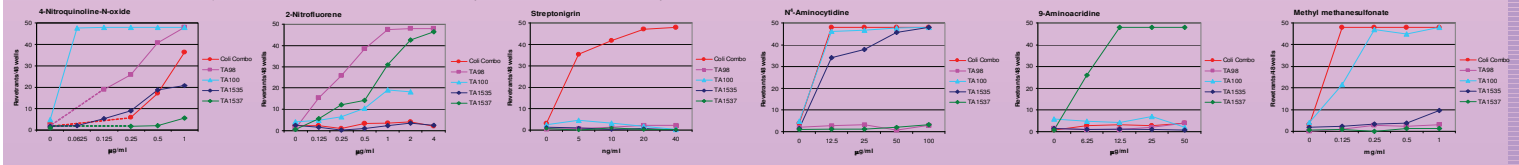


Figure 2:

Ames MPF™ PENTA I Assay: Performance of different strains in the presence of 6 reference compounds



Conclusions:

The new Ames MPF™ PENTA I assay allows to take advantage of the colorimetric microplate format while using the same *S. typhimurium* tester strains TA98, TA100, TA1535 and TA1537 and *E.coli* WP2 *uvrA* plus WP2[pKM101] that are used in the Ames plate incorporation test. The 384-well microtiter format requires about 3x less test compound, and considerable less consumables and hands-on-time.

The results confirm the usefulness of the liquid microplate format for bacterial mutagenicity testing and expand the range of available strains.

The use of E.coli WP2 uvrA plus WP2[pKM101] in the Ames MPF™ PENTA I assay allows the detection of additional mutagens compared to the use of the *S. typhimurium* strains only.

The Ames MPF™ PENTA I assay is therefore a rapid time- and resource-effective alternative to the Ames plate incorporation assay using the strains of *S. typhimurium and E.coli* mentioned in the 'OECD Guideline 471 for Testing of Chemicals'.

The Ames MPF™ 98/100 Assay:
Novel Mutagenicity Testing in Liquid Microplate Format using *S. typhimurium* TA98 and TA100

Sini Flückiger-Isler and Markus Kamber
Xenometrix, Gewerbestr. 25, CH-4123 Allschwil, Switzerland

Introduction:

Genetic toxicity testing has moved towards the earlier stages of drug discovery in order to identify genotoxic liabilities of new compounds in the pipeline. Scaled-down versions of the original Ames plate incorporation test using the *S. typhimurium* strains TA98 (frameshifts) and TA100 (base-pairs) are often used for this purpose. Because in early development many compounds are available in very small quantities, a liquid microplate version with these strains was developed to decrease compound consumption and to increase the throughput of the assay.

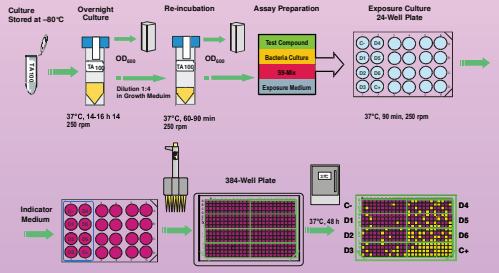
TA98 is already successfully used in the Ames II assay, in combination with TAMix, a mixture of strains to detect base-pair mutations. TA100 with its high spontaneous reversion rate was as yet not suitable for the microplate format with its 48-well upper limit.

We were able to decrease the spontaneous reversion rate of TA100 to a level low enough to be used in the microplate format without loss of sensitivity. The mutagenic responses to 14 reference compounds were compared in TA100 of the Ames MPF and in the TAMix of the Ames II test.

Test method:

The Ames MPF™ assay is performed in 384-well plates with the histidine auxotroph *Salmonella typhimurium* tester strains TA98 (frameshift mutations) and TA100 (base-pair substitutions). After overnight growth, dilution and re-incubation for about 1 hr, exposure with test chemicals is performed in 24-well plates (6 concentrations in triplicate, together with solvent and positive controls) in the absence and presence of S9 mix. After treatment, a specially formulated medium containing a pH indicator and lacking histidine is added. Each well of the 24-well plate is aliquoted into 48 wells of a 384 well-plate and incubated for two days to allow revertant bacteria to form colonies. Mutagenicity (bacterial growth) is measured colorimetrically by a color change (pH drop) from purple to yellow.

The data presented in this poster were done with 4 concentrations.



Results:

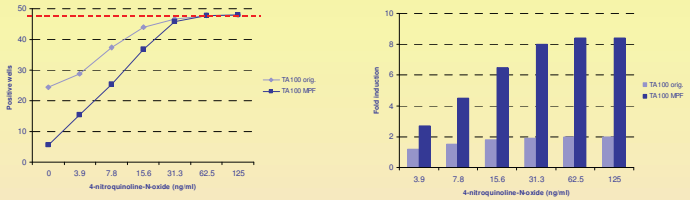


Figure 1: Comparison of the original TA100 strain with TA100 MPF.

Due to its greatly reduced spontaneous reversion rate the „Fold Induction over Zero Dose" is significantly higher and therefore suitable for the microplate format with a upper limit of 48 wells

	TA100 w/o S9	TA100 with S9	TAMix w/o S9	TAMix with S9
Methyl methanesulfonate	**2.7 µg/ml**		100 µg/ml	
N^4-Aminocytidine	**<<12.5 µg/ml**		12.5 µg/ml	
Formaldehyde	**7.5 µg/ml**		**7.5 µg/ml**	
4-Nitroquinoline-N-oxide	**<<7.8 ng/ml**	**0.5 µg/ml**	**<<7.8 ng/ml**	**0.5 µg/ml**
Pyrene-1,6-quinone	**0.4 µg/ml**		2.0 µg/ml	
1,6-Dinitropyrene	**<0.004 µg/ml**		<0.016 µg/ml	
Cyclophosphamide		**<200 µg/ml**		1000 µg/ml
Dimethylanthracene	**100 µg/ml**	20 µg/ml	500 µg/ml	**<20 µg/ml**
5-Azacytidine	---		**25 µg/ml**	
2-Aminoanthracene		**0.63 µg/ml**		**0.63 µg/ml**
Benzo(a)pyrene		**0.016 µg/ml**		0.4 µg/ml

not mutagenic -/+ S9: Pyrene, Anthracene, Ethylenediamine

Table 1:

Minimal Mutagenic Dose (MMD) in TA100 and TAMix

MMD was defined as >2-fold induction over baseline, which is the ratio of the mean number of positive wells for the dose concentration divided by the zero dose baseline. The zero dose baseline is obtained by adding one standard deviation to the mean number of positive wells of the zero dose (medium) control.

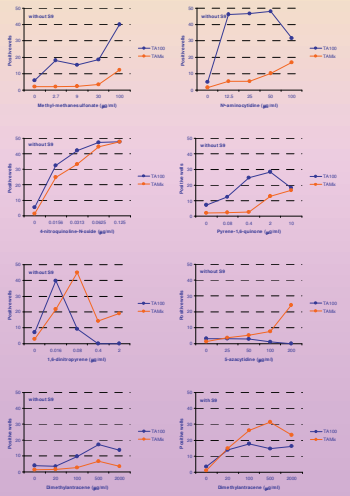


Figure 2: Comparison of TA100 and TAMix (Ames MPF vs. Ames II).

	TA100 MPF w/o S9	TA100 MPF with S9	TAMix w/o S9	TAMix with S9	TA100 plate incorp. w/o S9	TA100 plate incorp. with S9	References TA100 plate incorp.
N^4-Aminocytidine	+++		++		+++		1
Methyl methanesulfonate	+++		+		+++		2
1,6-Dinitropyrene	+++		+++		+++		3
Pyrene-1,6-quinone	++		++				
4-Nitroquinoline-N-oxide	+++	++	+++	+++	+++	+++	4
2-Aminoanthracene	---	+++	---	+++	---	+++	4
Dimethylanthracene	+	++	+	+++	+	++	5
Formaldehyde	++		++		++		6
Cyclophosphamide		+		+		+	5
Benzo(a)pyrene	---	++	---	+++	---	+	4
5-Azacytidine	---		++		?		7
Pyrene	---	---	---	---	---	---	4
Anthracene	---	---	---	---	---	---	5
Ethylenediamine	---	---	---	---	---	---	8

Table 2: Relative mutagenic potential of reference compounds as detected by TA100 MPF, TAMix and TA100 (plate incorporation format)

Scoring: Ames MPF and Ames II: Number of wells with revertant bacteria: +++ 30-48; ++ 15-29; + < 15 > MMD

Ames plate incorporation assay: +++ strong; ++ good; + weak; ? unclear

1) Negishi, K., Harada, C. et al. (1983) N4-aminocytidine, a nucleoside analog that has a high mutagenic activity. Nucleic Acid Research 11; no. 15; 5223-5233
2) Guadaño, A., de la Peña, E. et al. (1999) Development of a new bioluminescent mutagenicity assay based on the ames test. Mutagenesis 14; no. 4; 411-415
3) Kubo, T, Urano, K. and Utsumi, H. (2002) Mutagenicity characteristics of 255 environmental chemicals. J. Health Sci. 48; no. 6; 545-554
4) Hakura A., Shimada H. et al. (2005) Salmonella/human S9 mutagenicity test: a collaborative study with 58 compounds. Mutagenesis 20: no. 3; 217-228.
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7) Gee, P., Sommers, C.H. et al. (1998) Comparison of base-specific salmonella tester strains with the traditional strains for identifying mutagens: the results of a validation study. Mutation Research 412; 115-130
8) Dunkel V.C., Zeiger E. et al. (1985) Reproducibility of microbial mutagenicity assays: testing of carcinogens and noncarcinogens in Salmonella typhimurium and Escherichia coli. Environ. Mutagen. 7: Supp. 5; 1-248

Conclusions:

The new Ames MPF™ 98/100 test allows to take advantage of the microplate format while using the same *S. typhimurium* tester strains TA98 and TA100 that are used in the Ames plate incorporation test. The 384-well microtiter format requires about 6x less test compound and consumables, and considerable less hands-on time.

The comparison between TAMix and TA100 in the Ames II and Ames MPF microtiter format gave the following results: Seven compounds were more sensitive in TA100 than in TAMix, and both strains showed similar responses with three compounds. One compound was detected by TAMix only, and three chemicals showed no mutagenic activity in both strains.

A comparison of these compounds between TAMix and TA100 in the microplate format with published data of TA100 in the plate incorporation assay shows an excellent correlation.

The Ames MPF assay is therefore a rapid time- and recource-effective pre-registration alternative to the Ames plate incorporation assay using the same TA98 and TA100 strains of *S. typhimurium*.

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The Ames MPF™ Assays:
Novel Mutagenicity Testing in Liquid Microplate Format using *S. typhimurium* TA98, TA100, TA1535 and TA1537

Sini Flückiger-Isler and Markus Kamber
Xenometrix, Gewerbestr. 25, CH - 4123 Allschwil, Switzerland

Introduction:

Genetic toxicity testing has moved towards the earlier stages of drug discovery in order to identify genotoxic liabilities of new compounds in the pipeline. Because in early development many compounds are available in very small quantities, a liquid microplate version of the original Ames plate incorporation test with different S. typhimurium tester strains was developed to decrease compound consumption and to increase the through-put of the assay.

The complete Salmonella plate incorporation test includes at least 2 frameshift strains, usually TA98 and TA1537 or TA97, and at least two base-pair strains. TA100 is generally the most sensitive of all tester strains, but some mutagens are positive in TA1535 only. TA98 is already successfully used in the Ames II microplate assay, in combination with TAMix, a mixture of strains to detect specific base-pair mutations. Recently we were able to manage the high spontaneous reversion rate of TA100 such that it could be used instead of TAMix in the Ames MPF™ 98/100 without loss of sensitivity. TA1537 or TA1535 were until now not available in the microplate format.

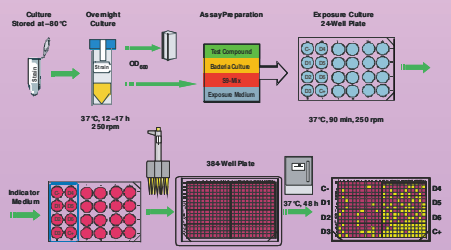
The mutagenic response to 24 reference compounds was examined with TA98, TA100, TA1537 and TA1535 in the microplate format, and the results were compared with published plate incorporation data. Depending on the strain tested and on the citation chosen, concordance between the two assay formats was 89 - 100%. Certain mutagens exclusively reverted TA1537 or TA1535, but not TA98 or TA100, irrespective of the assay format.

These new Ames MPF™ tests by Xenometrix using a liquid format and 384-well microplates offer a time and cost-effective pre-regulatory alternative to the plate incorporation method. As both formats use the same *Salmonella* strains, results can be compared with existing data sets. The new test kits include ready-to-use media and quality-controlled bacteria and allow rapid screening of a large number of compounds. They consume six times less test substances and consumables than the plate incorporation method, and reduce hands-on time.

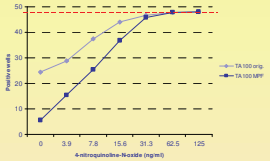
Test method:

The Ames MPF™ assays are performed in 384-well plates with the histidine auxotroph *Salmonella typhimurium* tester strains TA98 and TA1537 (frameshift mutations) and TA100 and TA1535 (base-pair substitutions). After overnight growth, exposure with test chemicals is performed in 24-well plates (6 concentrations in triplicate, together with solvent and positive controls) in the absence and presence of S9 mix. After treatment, a specially formulated medium containing a pH indicator and lacking histidine is added. Each well of the 24-well plate is aliquoted into 48 wells of a 384 well-plate and incubated for two days to allow revertant bacteria to form colonies. Mutagenicity (bacterial growth) is measured colorimetrically by a color change (pH drop) from purple to yellow.

The data presented in this poster were done with 4 concentrations.



Results:



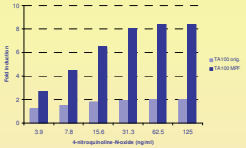


Figure 1: Comparison of the original TA100 strain with TA100 MPF.

Due to its greatly reduced spontaneous reversion rate the „Fold Induction over Zero Dose" is significantly higher and therefore suitable for the microplate format with a upper limit of 48 wells

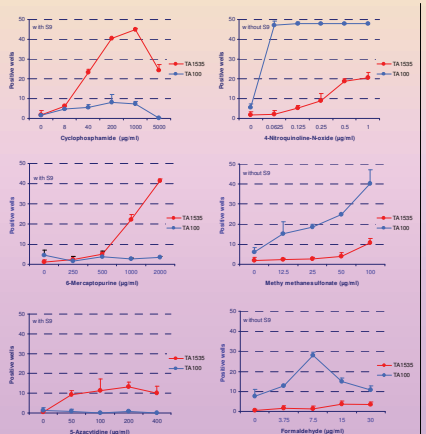


Figure 2:

Ames MPF™ basepair strains: Comparison of TA1535 (hisG46) and TA100 (hisG46[pKM101])

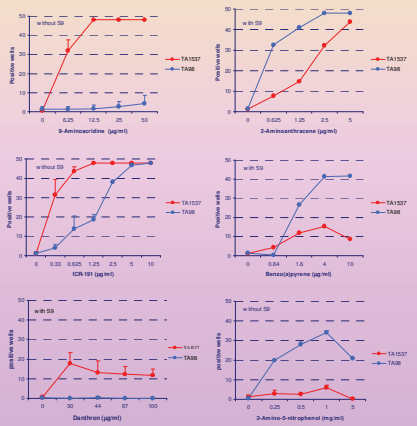


Figure 3:

Ames MPF™ frameshift strains: Comparison of TA98 (hisD3052[pKM101]) and TA1537 (hisC3076)

The newly available kit Ames MPF Penta I additionally includes the 2 E.coli strains wp2 uvrA and wp2 [pKM101]. Data for this kit will be presented on August 20-25 at the ICEM 2009 in Florence, Italy.

Compound	S9	Ames MPF				Ames plate incorporation (literature)			
		TA98	TA1537	TA100	TA1535	TA98	TA1537	TA100	TA1535
2-nitrofluorene	-	+++	+++	+	-	pos	pos	pos	neg
Pyrene	- +	- +	+ ++	- ?	- -	neg ?	neg ?/pos	neg ?	neg neg
Benzo(a)pyrene	+	+++	++	+++	-	pos	pos	pos	neg
1,6-dinitropyrene	-	+++	+++	+++	-	pos	pos	n.f.	n.f.
Pyrene-1,6-quinone	-	+++	+++	++	-	n.f	n.f	n.f.	n.f.
Anthracene	-/+	-	-	-	-	neg	neg	neg	neg
9,10-dimethylanthracene	- +	+++ +++	++ ++	++ ++	- -	pos	pos	pos	neg
2-aminoanthracene	+	+++	+++	+++	+++	pos	pos	pos	pos
Methyl methanesulfonate	-	-	-	+++	+	neg	neg	pos	pos/neg
4-nitroquinoline-N-oxide	-	+	+	+++	++	pos	pos	pos	pos
Cyclophosphamide	+	-	-	+	+++	neg	neg	pos	pos
5-azacytidine	+	+	-	-	+	neg	neg	neg/?	pos
6-mercaptopurine	- +	- -	- -	- -	+ +++	neg	neg	neg	pos
ICR-191	-	+++	+++	++	+	pos	pos	pos	neg
9-aminoacridine	-	-	+++	-	-	neg	pos	neg	neg
Proflavin	- +	+ +++	+++ +++	+ ++	- -	? pos	pos pos	neg pos	neg neg
Danthron	+	-	++	-	-	neg	pos	neg	neg
2-amino-5-nitrophenol	-	+++	+	-	-	pos	pos	w-pos	neg
N4-aminocytidine	-	-	-	+++	+++	neg	neg	pos	pos
Formaldehyde	-	+	-	++	-	pos	neg	pos	neg
Na-azide (3h exposure)	-	-	-	++	++	neg	neg	pos	pos
Ethylenediamine	-/+	-	-	-	-	neg	neg	neg	neg/pos
Primidone	-/+	N/A	N/A	-	-			neg	pos
Acetaldehyde oxime	-/+	N/A	N/A	-	-	neg	neg	neg/?	neg/pos

Table 1: Relative mutagenic potential of reference compounds as detected by Ames MPF™ 98, 100, 1535, and 1537. Comparison with published results in the Ames plate incorporation assay.

Scoring: Number of wells with revertant bacteria: +++ = 30 - 48; ++ = 15 - 29; + < 15 ≥ 2-fold induction over baseline

Concordances Ames MPF™ and Ames plate incorporation (from literature; conflicting results have been ignored):

TA98: 95% (19/20); TA1537: 100% (20/20); TA100: 95% (18/19); TA1535: 89% (17/19)

Conclusions:

The new Ames MPF™ assays allow to take advantage of the colorimetric microplate format while using the same *S. typhimurium* tester strains TA98, TA100, TA1535 and TA1537 that are used in the Ames plate incorporation test. The 384-well microtiter format requires about 6x less test compound and consumables, and considerable less hands-on-time.

The results confirm the usefulness of the liquid microplate format for bacterial mutagenicity testing and expand the range of available *S. typhimurium* strains.

The use of TA1535 and TA1537 allows the detection of additional mutagens compared to the use of TA100 and TA98 only.

Excellent concordances of 100 % (TA1537) 95% (TA98, TA100) and 89% (TA1535) between the microplate and the plate incorporation format were obtained.

The Ames MPF™ assays are therefore a rapid time- and resource-effective pre-registration alternative to the Ames plate incorporation assay using the same strains of *S. typhimurium*.

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Assessment of the performance of the Ames II™ assay: a collaborative study with 19 coded compounds

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Abstract

Nineteen coded chemicals were tested in an international collaborative study for their mutagenic activity. The assay system employed was the Ames II Mutagenicity Assay, using the tester strains TA98 and TAMix (TA7001–7006). The test compounds were selected from a published study with a large data set from the standard Ames plate-incorporation test. The following test compounds including matched pairs were investigated: cyclophoshamide, 2-naphthylamine, benzo(a)pyrene, pyrene, 2-acetylaminofluorene, 4,4′-methylene-bis(2-chloroaniline), 9,10-dimethylanthracene, anthracene, 4-nitroquinoline-*N*-oxide, diphenylnitrosamine, urethane, isopropyl-*N*(3-chlorophenyl)carbamate, benzidine, 3,3′-5,5′-tetramethylbenzidine, azoxybenzene, 3-aminotriazole, diethylstilbestrol, sucrose and methionine. The results of both assay systems were compared, and the inter-laboratory consistency of the Ames II test was assessed. Of the eight mutagens selected, six were correctly identified with the Ames II assay by all laboratories, one compound was judged positive by five of six investigators and one by four of six laboratories. All seven non-mutagenic samples were consistently negative in the Ames II assay. Of the four chemicals that gave inconsistent results in the traditional Ames test, three were uniformly classified as either positive or negative in the present study, whereas one compound gave equivocal results. A comparison of the test outcome of the different investigators resulted in an inter-laboratory consistency of 89.5%.

Owing to the high concordance between the two test systems, and the low inter-laboratory variability in the Ames II assay results, the Ames II is an effective screening alternative to the standard Ames test, requiring less test material and labor.
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Keywords: Ames II test; *Salmonella* mutagenicity test; Validation study

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1. Introduction

The value of the *Salmonella* mutagenicity assay has been clearly confirmed as a suitable primary test for the detection of potential mutagens and carcinogens, and since the mid-seventies the Ames assay [1,2] is used routinely as a screening assay to predict animal carcinogens.

The Ames II assay is a liquid microtiter modification of the Ames test and consists of the 'strains' TAMix and TA98. TAMix is a mixture of the *Salmonella typhimurium* strains TA7001, TA7002, TA7003, TA7004, TA7005 and TA7006 [3]. The genetic complementation among the six TA700*x* strains (where $x = 1, 2, 3, 4, 5,$ and 6) is low enough such they may be combined in a single assay to facilitate screening for mutagens. The strains in TAMix (base-pair substitutions) are like TA98 (frameshift mutation), histidine auxotrophs and mutagenesis will cause reversion to histidine prototrophy. Like the traditional strains, the genetic background of the TA700x series of strains has been modified to improve the sensitivity of their reversion by many classes of compound. The *uvrB* gene that is involved in excision repair has been deleted to allow lesions in the DNA to accumulate. The selection pressure to mutate or revert is facilitated so that less compound is needed to see an effect. The *galE503* mutation reduces the effectiveness of epimerase responsible for the inter-conversion of UDP-galactose and UDP-glucose. This inter-conversion is necessary for the synthesis of a complete cell wall, thus the point mutation in the epimerase allows a higher permeability of larger compounds into the cell and gives a population of cells which have a '*rough*' phenotype (*rfa*). The tester strains carry the plasmid pKM101, which has the *umuDC* homologues, *mucA/B* and the β-lactamase gene that confers ampicillin resistance. These gene products increase the cell's ability to perform mutagenic lesion bypass repair during DNA replication.

This study had two goals: (1) to corroborate the use of the Ames II test as a suitable alternative screening assay [4,5] to the traditional Ames plate-incorporation method, and (2) to test the Ames II assay system for its reproducibility among different laboratories. The 19 compounds included in this study were selected on the basis of traditional Ames data published as a report of the International Collaborative Program for the Evaluation of Short-Term Tests for Carcinogens (ICPESTTC study) [6]. The chemicals selected were either Ames-positive, -negative or equivocal: among the compounds that were positive in the traditional Ames assay, weak and strong mutagens were chosen, and the necessity of metabolic activation (S9 mix) for a positive response as well as the target site (frameshift mutation versus base-pair substitution) were considered. The equivocal chemicals that were chosen gave either inconsistent results in the ICPESTTC study or are known to be difficult to detect in bacterial mutagenesis assays. Although the discrimination between carcinogens and non-carcinogens played a secondary role in the present study, some chemical 'pairs' (carcinogens and their non-carcinogenic analogs) were included.

The 19 chemicals (Table 2) were coded at random before being distributed among nine independent laboratories, which allowed an opportunity for an inter-laboratory comparison of the Ames II system. Each compound was tested by 4–6 different investigators. The following companies participated in this study: Aventis Pharma Deutschland GmbH (Hattersheim, DE), BASF AG (Ludwigshafen, DE), Boehringer Ingelheim (Biberach, DE), Johnson&Johnson Pharmaceutical Research&Development (Beerse, BE), Novartis Consumer Health (Nyon, CH), Schering AG (Berlin, DE), Servier Group (Orléans-Gidy, FR), Federal Environmental Agency (Bad Elster, DE) and Xenometrix by Endotell GmbH (Allschwil, CH).

2. Materials and methods

2.1. Bacterial strains

The Ames II test was performed with *S. typhimurium* TA98 and TAMix [3]. TAMix consists of the strains TA7001–7006 in equal proportions and was treated as if it were an individual strain. The tester strains are characterized in Table 1.

Freshly thawed frozen strains of 10 μl were inoculated in 10 ml of growth medium (Xenometrix by Endotell GmbH) and the cultures were grown overnight (12–17 h) at 37 °C in an environmental shaker at 250 rpm in the presence of 50 μg/ml ampicillin (Xenometrix by Endotell GmbH).

Table 1
Bacterial strains used, and the mixture

Strain	Genotypes	Mutation[a]
TA98	*hisD3052 Δara9 Δchl008 (bio chl uvrb gal)rfa1004/pKM101*	Frameshifts
TAMix	TA7001, TA7002, TA7003, TA7004, TA7005, TA7006	Base-pair
TA7001	*hisG1775 Δara9 Δchl004 (bio chlD uvrb chlA)galE503 rfa1041/pKM101*	A:T → G:C
TA7002	*hisC9138 Δara9 Δchl004 (bio chlD uvrb chlA)galE503 rfa1041/pKM101*	T:A → A:T
TA7003	*hisG9074 Δara9 Δchl004 (bio chlD uvrb chlA)galE503 rfa1041/pKM101*	T:A → G:C
TA7004	*hisG9133 Δara9 Δchl004 (bio chlD uvrb chlA)galE503 rfa1041/pKM101*	G:C → A:T
TA7005	*hisG9130 Δara9 Δchl004 (bio chlD uvrb chlA)galE503 rfa1041/pKM101*	C:G → A:T
TA7006	*hisC9070 Δara9 Δchl004 (bio chlD uvrb chlA)galE503 rfa1041/pKM101*	C:G → G:C

[a] Mutation detected by this strain.

2.2. Test chemicals

Nineteen chemicals (Table 2) were selected for this study from 42 compounds described in the ICPESTTC report [6]. If possible, chemical pairs were chosen, i.e. carcinogens and non-carcinogens with closely related chemical structure. The structures of the test compounds are given in Appendix A. Excluded were chemicals that were not easily available, unstable, gaseous or liquid.

The 19 chemicals selected included 11 carcinogens and 8 non-carcinogens of which 8 were mutagenic, 7 non-mutagenic and 4 with conflicting responses in the different laboratories of the ICPESTTC study using the traditional Ames assay.

CAS numbers, carcinogenicity and mutagenicity as classified in the ICPESTTC report, suppliers and purity of the chemicals are listed in Table 2. The samples were coded at random by an independent person at Xenometrix by Endotell GmbH prior to shipping to the participating laboratories. With three exceptions, the chemicals were shipped in the supplier vials after the original labels had been removed. All participants received identical lot numbers. After receipt,

Table 2
Chemicals tested

Chemical	CAS no.	MW[a]	Carcinogenicity[b]	Mutagenicity[b]	Supplier	Purity (%)
2-Acetylaminofluorene	53-96-3	223.3	+	+	Sigma	Unknown
3-Amino-1,2,4-triazole	61-82-5	84.1	+	−	Sigma	95
Anthracene	120-12-7	176.2	−	−	Sigma	99+
Azoxybenzene	495-48-7	198.2	−	?	Riedel-de Haën	99+
Benzidine	92-87-5	184.2	+	+	Riedel-de Haën	99+
Benzo(a)pyrene	50-32-8	252.3	+	+	Fluka	98
Cyclophosphamide	6055-19-2	279.1	+	+	Aldrich	98+
Diethylstilbestrol	56-53-1	268.3	+	−	Riedel-de Haën	99+
9,10-Dimethylanthracene	781-43-1	206.3	+	+	Fluka	99
Diphenylnitrosamine	86-30-6	198.2	−	?	Fluka	97
Isopropyl-*N*(3-chlorophenyl) carbamate	101-21-3	213.7	−	−	Sigma	95
L-Methionine	63-68-3	149.2	−	−	Sigma	98
4,4-Methylene-bis(2-chloroaniline)	101-14-4	267.2	+	+	Fluka	99+
2-Naphthylamine	91-59-8	143.2	+	+	Sigma	Unknown
4-Nitroquinoline-*N*-oxide	56-57-5	190.2	+	+	Aldrich	98
Pyrene	129-00-0	202.3	−	?	Fluka	99
D-Sucrose	57-50-1	342.3	−	−	Sigma	99+
Tetramethylbenzidine	54827-17-7	240.5	−	−	Fluka	98
Urethane	51-79-6	89.1	+	?	Aldrich	99

(+) Positive; (−) negative; (?) equivocal.
[a] Molecular weight.
[b] Assessment according to the ICPESTTC study.

Table 3
Positive control chemicals used in the Ames II assay

Ames II strain	S9	Control chemical	Concentration (μg/ml)
TAMix	−	4-Nitroquinoline-*N*-oxide	0.5
TA98	−	2-Nitrofluorene	2.0
TAMix and TA98	+	2-Aminoanthracene (2-AA)	5.0

the chemicals were stored according to the directions on the label. Fresh, 25× concentrated stock solutions were prepared in DMSO immediately before use by each laboratory and then kept at −20 °C for potential repeat testing. The solvent was used at a final concentration of 4% in the assay. The investigators handled all compounds as if they were carcinogenic and mutagenic.

2.3. Positive controls

All investigators included positive control chemicals in each experiment. The following positive controls were used in assessing the performance of the Ames II assays (Table 3). Each participant prepared his own positive control chemicals as a 25× stock in DMSO.

2.4. Metabolic activation

The Ames II assays performed in this study were carried out in the presence and absence of Aroclor 1254-induced rat liver S9 (Moltox, USA). The biochemical and metabolic characterization of the S9 fraction used is available. The S9 mix stock preparation was made immediately prior to use, and stored on ice during preparation. The final concentration in the assay was 4.5%. S9 use and preparation are described in the Ames II instruction manual.

2.5. Study design

The individual chemicals should be tested by the different laboratories under as similar conditions as possible. The investigators were asked to strictly follow the Ames II instruction manual, and to use a prescribed dosing protocol, if feasible. All participants received identical batches of strains, media, S9 and chemicals. Unless stated otherwise, all procedures were performed manually.

2.5.1. Repeat testing

In general, experiments that gave clear positive or negative results were not repeated. However, the large majority of investigators repeated experiments with weak or borderline result at least once. One laboratory tested all chemicals only once due to restricted material.

2.5.2. Test concentrations

The test protocol was designed for a total of six concentrations, plus a negative (solvent) control and a positive control. Each culture had to be treated and dispensed into microtiter plates in triplicate. For a first screen, the compounds should be tested without any determination for viability or optimization for dose. The highest and the lowest dose level were 5000 and 4 μg/ml, respectively, and the intermediate doses were spaced at two- to five-fold intervals.

Six of the nine investigators strictly followed the protocol, and two used solubility limits to choose the maximum test concentrations. One group (P1) performed the Ames II assay manually as well as with robotics. The robotic system required some protocol changes, namely a different dose range, a lower top dose (1000 μg/ml), and only two replicates per dose and chemical. Another group (P4) used its own internally validated setup for an automated system which differed from the prescribed protocol in that: (1) a 10 times lower top dose (500 μg/ml) was used, (2) the triplicate values derived from three different overnight cultures, (3) there was no agitation during the 90 min of exposure (see Section 2.5.3 liquid exposure), and (4) the plate scoring was performed through spectrophotometry.

2.5.3. Liquid exposure

Absence of S9 fraction: Into 1-well of a 24-well plate (one well/strain/dose/replicate), 0.215 ml of Exposure Medium (Xenometrix by Endotell GmbH) and 0.025 ml of culture were aliquoted.

Presence of S9 fraction: Into 1-well of a 24-well plate (one well/strain/dose/replicate), 0.1775 ml of Exposure Medium, 0.025 ml of culture and 0.0375 ml of 30% S9 mix were aliquoted. Both proceedings gave a total volume of 0.240 ml. To each of these cultures,

0.01 ml of test chemical, diluted to the appropriate concentration was added, to give a total volume of 0.250 ml. This mixture was incubated for 90 min at 37 °C with agitation at 250 rpm.

At the conclusion of the 90-min incubation, each well of the 24-well plates containing the chemically treated cultures received 2.8 ml of Indicator Medium (Xenometrix by Endotell GmbH). The cultures were mixed gently with the histidine-deficient Indicator Medium that selects for prototrophic reversion before being distributed in 0.05 ml aliquots to 48 wells of a 384-well microtiter plate. One plate was used per strain and replicate. The plates were then incubated at 37 °C for 48 h. Bromocresol purple, an essential constitution of the Indicator Medium, turns yellow as the pH drops ($pK_1 = 5.2$) by catabolic activity of revertant cells which grow in the absence of histidine.

2.5.4. Determination of positive wells

The number of positive (yellow) wells out of 48 wells per replicate and dose was compared with the number of spontaneous revertants obtained in the negative control section. The average number of wells containing revertants per culture and concentration was calculated from the triplicate sections, and the increases above the zero dose were determined at each concentration of the test chemicals.

After completion of the study the investigators sent back their raw data together with a positive or negative classification of the chemicals tested according to their own evaluation criteria.

2.5.5. Final assessment

As there were different criteria for judging positive and negative responses among the investigators, a harmonized evaluation method was used for the collected data. The following factors for calculations were considered [5]:

- *1F* is the fold increase of bacterial revertant colonies relative to the revertant colony number at zero dose. It was determined by dividing the mean number of positive wells at each dose by that of the actual zero dose group. If the mean of spontaneous mutation frequencies was below 1 it was set to 1.
- *2F* is the fold increase of revertant wells in dose groups relative to the baseline of the actual zero dose group. The baseline derived from the mean of spontaneous number of positive wells plus 1 standard deviation.
- *3F* is the fold increase of revertant wells in dose groups relative to a separate baseline that derives from the mean of spontaneous revertants of a run. A run includes all experiments with different chemicals that were performed on the same day with the same overnight culture. The baseline derived from the mean of the accumulated replicates for zero dose controls of each run, plus 1 standard deviation from the distribution of these spontaneous data.

The calculation of reversion events based on the baseline data gives a more reliable information about the variation/deviation in spontaneous positive wells and therefore diminishes the influence of outlying data in dose groups. Xenometrix Inc., USA, recommended this calculation method.

A revertant yield greater than two times the baseline level 3F obtained in the triplicate values of a given dose was classified as an increase in revertant yield of the assay. Multiple responses of greater than two-fold the baseline level led to the test compound being classified as a clear positive.

The results were presented in a round table session after all data had been returned. If the final classification of a chemical obtained by the investigators methods differed from that obtained with the final evaluation method, the different criteria were discussed in detail and consent among the groups was found.

The results of the laboratory that did not follow the protocol instructions (investigator P4) were not included in the final evaluation method described above, since the experimental design did not allow the generation of baselines. These triplicate values derived from experiments with single replicates per chemical and dose performed on three different days. The results of this investigator are based on his own criteria and are marked with a special symbol (×) in the following figures.

After the study, laboratory P1 looked into 8 of the 9 remaining chemicals that it had not received for testing (Codes 6, 7, 8, 9, 10, 16, 17, 18). These supplementary results, performed manually as well as with robotics, are commented on under the specific codes in Section 3, but they are not considered in Section 4.

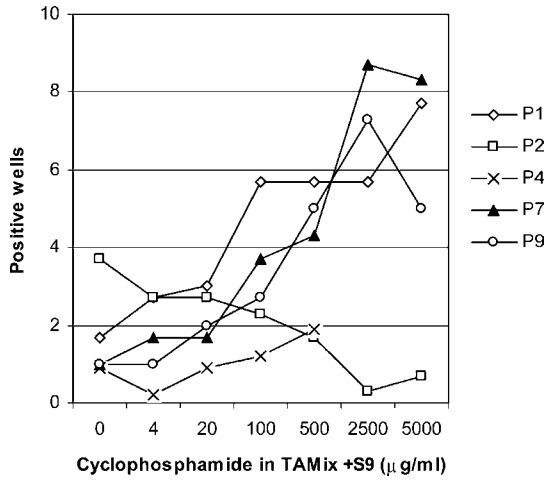


Fig. 1. Cyclophosphamide-induced reversion events in TAMix in the presence of S9 mix. P: participating laboratory; each number represents a specific company. Positive wells: the number of wells out of 48, where mutation occurred (see Section 2). Factors 3*F* greater than 2.0 were observed by P1, P7 and P9 at cyclophosphamide concentrations of 500 μg/ml and higher.

3. Results and discussion

The results for the positive and equivocal test chemicals generated in the present study are shown in Figs. 1–12. The figures represent the raw data obtained by the different laboratories. For representation reasons, the *y*-axis of the strong mutagens (maximum 48 positive wells) differ from those of the weak mutagenic and equivocal compounds. Clearly negative results are not shown graphically. The robotic results of laboratory P1 are not shown in the following figures since another dose range was used. If there were discrepancies between the manual and robotic system, they will be commented on under the chemicals concerned.

3.1. Code 1: cyclophosphamide

Cyclophosphamide was mutagenic for TAMix with S9 mix in 3 of 5 laboratories (P1, P7 and P9). The positive results were consistently weak and were observed at concentrations of around 500 μg/ml and higher (Fig. 1). Using the robotic system with 1000 μg/ml as top concentration, laboratory P1 observed an equivocal effect in the first test and a positive result in a second experiment in TAMix plus S9 mix. Laboratory P4 that initially assigned a negative response using 500 μg/ml as top dose observed a weak positive result in TAMix plus S9 on re-testing at higher concentrations after the study (not shown). The group that did not register a positive response (P2) tested at concentrations up to 5000 μg/ml.

As expected from the standard Ames test [6], cyclophosphamide was not mutagenic in the absence of metabolic activation and no revertant increase was seen in TA98.

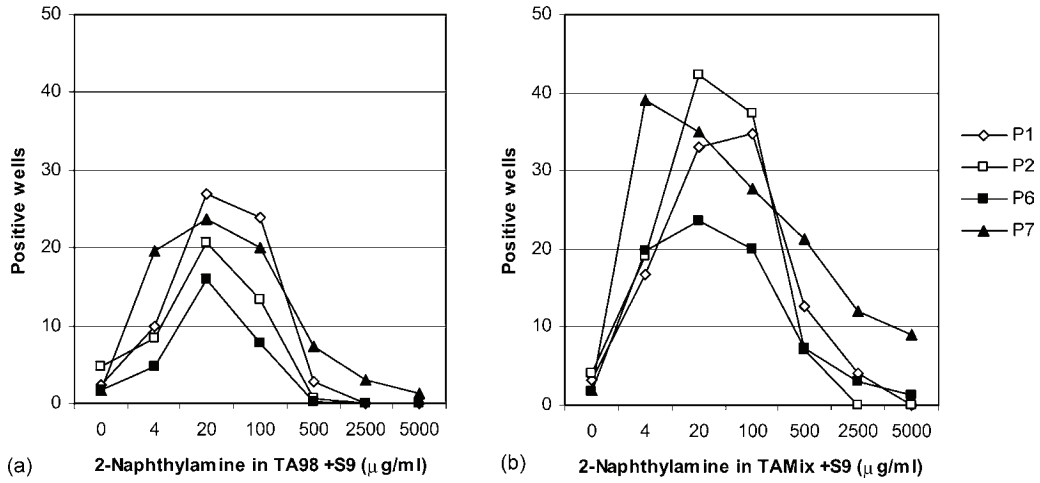


Fig. 2. 2-Naphthylamine-induced mutagenicity in the presence of metabolic activation: (a) strain TA98 and (b) strain TAMix.

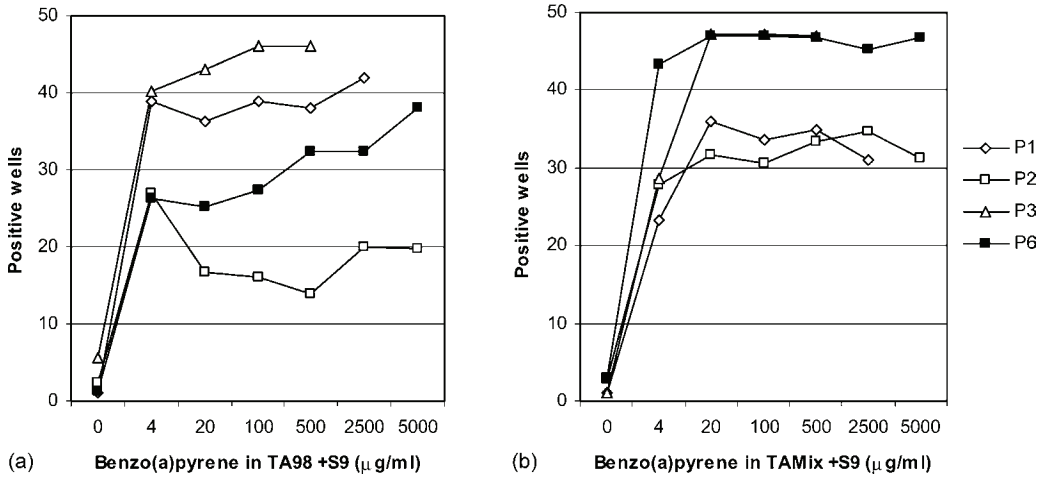


Fig. 3. Benzo(a)pyrene-induced reversion events in the presence of S9: (a) strain TA98 and (b) strain TAMix.

Cyclophosphamide is a strong alkylating agent but a weak bacterial mutagen in the traditional Ames base-pair strains of *S. typhimurium* in the presence of metabolic activation [6]. The degree of positive responses varied and concentrations 500 μg/plate and higher were necessary to demonstrate a significant effect.

3.2. Code 2: 2-naphthylamine

All four laboratories that tested the compound found 2-naphthylamine to be positive in TA98 and TAMix. The responses were more pronounced in TAMix than in TA98 and the presence of S9 mix was absolutely required for the mutagenic effect (Fig. 2). The positive responses were observed already at the lowest doses tested (4 μg/ml) and reached a maximum at 20–100 μg/ml. 2-Naphthylamine was toxic for both strains at concentrations of 500 μg/ml and higher. The dose response curves obtained by the individual investigators were comparable.

The results are in agreement with those of the ICPESTTC study [6] where 2-naphthylamine was consistently mutagenic in the presence of metabolic activation in *S. typhimurium*.

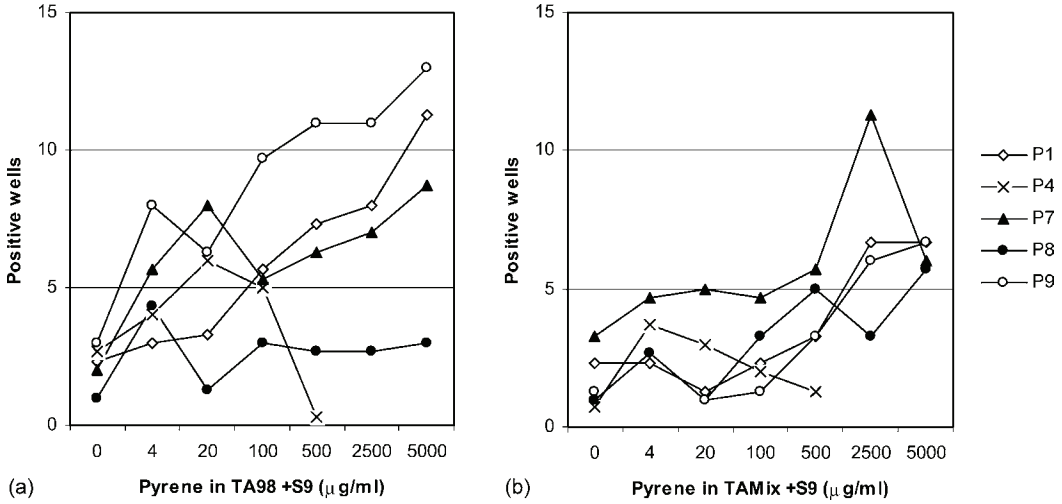


Fig. 4. Dose response curve of pyrene with TA98 and TAMix in the presence of S9 mix: (a) strain TA98 and (b) strain TAMix.

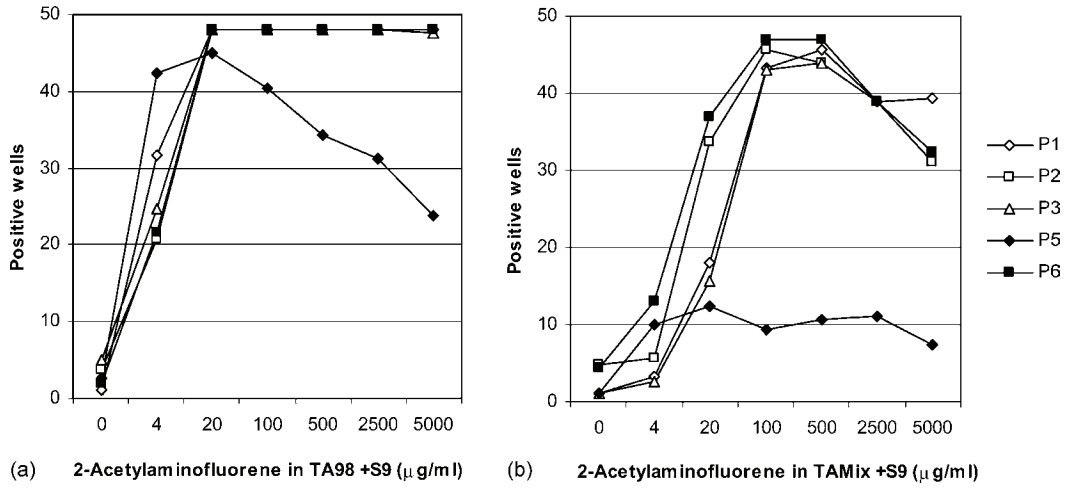


Fig. 5. 2-Acetylaminofluorene-induced mutagenicity in the presence of S9: (a) strain TA98 and (b) strain TAMix.

3.3. Code 3: benzo(a)pyrene

Benzo(a)pyrene gave consistently positive results in the Ames II test and S9 mix was likewise typically required for this activity. The maximum response was observed between 4 and 100 μg/ml (Fig. 3). One laboratory (P2) repeated the test with a lower dose range and observed a mutagenic effect beginning at 0.5 μg/ml (not shown).

The Ames II results for benzo(a)pyrene are in line with those of the ICPESTTC study [6].

3.4. Code 11: pyrene

Pyrene was a weak mutagen in the Ames II assay in 4 of 5 laboratories (P1, P7, P8 and P9), and S9 mix was typically required for this effect (Fig. 4). Laboratory P4 judged its results in the presence of S9 mix as equivocal. In general, higher concentrations were required for activity in TAMix (2500–5000 μg/ml) than in TA98 (20–100 μg/ml). When using the robotic system with a top dose of 1000 μg/ml, group P1 obtained a clear positive result only in TA98 (not

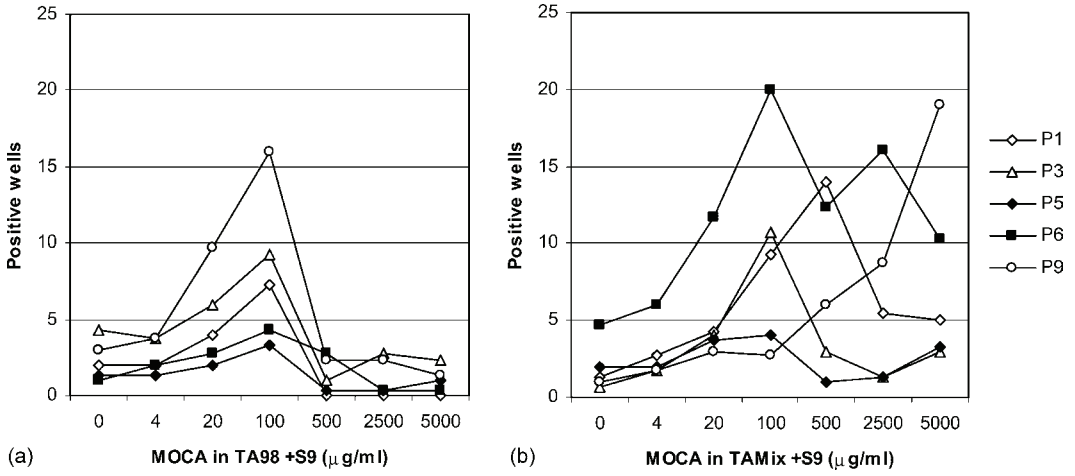


Fig. 6. 4,4′-methylene-bis(2-chloroaniline)-induced reversion events in the presence of S9 mix: (a) strain TA98 and (b) strain TAMix.

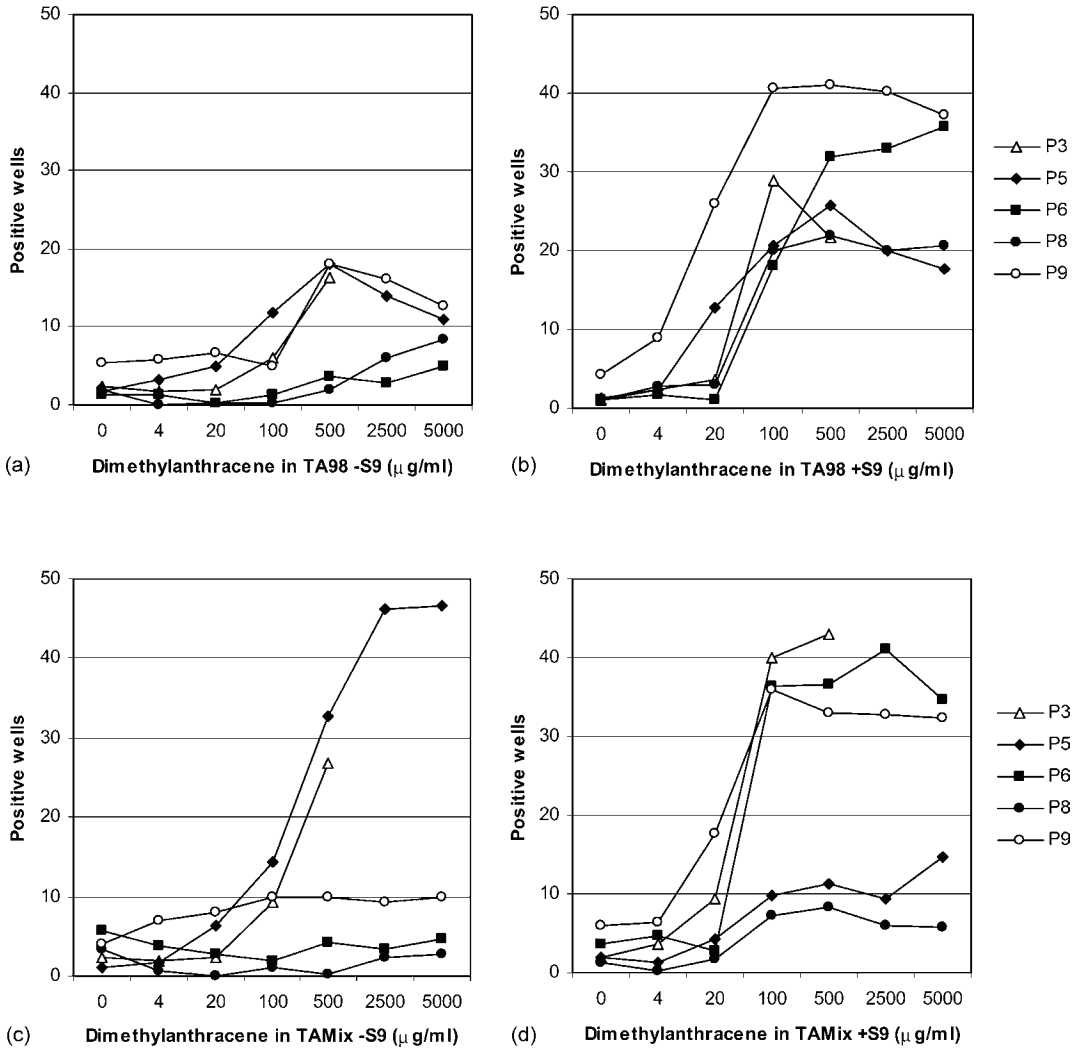


Fig. 7. 9,10-Dimethylanthracene-induced mutagenicity in the Ames II assay: (a) TA98 without S9; (b) TA98 with S9; (c) TAMix without S9 and (d) TAMix with S9.

shown). Another group (P8) demonstrated a positive result only in TAMix due to a high daily baseline level in TA98.

Pyrene, the non-carcinogenic analog of benzo(a)-pyrene (Code 3) gave equivocal results in the ICPESTTC study. The majority of laboratories did not detect mutagenicity in the traditional Ames test, and where positive effects were seen they were variable. However, it had been considered a mutagen that was difficult to detect because of differences in protocol or evaluation criteria [6].

3.5. Code 4: 2-acetylaminofluorene

2-Acetylaminofluorene was consistently mutagenic in the Ames II test, and S9 mix was typically required (Fig. 5). Maximum responses were observed at 20 and 100 μg/ml for TA98 and TAMix, respectively. All laboratories observed precipitation of 2-acetylaminofluorene at the two highest concentrations. Laboratory P5 attributed the decrease of positive wells in TA98 (100 μg/ml and higher), and the weak positive responses in TAMix to a toxic effect

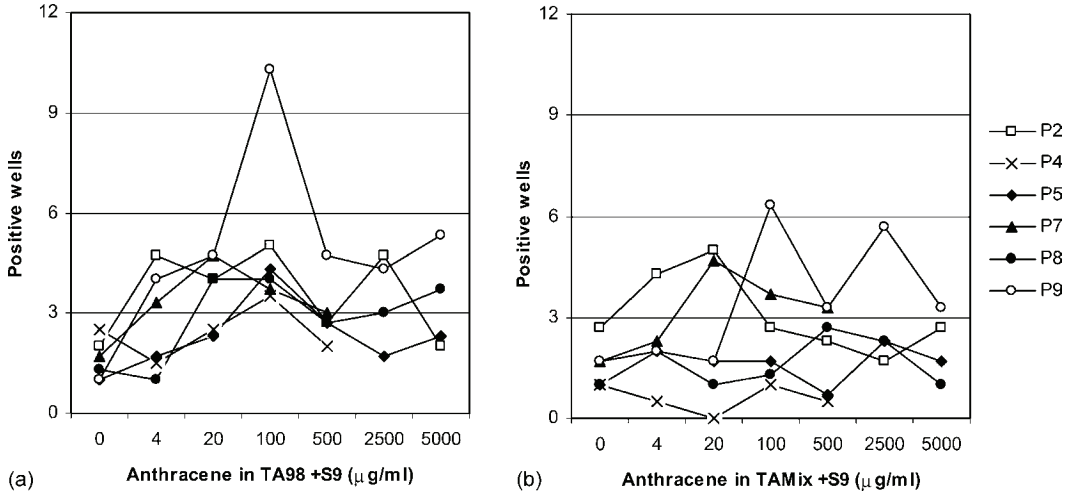


Fig. 8. Anthracene-induced reversion events in the Ames II assay in the presence of S9 mix: (a) strain TA98 and (b) strain TAMix.

of 2-acetylaminofluorene, beginning at 100 μg/ml. 2-Acetylaminofluorene was a clear mutagen in the traditional Ames test in the presence of S9 mix [6].

3.6. Code 5: 4,4′-methylene-bis(2-chloroaniline) (MOCA)

All laboratories except one (P5) demonstrated mutagenicity of MOCA in the Ames II assay in TA98 and TAMix, and the positive responses were observed in the presence of S9 only. In TA98, they were weaker, and in one case even borderline (P6, factor $3F = 2.1$), with a maximum at 100 μg/ml, due to toxicity at higher concentrations (Fig. 6a). In TAMix, the positive responses were generally more pronounced and the maximum effects varied between 100 and 5000 μg/ml (Fig. 6b).

These results agree very well with those of the ICPESTTC study [6], where the *Salmonella* reversion test was positive in TA100 and TA98 in the presence of metabolic activation. Activity in TA98 was also limited to doses of around 100 μg/plate, because higher doses were reported to be toxic in this strain.

3.7. Code 6: 9,10-dimethylanthracene

9,10-Dimethylanthracene gave positive results in all laboratories. S9 mix was not required for TA98, whereas for TAMix it was essential in three of five laboratories (P6, P8 and P9). With one exception in TAMix (P5, Fig. 7c and d), the positive responses were higher with S9, indicating that the metabolic activation enhances the activity of 9,10-dimethylanthracene. Laboratory P1 confirmed the positive responses in its supplementary test with clearly more pronounced effects in the presence of S9 (not shown). The results agree with those of the traditional Ames test [6], where all laboratories except two obtained a positive result, although most required S9 mix for activity in TA98.

Due to poor solubility in DMSO and sticky consistency of the compound in the microtiter assay, the onset of the dose responses and the intensity of the positive effects varied considerably among the different investigators.

3.8. Code 18: anthracene

Five laboratories classified anthracene, the non-carcinogenic analog of 9,10-dimethylanthracene, non-mutagenic (Fig. 8). One laboratory (P9) obtained reproducibly positive results in TA98 and to a lesser extent in TAMix at 100 μg/ml, both in the presence of S9 mix. Laboratory P1 that tested anthracene after the study, obtained a weak positive response (factor $3F = 2.3$) in TA98 plus S9 mix in the manual (500 μg/ml) as well as in the robotic system (100 μg/ml, not shown).

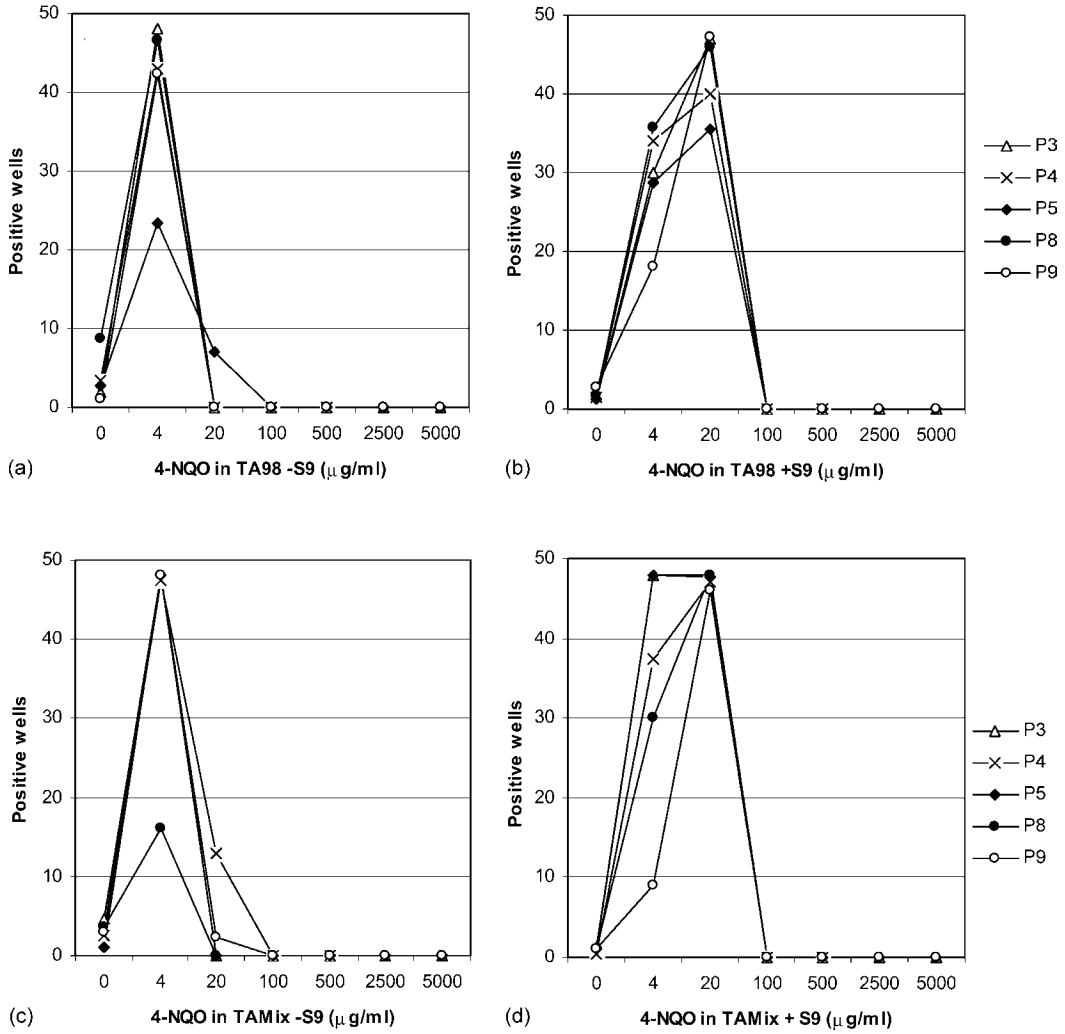


Fig. 9. 4-NQO-induced mutagenicity in the presence and absence of S9 mix: (a) TA98 without S9; (b) TA98 with S9; (c) TAMix without S9 and (d) TAMix with S9.

The overall consensus was that anthracene is not mutagenic, as it was in the traditional Ames test [6], where only 2 out of 15 participants obtained a positive result.

3.9. Code 7: 4-nitroquinoline-N-oxide (4-NQO)

4-NQO was highly mutagenic in the Ames II test for both TA98 and TAMix in all laboratories that tested the chemical, and there was no requirement for metabolic activation (Fig. 9). The doses chosen in this study were extremely toxic; without S9 toxicity started at 20 μg/ml and with S9 at 100 μg/ml. Higher concentrations caused cell death. These results were confirmed by laboratory P1 after the study (not shown). One laboratory (P9) repeated the test with a lower dose range in which mutagenicity started at 0.16 and 0.8 μg/ml in the absence and presence of S9, respectively (not shown). In the traditional Ames assay [6], TA98 and TA100 were the most useful strains for detecting 4-NQO activity, and S9 mix was, in general, not necessary for a mutagenic effect.

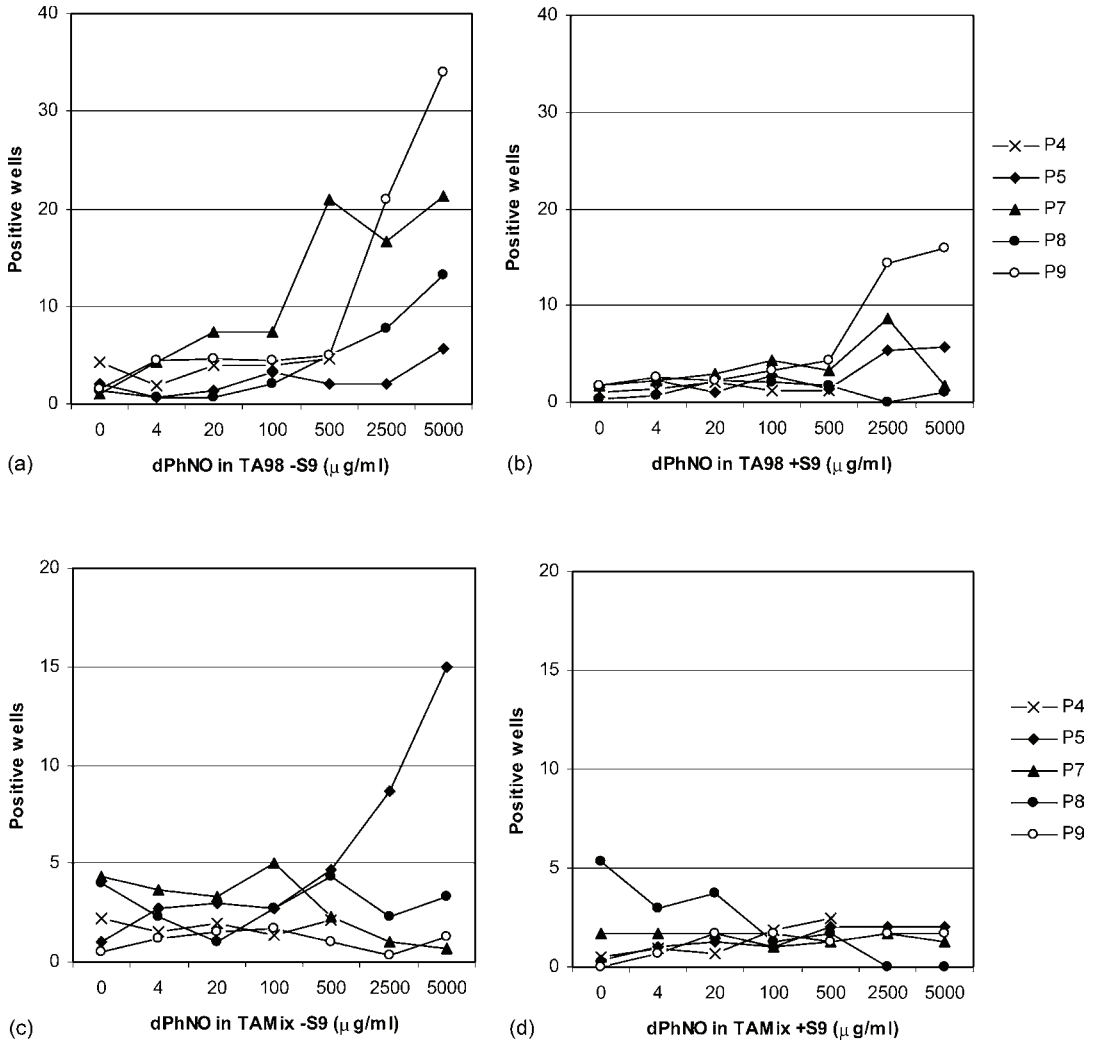


Fig. 10. Diphenylnitrosamine-induced mutagenicity in TA98 in the absence (9a) and presence (9b) of S9 mix.

3.10. Code 8: diphenylnitrosamine (dPhNO)

Diphenylnitrosamine was mutagenic in all laboratories that tested the chemical at concentrations higher than 500 μg/ml. But as in the ICPESTTC study [6], there was little consistency in the pattern of results and the scale of positive responses. Three laboratories (P7, P8 and P9) found diphenylnitrosamine mutagenic in TA98 without S9 mix (Fig. 10a), and three (P5, P7 and P9) found it positive in TA98 with S9 (Fig. 10b), one of which (P5) obtained also significant responses in TAMix in the absence of S9 (Fig. 10c). Investigator P4 that tested with a top dose of 500 μg/ml obtained an equivocal result in TAMix with S9 mix (Fig. 10d). The positive responses in TA98 without S9 were dose-dependent with an onset of around 500 μg/ml. Of the two laboratories that re-tested diphenylnitrosamine (P7 and P9), only one (P9) could reproduce the positive result with TA98 in the presence of S9. Laboratory P1 obtained a positive, dose-related effect in TA98 without S9 mix in the manual as well as in the robotic system in its supplementary test (not shown).

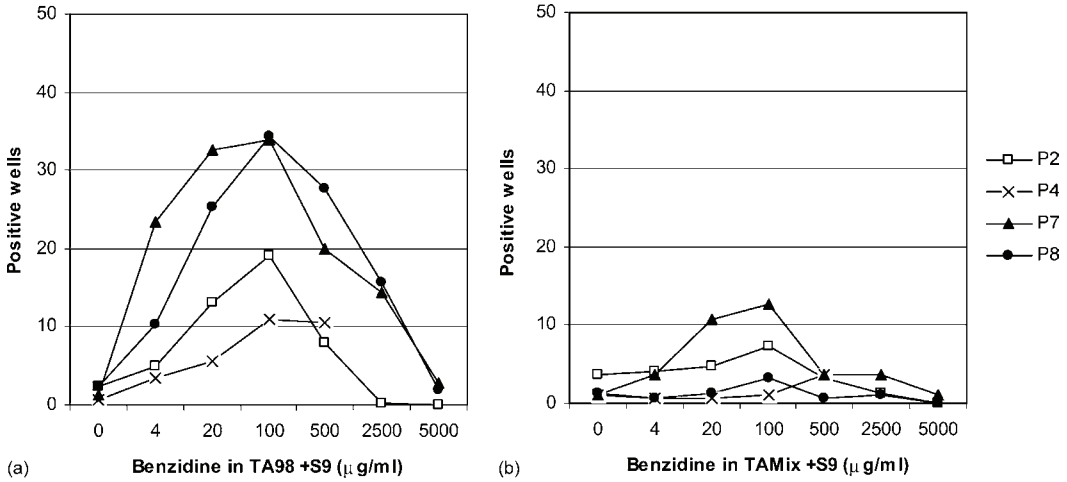


Fig. 11. Benzidine-induced mutagenicity in the presence of S9 mix: (a) strain TA98 and (b) strain TAMix.

Although diphenylnitrosamine appeared to be a weak mutagen in several laboratories that participated in the ICPESTTC study [6], it has been considered non-mutagenic in the final ICPESTTC report due to inconsistency and irreproducibility of the positive results.

3.11. Code 9: urethane

In the present study, no mutagenic response was obtained in all four laboratories that tested urethane (P4, P7, P8 and P9). Urethane was also negative in the robotic system in the supplementary test of laboratory P1, but it was clearly positive at 500 and 2500 μg/ml in TA98 and TAMix without S9 mix when tested manually (not shown).

Urethane is a carcinogen that is known to be difficult to detect in bacterial mutagenesis assays, and it has been described to be non-mutagenic in *Salmonella* [7]. In the ICPESTTC study [6], a mutagenic response was not obtainable in the majority of laboratories that tested urethane in the *Salmonella* reversion mutation assay.

3.12. Code 17: isopropyl-N(3-chlorophenyl) carbamate (IsoPC)

Neither in the present Ames II study nor in the standard plate-incorporation test [6], any mutagenic activity of IsoPC, the non-carcinogenic analog of urethane was shown. IsoPC was toxic in the Ames II assay in all laboratories (P2, P3, P4, P5, P8, P9 and P1 after the study) at concentrations of 500 μg/ml and higher.

3.13. Code 10: benzidine

All four laboratories that tested benzidine (P2, P4, P7 and P8) found it to be mutagenic in TA98 (Fig. 11a). All groups obtained a similar dose response curve, and S9 mix was essential for demonstration of activity. One laboratory (P7) obtained a mutagenic result also in TAMix in the presence of S9 mix (Fig. 11b). The supplementary tests of laboratory P1 confirmed the results obtained by the other participants with a clear positive response in TA98 plus S9 mix and a weak mutagenic effect in TAMix in the presence of S9 (not shown). These results are in agreement with those of the ICPESTTC study [6], where TA98 and TA100 were the most useful strains for detecting benzidine mutagenicity in the presence of S9 mix.

3.14. Code 15: 3,3′-5,5′-tetramethylbenzidine (TMB)

TMB, the non-carcinogenic analog of benzidine was negative in the Ames II assay in all six laboratories that

tested the chemical (P1, P2, P3, P5, P6 and P8). It was also considered to be non-mutagenic in the traditional Ames assay [6].

3.15. Code 12: azoxybenzene

Azoxybenzene did not result in significant responses in three groups out of five that tested the chemical (Fig. 12). Two groups (P1 and P7) obtained a positive response in TA98 in the presence of S9 mix. One of them (P1) had a weak mutagenic effect at 100 μg/ml in the manual but not in the robotic system, and the other (P7) at 20 and 100 μg/ml. The latter positive result was confirmed upon repeating the test after the study with a dose response from 20 to 500 μg/ml. Laboratory P4 judged azoxybenzene negative according to its proper evaluation criteria.

Azoxybenzene gave equivocal results in the ICPESTTC study [6]. In those laboratories where a mutagenic effect was observed, S9 mix was essential. It has therefore been suggested that the capacity of S9 mix may be critical for demonstration of azoxybenzene mutagenicity. In the present study, it was mainly negative. The concentration of S9 mix in the Ames II test is 4.5% and therefore considerably lower than the 10 and 30% used in the traditional Ames assay. The lower S9 concentration may have been the reason that the majority of the laboratories in the present study did not identify azoxybenzene as a mutagen.

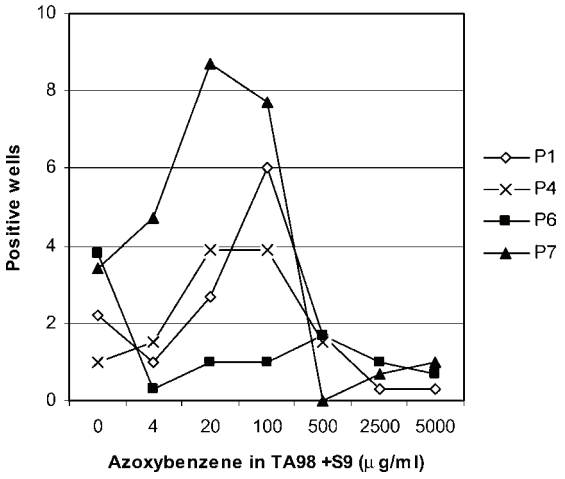


Fig. 12. Azoxybenzene-induced reversion events in TA98 with S9 mix.

3.16. Code 13: 3-aminotriazole

3-Aminotriazole is a carcinogen that was not found to be mutagenic in the Ames II assay, tested by five laboratories (P1, P3, P6, P7 and P9). One group (P7) obtained a weak positive result with a fold induction of 2.1 over the baseline (factor 3*F*) at the highest dose (5000 μg/ml), and this result was confirmed (factor $3F = 2.6$) upon re-testing after the study. Based on the calculation criteria used in this study, the aminotriazole result of this laboratory was judged equivocal. It has also been concluded in the ICPESTTC study [6] that the carcinogen 3-aminotriazole was negative in *S. Typhimurium*.

3.17. Code 14: diethylstilbestrol (DES)

Diethylstilbestrol was consistently non-mutagenic in the Ames II assay, tested by P1, P2, P3, P5 and P9, which is in agreement with the results obtained with the traditional Ames test [6]. Diethylstilbestrol is a carcinogen acting by a mechanism not involving DNA damage, and is therefore difficult to be detected in bacterial mutagenesis assays [7].

3.18. Code 16: sucrose

Sucrose was consistently negative in the Ames II test performed by the laboratories P2, P3, P5, P6, P8, and P1 after the study, which corresponds to the standard Ames test [6].

3.19. Code 19: methionine

None of the laboratories (P2, P4, P7 and P8) obtained a mutagenic effect with methionine, confirming the results of the ICPESTTC study [6].

4. Conclusions

The present Ames II study revealed an overall agreement of 84.2% (16 of 19 compounds, Fig. 13a–p) with

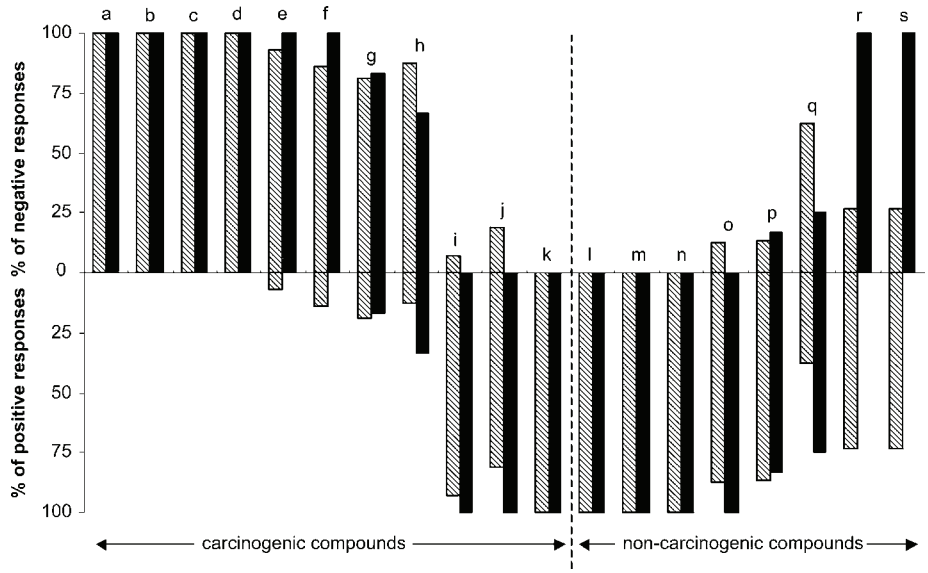


Fig. 13. Relative performances of the traditional Ames (light bars) and the Ames II (black bars) assays. Responses have been normalized (%) because of different group sizes. Questionable responses have been ignored. (a) benzo(a)pyrene; (b) 2-acetylaminofluorene; (c) 4-nitroquinoline-*N*-oxide; (d) benzidine; (e) 2-naphthylamine; (f) 9,10-dimethylanthracene; (g) 4,4-methylene-bis(2-chloroaniline); (h) cyclophosphamide; (i) diethylstilbestrol; (j) urethane; (k) aminotriazole; (l) 3,3′-5,5′-tetramethylbenzidine; (m) sucrose; (n) isopropyl-*N*(3-chlorophenyl)carbamate; (o) methionine; (p) anthracene; (q) azoxybenzene; (r) diphenylnitrosamine; (s) pyrene. (a–h) mutagenic in the traditional Ames (ICPESTTC study); (i and k–p) negative in the traditional Ames; (j and q–s) equivocal in the traditional Ames; (a–k) carcinogenic compounds; (l–s) non-carcinogenic compounds.

the standard Ames results of the ICPESTTC study [6]. All eight mutagenic chemicals that were selected from the ICPESTTC report (Fig. 12a–h) were also positive in the Ames II test, except cyclophosphamide (h) which was found to be positive in 4 of 6 laboratories in the present study. All seven Ames-negative compounds were also clearly negative in the Ames II test (Fig. 13i and k–p).

Table 4
Inter-laboratory consistency

Participant	Code no.																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
P1	+	+	+	+	+						+	?	−	−	−				
P2	−	+	+	+						+					−	−	−	−	−
P3			+	+	+	+	+						−	−	−	−	−		
P5				+	−	+	+	+						−	−	−	−	−	
P6		+	+	+	+	+						−	−	−	−	−			
P7	+	+						+	−	+	+	+	?					−	−
P8						+	+	+	−	+	+					−	−	−	−
P9	+				+	+	+	+	−		+			−	−		−	+	
P1 (Robot)	+	+	+	+	+						+	−	−	−	−				
P4 (Robot)	−						+	?	−	+	?	−					−	−	−
Consent	+	+	+	+	+	+	+	+	−	+	+	−	−	−	−	−	−	−	−
% agreement	67	100	100	100	83	100	100	100	100	100	100	75	100	100	100	100	100	83	100

(P) participating laboratories 1–9; (+) positive; (−) negative; (?) questionable. The test chemicals are listed by code numbers.

Disagreement between standard Ames and Ames II results was observed in 2 of the 4 substances that gave inconsistent results in the ICPESTTC study: Pyrene (s) was weakly but consistently positive in the present study. Although the chemical was negative in the majority of laboratories participating in the ICPESTTC study, it has been considered to be a mutagen that is difficult to detect, mainly because of differences in protocol or evaluation criteria. Diphenylnitrosamine (r) has been considered non-mutagenic in the ICPESTTC report due to inconsistency and irreproducibility of the positive results. It was consistently mutagenic in the Ames II assay but also here, the pattern of positive responses varied among the different laboratories.

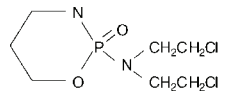
Table 4 summarizes the Ames II assay results of the 19 coded compounds obtained by the different participants. All laboratories agreed to 100% in 12 of the 19 chemicals, and if the questionable results are ignored, the 100% agreement increases to 15 compounds. Furthermore, all except one investigator came to the same conclusion for another two test chemicals (Codes 5 and 18) which results in an inter-laboratory consistency of 89.5% (17/19). As with the traditional Ames assay [6], inconsistent results were obtained for Code 12, azoxybenzene (1 positive, 1 questionable and 3 negative results). Cyclophosphamide (Code 1) was identified correctly by 4 of 6 investigators.

The present international collaborative study, in 9 laboratories with 19 chemicals, shows that: (1) the Ames II test results are well reproducible among the different laboratories and (2) that the sensitivity of both Ames assays, the Ames II and the traditional Ames, are comparable. The Ames II assay is therefore as effective as the standard Ames test for screening new substances for their genotoxic potential.

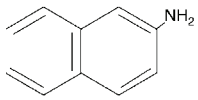
A screening assay should be performed with a relatively high throughput as there is an increased need to screen many compounds efficiently and in a cost-effective manner in the early phase of development. The Ames II assay meets these criteria. It offers a higher speed format than the traditional Ames assay even when performed manually. The simplicity of the protocol allows employing automated pipetting stations to perform the bulk of labor. The mix of the six new strains (TAMix) allows to record all possible base-pair substitutions in one culture. As the Ames II is a colorimetric assay, it is easy to score. The assay consumes a substantially lower amount of test chemical for yielding information useful in making decisions about a given compound.

Appendix A. Structural formulae of the test chemicals

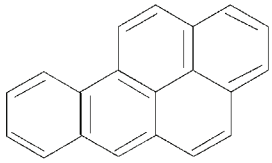
Carcinogenic/non-carcinogenic pairs are placed next to each other. Code numbers are in brackets.



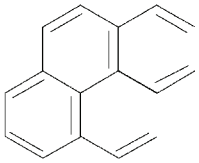
Cyclophosphamide (1)



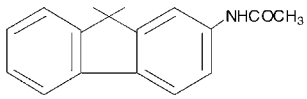
2-Naphthylamine



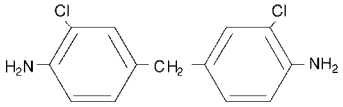
Benzo(a)pyrene (3)



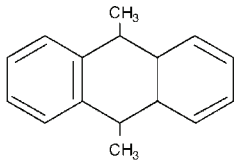
Pyrene (11)



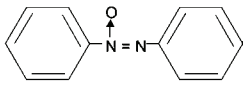
2-Acetylaminofluorene (4)



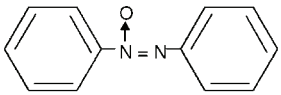
MOCA (5)



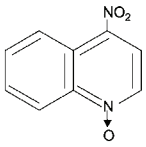
9,10-Dimethylanthracene (6)



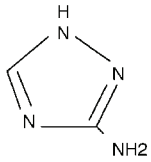
Azoxybenzene (12)



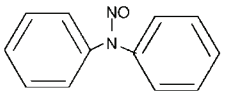
Azoxybenzene (12)



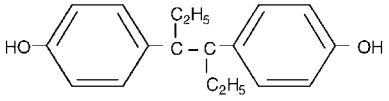
4-NQO (7)



3-Aminotriazole (13)

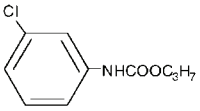


dPhNO (8)

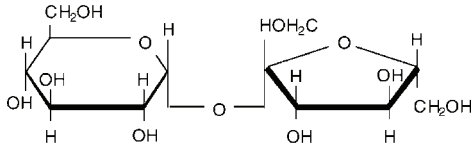


Diethylstilbestrol (14)

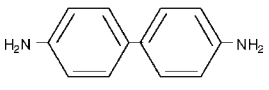
Urethane (9)



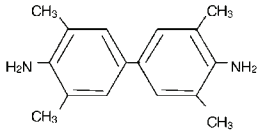
IsoPC (17)



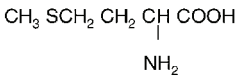
Sucrose (16)



Benzidine (10)



TMB (15)



Methionine (19)

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Comparison of the Ames II and traditional Ames test responses with respect to mutagenicity, strain specificities, need for metabolism and correlation with rodent carcinogenicity

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The Ames II *Salmonella* mutagenicity assay procedure was used to test 71 chemicals, and the results were compared with those from the traditional Ames *Salmonella* test using the NTP database as the reference. All Ames II tests were performed using a fluctuation procedure in microplate format, using TAMix for the detection of base pair substitutions and TA98 to detect frameshift mutations. There was 84% agreement between the two procedures in identifying mutagens and non-mutagens, which is equivalent to the intra- and interlaboratory reproducibility of 87% for the traditional test. The two tests also performed similarly in their predictions of rodent carcinogenicity.

Introduction

The *Salmonella* strains and microfluctuation test procedure that is used in the Ames II assay were developed by Gee *et al*. (1) and was originally designed to serve both as a screen for mutagenic substances and, at the same time, allow the identification of the specific base pair substitution mutations produced. The test comprises six histidine mutant *Salmonella* tester strains, TA7001–TA7006, each with a different base pair substitution histidine mutation. Each of these mutants can be reverted only by a specific transition or transversion, so that all possible base pair changes can be detected and identified. Because the spontaneous reversion frequencies of these strains is low, they can be mixed together (TAMix) and tested in liquid cultures in multiwell plates using a colorimetric readout. Because these strains are not responsive to frameshift mutations, *Salmonella* strain TA98 is run in parallel when screening chemicals. Important advantages of this test system are that it can be used with much less test chemical than in the standard plate or pre-incubation tests, requires less hands-on time, needs less S9 and plasticware and can be automated. Although the Ames II procedure is a version of the fluctuation test mentioned in the Organization for Economic Co-operation and Development (OECD) guidelines 471 (2), it does not strictly conform to the guidelines used for regulatory approval of products because it uses different—albeit functionally comparable—*Salmonella* tester strains for the detection of base pair mutations. The procedure has, however, found its use as an early mutagenicity screening procedure with pharmaceutical and chemical companies, as well as in the field of environmental screening.

There are many levels at which the performance of a bacterial test method can be evaluated with respect to the performance of a different test method, including a simple, overall agreement or disagreement; agreement or disagreement with regard to the genetic endpoint, and whether metabolic activation is required for activity; comparisons of the active test chemical concentration ranges and with respect to the effect the test is designed to predict, i.e. cancer. Two previous studies (3,4) have compared the performance of the Ames II assay to that of the traditional Ames test procedure [i.e. the procedure with the traditional strains, as described in (5) and (6)] to validate its use as an alternative to the traditional Ames test procedure.

This manuscript compares the results of testing using the Ames II liquid, multiwell test procedure with the results for the same chemicals in the NTP database using the traditional Ames *Salmonella* pre-incubation test. The two procedures are compared at a number of levels: (a) the overall agreement of test results, i.e. positive or negative, regardless of the tester strain used or the presence of metabolic activation; (b) agreement as to whether the substance produces base pair substitution or frameshift mutations, or both, (c) whether exogenous metabolic activation (S9) is required for a positive response; and (d) the relative predictivities of the two procedures for rodent cancer.

Materials and methods

The Ames II test

Bacterial strains. The strains used in both test procedures are listed in Table I; the TA7001–7006 strains are described in more detail by Gee *et al*. (1).

Strain TA98 is the only tester strain in common between the two procedures. The TA7000 series of strains were mixed in equal proportions as TAMix culture and preserved frozen in 15% glycerol at −80°C. TAMix and TA98 were grown overnight (12–15 h) in Growth Medium (Xenometrix, Allschwil, Switzerland) at 37°C in an environmental shaker set at 250 r.p.m. (New Brunswick Scientific Co., Edison, NJ) in the presence of 25 μg/ml ampicillin (Xenometrix).

Liquid exposure. The chemicals were tested in TAMix and TA98 using a modified liquid fluctuation test. In the absence of S9 fraction, 0.190 ml of Ames II Exposure medium (Xenometrix) per well of a 24-well plate and 0.050 ml of each overnight culture per well were mixed gently. Each test chemical was added in 0.010 ml aliquots. In experiments with Aroclor 1254-induced rat liver S9 fraction (Moltox, Boone, NC), the aliquot of Exposure medium was decreased to 0.152 ml to accommodate 0.038 ml of the S9 reagent. This provided a final concentration of 4.5% S9 fraction. The S9 mix contained 33 mM KCl, 8 mM $MgCl_2$, 5 mM glucose-6-phosphate, 4 mM nicotinamide adenine dinucleotide phosphate, 102 mM NaH_2PO_4 buffer (all Sigma) and 30% S9 (Moltox). The 24-well plates were incubated at 37°C for 90 min, with shaking at 250 r.p.m.

Prototrophic selection. After the 90 min incubation, the 24-well plates were removed from the incubator and transferred to the platform of a robotics station. An aliquot of 2.8 ml of histidine-deficient Ames II Reversion Indicator medium (Xenometrix) was dispensed by the automated pipet arm of the robotics station (Hamilton Co., Reno, NV) into each well of the 24-well plates containing chemically treated cultures. This effectively diluted any remaining histidine in the Exposure medium to prevent the growth of the auxotrophic population. The indicator medium which selects for prototrophic reversion was mixed gently several times in the robotics station. Then, each well of a 24-well microtiter

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plate was distributed in 50 µl aliquots over 48 wells of a 384-well microtiter plate by the robotics pipeting station. Each column (four wells) of the 24-well plate was transferred into one half of a 384-well plate, effectively dividing each sample among 48 wells of the plate. Therefore, one plate was used per strain per replicate. The 384-well microtiter plates were sealed in Ziploc® plastic bags to prevent evaporation and incubated at 37°C for 48 h.

Data acquisition. A pH indicator dye in the Indicator Media turns yellow as the pH drops (pKa ≅ 5.2) as catabolites accumulate from the metabolicaly active revertant cells which grow in the absence of histidine. The number of yellow positive wells out of a total of 48 wells is an indication of the frequency of reversion per replicate per dose and was compared to the number of spontaneous revertant wells obtained in the solvent control sections. Each 48-well section of the 384-well plates was scored for the number of revertant wells (yellow) by an SLT Spectra Image plate reader (Tecan U.S., Research Triangle Park, NC) at optical density $(OD)_{492\ nm}$ normalized at $OD_{623\ nm}$ as a reference wavelength. The optical density was digitized by the SLT data Capture software and exported to Microsoft Excel. The data were organized further into summary tables per compound code for its classification.

The initial screen was performed without replicates. With single data points, the emphasis for evaluating test compounds must be on dose dependency rather than individual data points. A single, isolated data point above the chosen baseline threshold of 'zero dose plus 1 SD' has little significance and does not qualify to label a compound 'positive' or 'weak positive'. However, an isolated data point >4-fold over the baseline at the highest concentration tested could indicate the beginning of a dose-dependent response and was therefore classified as 'possibly positive'. The rules observed in the evaluation of compounds that were tested only once are summarized in Table II.

Ten compounds were chosen to be retested in triplicates to allow for statistical analysis and to evaluate the robustness of the original screen. The data from the initial tests were taken into account when test concentrations were chosen for the repeated experiments. The average number of wells containing revertants per culture per dose was calculated from the triplicates, and the increases above the zero dose baseline (mean of zero dose plus 1 SD) were determined at each dose of test chemical. If the zero dose baseline was <1, it was set to 1. Results from this triplicate experiment were used for evaluation rather than the less reliable single-point measurements.

Detailed evaluation data are available at the Xenometrix homepage (www.xenometrix.ch)

Table I. Salmonella tester strains used

Strain	Responds to	Ames II	Ames
TA7001	T:A > C:G	●	
TA7002	T:A > A:T	●	
TA7003	T:A > G:C	●	
TA7004	C:G > T:A	●	
TA7005	C:G > A:T	●	
TA7006	C:G > G:C	●	
TA98	Frameshift	●	●
TA100	Base pair substitution		●
TA1535	Base pair substitution		●
TA97	Frameshift		●

●, Strain used.

Table II. Evaluation criteria of compounds with single data points

For each compound dilution series		
No. of wells >2× baseline	No. of wells >4× baseline	Compound label
0	0	Negative
1	0	Negative
0	1	EQ/possibly positive[a]
1	+1, not adjacent	EQ/possibly positive[a]
1	+1, adjacent	Positive
2, adjacent	0	Weak positive
2, adjacent	>0, any	Positive
2, non-adjacent	0	EQ
2, non-adjacent	1, non-adjacent	Weak positive
2, any	>0, adjacent	Positive
3+, any	0	Weak positive
3+, any	>0, any	Positive

EQ, equivocal.
[a]Possibly positive if >4× baseline at highest concentration tested.

Traditional Ames test

All traditional Ames test data were taken from the US-NTP database; these results and supporting data are publicly available online at NTP (7). All chemicals were tested under code in a pre-incubation procedure using Aroclor 1254-induced rat and hamster S9 preparations. The detailed methods used to generate the data and evaluate the results are described in (8) and (9). Strains TA102 and TA104 which, unlike strains TA100 and TA1535, respond to mutagens reacting with A:T sites were not routinely used when the NTP data were being generated. As a consequence, chemicals that induce mutation only at A:T sites would not have been detected. Chemicals positive in the NTP tests only with hamster S9 were not judged positive for the purposes of this comparison.

Selection of chemicals

The chemicals chosen cover a wide range of structures, activities and uses (Table III). The 71 test compounds were coded to ensure a blinded design. All coded chemicals were handled by the experimentalist as if they were carcinogenic and mutagenic. In its high-throughput screening format, the assay incorporated no replicates and there was no pre-assay for dose range determination. Therefore, a broad range using seven concentrations, commonly over half-log increments, was used.

In the absence of S9 fraction, the positive control chemicals used for TAMix and TA98 were 4-nitroquinoline-*N*-oxide (4-NQO) at 0.5 µg/ml and 2-nitrofluorene (2-NF) at 0.5 µg/ml, respectively. 2-Aminoanthracene (2-AA) at 5.0 µg/ml was used as the positive control for all S9 activation experiments. The stability and reproducibility of the Ames II format are demonstrated by the values for the negative (solvent) and positive controls between the individual runs for the compound testing: 1.2 ± 0.6 (TAMix, solvent −S9), 0.8 ± 0.4 (TAMix, solvent +S9; 1.7 ± 1.5 (TA98, solvent −S9); 1.3 ± 0.7 (TA98, solvent +S9); 45.4 ± 8.1 (TAMix, 4-NQO −S9); 47.7 ± 0.8 (TAMix, 2-AA +S9); 46.4 ± 4.7 (TA98, 2-NF −S9); 46.5 ± 5.1 (TA98, 2-AA +S9).

The chemicals tested in the Ames II procedure that have corresponding test results from the traditional Ames test in the NTP (7) database are listed in Table III with the solvent used and the concentration range in the Ames II test. Not all chemicals tested in the Ames procedures were tested in the cancer assay.

Results and discussion

Table IV contains the test results from the 71 chemicals that were tested in both the Ames II and the NTP *Salmonella* procedures.

Comparison of Ames and Ames II responses in the identification of mutagens

As a consequence of the differences in protocol between the two procedures, and the potential differences in sensitivity between the TA7001-7006 strains and the TA100 and TA1535 strains used in the Ames assay, it was not surprising to find differences in the responses (positive or negative) and patterns of responses (e.g. S9 requirement, responding strains). There were four chemicals whose responses in the NTP database are different from the Ames II responses for reasons related to specific aspects of the different protocols used.

- Direct blue 1 requires reductive metabolism for a positive response in *Salmonella*. When it originally tested as negative by the NTP, the test was repeated using an flavin mononucleotide reduction procedure, resulting in a positive response. Because reductive metabolism was not used for the Ames II tests, the original NTP test negative response was used for the comparison.
- *o*- and *p*-Toluidine were positive in the NTP protocol only with hamster S9 and would have been declared non-mutagenic if only rat S9 had been used, as in most standard

Table III. Chemicals tested

Code No.	CAS number	Chemical name	Solvent	Concentrations (μg/ml)[a]	
				−S9	+S9
34	60-35-5	Acetamide	H_2O	4.44–4400	4.44–4400
97	53-96-3	2-Acetylaminofluorene	DMSO	4.44–4400	0.49–492
12	3761-53-3	Acid Red 26	DMSO	1.92–1920	1.92–1920
18	107-02-8	Acrolein	DMSO	5.00–5000	4.44–4400
89	79-06-1	Acrylamide	H_2O	4.44–4400	4.44–4400
110	107-13-1	Acrylonitrile	DMSO	4.44–4400	4.44–4400
58	117-79-3	2-Aminoanthraquinone	DMSO	1.92–1920	1.92–1920
107	92-67-1	4-Aminobiphenyl	DMSO	4.44–4400	4.44–4400
115	92-36-4	2-(4-Aminophenyl)-6-methylbenzothiazole	DMSO	4.44–4400	0.05–49.2
21	62-53-3	Aniline	DMSO	4.44–4400	4.44–4400
108	90-04-0	*o*-Anisidine	DMSO	5.00–5000	4.44–4400
118	120-12-7	Anthracene	THF	1.20–1200	1.20–1200
68	71-43-2	Benzene	DMSO	4.44–4400	4.44–4400
11	92-87-5	Benzidine	DMSO	4.44–4400	0.59–492
114	431-03-8	2,3-Butanedione	DMSO	5.00–5000	4.44–4400
127	3068-88-0	Beta-butyrolactone	DMSO	5.00–5000	4.44–4400
128	96-48-0	Gamma-butyrolactone	DMSO	4.44–4400	4.44–4400
67	120-80-9	Catechol	H_2O	4.44–4400	4.44–4400
39	107-20-0	Chloroacetaldehyde	DMSO	4.44–4400	4.44–4400
41	548-62-9	Crystal violet	H_2O	0.96–960	0.96–960
62	117-10-2	Danthron	DMSO	0.49–492	0.49–492
75	101-80-4	Diaminodiphenyl ether	DMSO	4.44–4400	0.49–492
92	95-80-7	2,4-Diaminotoluene	DMSO	4.44–4400	4.44–4400
93	823-40-5	2,6-Diaminotoluene	DMSO	4.44–4400	1.52–1516
9	119-90-4	*o*-Dianisidine	DMSO	4.44–4400	0.50–500
13	107-06-2	1,2-Dichloroethane	DMSO	4.44–4400	4.44–4400
14	78-87-5	1,2-Dichloropropane	DMSO	4.44–4400	4.44–4400
104	60-11-7	*p*-Dimethylaminoazobenzene	DMSO	1.92–1920	0.20–200
112	79-44-7	Dimethylcarbamoyl chloride	DMSO	4.44–4400	4.44–4400
27	540-73-8	1,2-Dimethylhydrazine	DMSO	5.00–5000	5.00–5000
90	121-14-2	2,4-Dinitrotoluene	DMSO	4.44–4400	0.15–151.6
91	606-20-2	2,6-Dinitrotoluene	DMSO	4.44–4400	14–1000
8	2610-05-1	Direct blue 1	H_2O	1.28–1280	1.28–1280
101	62-50-0	Ethyl methanesulfonate	DMSO	4.44–4400	4.44–4400
3	50-00-0	Formaldehyde	DMSO	4.44–4400	4.44–4400
5	111-30-8	Glutaraldehyde	DMSO	5.00–5000	4.44–4400
4	107-22-2	Glyoxal	DMSO	4.44–4400	4.44–4400
109	680-31-9	Hexamethylphosphoramide	DMSO	5.00–5000	4.44–4400
1	5341-61-7	Hydrazine dihydrochloride	H_2O	4.44–4400	4.44–4400
66	123-31-9	Hydroquinone	H_2O	1.92–1920	1.92–1920
7	7803-49-8	Hydroxylamine	DMSO	4.44–4400	4.44–4400
25	100-61-8	*N*-methylaniline	DMSO	4.44–4400	4.44–4400
94	598-55-0	Methyl carbamate	DMSO	4.44–4400	4.44–4400
100	56-49-5	3-Methylcholanthrene	DMSO	0.10–100	0.10–100
73	101-14-4	4,4′-Methylene-bis(1-chloraniline)	DMSO	4.44–4400	0.15–151
77	101-77-9	4,4′-Methylenedianiline	DMSO	4.44–4400	1.52–1520
17	78-94-4	Methyl vinyl ketone	DMSO	4.44–4400	4.44–4400
63	90-94-8	Michler's ketone	DMSO	0.97–972	0.97–972
102	134-32-7	1-Naphthylamine	DMSO	4.44–4400	4.44–4400
103	91-59-8	2-Naphthylamine	DMSO	4.44–4400	4.44–4400
129	75-52-5	Nitromethane	DMSO	5.00–5000	4.44–4400
130	79-46-9	2-Nitropropane	DMSO	5.00–5000	4.44–4400
65	108-95-2	Phenol	DMSO	4.44–4400	4.44–4400
71	88-99-3	Phthalic acid	DMSO	4.44–4400	4.44–4400
69	85-44-9	Phthalic anhydride	DMSO	4.44–4400	4.44–4400
6	542-78-9	Propanedial	H_2O	4.44–4400	4.44–4400
20	107-18-6	2-Propen-1-ol	DMSO	4.44–4400	4.44–4400
111	26628-22-8	Sodium azide	H_2O	4.44–4400	4.44–4400
135	151-21-3	Sodium lauryl sulphate	H_2O	4.44–4400	0.44–444
36	54827-17-7	3,3′,5,5′-Tetramethylbenzidine	DMSO	4.44–4400	4.44–4400
72	101-61-1	*N*,*N*,*N*′,*N*′-tetramethyl-4,4′-methylenedianiline	DMSO	1.92–1920	1.92–1920
74	139-65-1	4,4′-Thiodianiline	DMSO	5.00–5000	5.00–5000
126	62-56-6	Thiourea	H_2O	5.00–5000	4.44–4400
23	108-44-1	*m*-Toluidine	DMSO	4.44–4400	4.44–4400
22	95-53-4	*o*-Toluidine	DMSO	4.44–4400	4.44–4400
24	106-49-0	*p*-Toluidine	DMSO	4.44–4400	4.44–4400
29	78-40-0	Triethyl phosphate	DMSO	5.00–5000	4.44–4400
28	512-56-1	Trimethyl phosphate	DMSO	5.00–5000	4.44–4400
31	126-72-7	Tris(2,3-dibromopropyl) phosphate	DMSO	5.00–5000	5.00–5000

Table III. *Continued*

Code No.	CAS number	Chemical name	Solvent	Concentrations (μg/ml)[a]	
				−S9	+S9
30	115-96-8	Tris(2-chloroethyl) phosphate	DMSO	5.00–5000	5.00–5000
95	51-79-6	Urethane	H_2O	4.44–4400	4.44–4400

CAS, Chemical Abstracts Service; DMSO, dimethyl sulfoxide; THF, tetrahydrofuran.
[a]Concentration range tested in Ames II.

testing protocols. Because hamster S9 was not used in the Ames II tests, only the negative NTP rat S9 results were used for purposes of this comparison. In the Ames II protocol, these chemicals scored 'weakly positive' and possibly positive with TAMix +S9.

- 2,3-Butanedione was positive only in strain TA97 in the NTP tests. Because this frameshift strain was not used here, and responds to a different spectrum of mutagens than TA98, chemicals that are mutagenic only TA97 (or TA1537) would not be expected to be detected in the Ames II procedure.

Table V summarizes the comparisons of the two test procedures (detailed in Table IV) with respect to their performance for identifying mutagens and non-mutagens. Of the 71 chemicals tested, seven gave equivocal results in one or the other test (three in Ames II and four in the NTP protocol). Similarly, two chemicals labelled possibly positive (+?) in Ames II were not included for the following comparison. For the remaining 62 chemicals, the results were concordant (positive in both or negative in both) for 52 (84%). This concordance between the different assays is comparable to the intra- and interlaboratory reproducibility of 87% (pair-wise concordance) in the NTP Ames test procedure (10). Of the chemicals that were not concordant, 7/10 (70%) were positive using the traditional Ames test procedure and negative in Ames II procedure (Table IV). The three chemicals that were positive in Ames II and negative in the NTP tests (1,2-dimethylhydrazine, phenol and *o*-toluidine) were not tested by the NTP in *Salmonella* strains designed to respond to agents that act specifically at A:T sites (TA102 and TA104). Further testing will be needed to determine if the positive responses of these chemicals in the Ames II procedure is due to mutagenicity of the A:T-sensitive strains (TA7001, 7002 and 7003). Of the seven chemicals (Acid Red 26, 2,3 butanedione, danthron, 1,2-dichloropropane, glutaraldehyde, methyl vinyl ketone and sodium azide) positive only with the traditional Ames protocol, five were considered weak positives. The non-carcinogenic sodium azide requires bacterial metabolism for mutagenicity (11) which could explain the negative result in the Ames II being due to the limited 90-min exposure time with this protocol. Two of the three chemicals that were positive in Ames II but negative in the standard protocol are carcinogens, whereas among the seven chemicals that were only positive in the standard protocol, three are carcinogens.

The patterns of the responses in the different tests with respect to S9 requirements and strain specificities were also compared. Among the chemicals testing positive in both procedures, 87% (27/31) agreed on the requirement for S9, 19 chemicals required S9 and 8 were mutagenic in both procedures without metabolic activation. Of the four chemicals for which there was disagreement regarding the need for S9, three required it in the Ames II procedure and one in the NTP procedure.

As noted above, two of the chemicals (*o*- and *p*-toluidine) were positive in the NTP tests only with hamster S9; one of these, *o*-toluidine, was weakly mutagenic in the Ames II test with S9. For the purposes of this test-to-test comparison, only the negative NTP rat S9 result was used. The other chemical, *p*-toluidine, was weakly positive only with hamster S9 and did not give a clear result (possibly positive, +?) in Ames II. Due to this unclear result, it was not used for this test-to-test comparison.

The mutation specificities were also compared regardless of S9 requirement. There was agreement for 74% (23/31) of the chemicals that were judged positive in the two procedures; 17 chemicals mutated both the base pair substitution and the frameshift strains and 6 mutated only the base pair substitution strains. One chemical, 2,3-butanedione, that was non-mutagenic in Ames II was weakly mutagenic only in frameshift strain TA97, which is not used in the Ames II procedure; it was considered to be a mutagen for this compilation. Interestingly, there were three chemicals with complete disagreement on the mutagenic specificity; Michler's ketone was mutagenic only in TAMix in Ames II and only in TA98 in the traditional test, and acrylonitrile and 2,4-dinitrotoluene were mutagenic in the Ames II procedure only in TA98, but only in TA100 in the traditional test.

Beyond this, it is difficult to compare the strain-specific responses of the two procedures. In a number of cases, a substance that was judged mutagenic only in the base pair substitution or frameshift strains in one procedure was judged mutagenic in both strain types in the other. In many of those situations, where only the base pair substitution or frameshift response was judged positive in the one procedure, the corresponding response in the other procedure was equivocal or was too weak to be considered significant. It should also be noted that this comparison between the two methods is limited by the fact that the suppliers of many of the chemicals used in the two assays was different, and the purity of the chemicals at the time of the assays was also probably different. This and differences in the S9 batches used could account for some of the differences seen between the weak and negative responses. This makes the high concordance between the two test systems even more significant.

Comparison of the Ames and Ames II responses for the identification of carcinogens

The two test procedures were also compared for their ability to correctly identify rodent carcinogens as listed in the CPDP database (12,13). The chemicals that have test data from both the *Salmonella* test procedures and the rodent cancer test are listed in Table IV. Table VI summarizes and compares the

Table IV. Summary of rodent cancer test results, mutagenicity test responses and comparison of strain and Aroclor 1254-induced S9 requirements in the Ames II and NTP *Salmonella* (Ames) procedures

Chemical name	Cancer	Ames II	S9	TAMix	TA98	NTP Ames	S9	TA100, 1535	TA98
Acetamide	+	−				−			
2-Acetylaminofluorene	+	+	y	+	+	+	y	+	+
Acid Red 26	+	−[a]				w+	y	+	+
Acrolein	−	−				E	y	E	−
Acrylamide	+	−				E	y	−	E
Acrylonitrile	+	w+	y	−	+	+	y	+	−
2-Aminoanthraquinone	+	+	y	−	+	+	y	+	+
4-Aminobiphenyl	+	+	y	+	+	+	y	+	+
2-(4-Aminophenyl)-6-methylbenzothiazole		+	y	+	+	+	y	+	+
Aniline	+	−				−			
o-Anisidine	+	−				−			
Anthracene		+	y	−	+	w+	y	+	−
Benzene	+	−				−			
Benzidine	+	+	y	−	+	+	y	+	+[b]
2,3-Butanedione		−				w+	n	−	
Beta-butyrolactone	+	+	n	+	+	+	n	+	+
Gamma-butyrolactone	−	−[a]				−			
Catechol	+	−				−			
Chloroacetaldehyde		E	n	E	−	+	n	+	−
Crystal violet	+	−				E	y	−	E[b]
Danthron	+	−				w+	y	+	−
Diaminodiphenyl ether	+	+	y	+	+	+	y	+	+
2,4-Diaminotoluene	+	+	y	+	+	+	y	+	+
2,6-Diaminotoluene	−	+	y	+	+	+	y	+	+
o-Dianisidine	+	+	y	−	+	+	y	+	+
1,2-Dichloroethane	+	w+	n	+	−	+	y	+	−
1,2-Dichloropropane	+	−				w+	n	+	−
p-Dimethylaminoazobenzene	+	+	y	+	+	+	y	+	+
Dimethylcarbamoyl chloride	+	+	n	+	+	+	n	+	+
1,2-Dimethylhydrazine	+	+	n	+	−	−			
2,4-Dinitrotoluene	+	+	n	−	+	+	n	+	−
2,6-Dinitrotoluene	+	+[a]	n	+	+	+	n	+	+
Direct blue 1		−[a]				−[c]			
Ethyl methanesulfonate	+	+	n	+	−	+	n	+	−
Formaldehyde	+	+	n	+	−	+	n	+	−
Glutaraldehyde	−	−				w+	n	+	−
Glyoxal		+	n	+	E	+	n	+	+
Hexamethylphosphoramide	+	+?	y	+?	−	−			
Hydrazine dihydrochloride	+	w+[a]	y	+	−	+	n	+	−
Hydroquinone	+	−				−			
Hydroxylamine		−				−			
N-Methylaniline		E	+	−	E	−			
Methyl carbamate	+	−				−			
3-Methylcholanthrene	+	+	y	+	+	+	y	+	+
4,4′-Methylene-bis(1-chloraniline)	+	+	y	+	+	+	y	+	+
4,4′-Methylenedianiline	+	+	y	+	+	+	y	+	+
Methyl vinyl ketone		−				+	y	−	+
Michler's ketone	+	+	y	+	−	+	y	−	+
1-Naphthylamine	−	+[a]	y	+	−	+	y	+	+
2-Naphthylamine	+	+	y	+	+	+	y	+	+
Nitromethane	+	−				−			
2-Nitropropane	+	+	y	+	+	+	n	+	+
Phenol	−	w+	y	−	+	−			
Phthalic acid		E	n	−	E	−			
Phthalic anhydride	−	−[a]				−			
Propanedial		−				−			
2-Propen-1-ol		−[a]				−			
Sodium azide	−	−				+	n	+	−
Sodium lauryl sulphate		−				−			
3,3′,5,5′-Tetramethylbenzidine		−				−			
N,*N*,*N*′,*N*′-Tetramethyl-4,4′-methylenedianiline	+	−				−			
4,4′-Thiodianiline	+	+	n	+	+	+	n	+	+
Thiourea	+	−				−			
m-Toluidine	+	−				−			
o-Toluidine	+	w+[a]	y	+	−	−	y[d]	+	−
p-Toluidine	+	+?	y	+?	−	−	y[d]	+	−
Triethyl phosphate		−				−			
Trimethyl phosphate	+	w+	y	+	−	+	n	+	−
Tris(2,3-dibromopropyl) phosphate	+	+	y	+	+	+	y	+	+

Table IV. *Continued*

Chemical name	Cancer	Ames II	S9	TAMix	TA98	NTP Ames	S9	TA100, 1535	TA98
Tris(2-chloroethyl) phosphate	+	−[a]				−			
Urethane	+	−[a]				E	y	E[d]	

Cancer: summary cancer results in rats and/or mice (7,12). Ames II: fluctuation (micro-well) assay using TAMix (combination of strains TA7001–7006) and TA98, with and without rat S9. Ames: NTP *Salmonella* pre-incubation test protocol using TA98, TA100 (all chemicals), TA1535, TA97 (all negative and some positive chemicals), with and without rat and hamster S9. +, mutagenic, carcinogenic; w+, weakly mutagenic; −, not mutagenic, carcinogenic; E, equivocal response; +?, possibly mutagenic; n, positive without S9; y, only positive with S9.
[a]Based on triplicate data.
[b]Positive in TA97/TA1537, negative in TA98.
[c]Positive only with reductive metabolism.
[d]Only with hamster S9.

predictivity of Ames II with the traditional Ames test for the 56 chemicals tested in both systems and for rodent carcinogenicity.

The Ames (NTP) and Ames II test procedures had similar predictivities (concordance) for the rodent carcinogenicity results, with 34 and 33 chemicals (61 and 59%) correctly predicted, respectively. The differences in predictivity between the two procedures are small and not significant because of the relatively low number of total chemicals and the very low number (eight) of non-carcinogens in this database. The predictivities obtained in this study can be compared with the previously published compilation of 363 chemicals (14) that include the 56 presented here (Table VII). The major difference between the two databases is in their different proportions of non-carcinogens (14% for the chemicals reported here versus 44%). This disparity, considering the low number (eight) of non-carcinogens in this study, tends to magnify small differences in predictivity of non-carcinogens, i.e. the specificity and false-positive rates.

In addition to the agreement in predictivity between the two procedures, the predictions of the carcinogenicity of the individual chemicals were consistent. The two tests agreed almost completely in their predictions of carcinogens (30 and 28) and non-carcinogens (4 and 5) and on their incorrect predictions of 22 and 23 chemicals. Of the nine chemicals on which there was no agreement between the two tests (equivocals and possibly positives not counted), the NTP Ames test correctly predicted five and the Ames II test correctly predicted four chemicals (Table IV).

Some of the carcinogens in Table IV are not considered DNA reactive and therefore would not be expected to be identified by a bacterial point mutation assay like the Ames test. These chemicals include acetamide, aniline, methyl carbamate and thiourea.

Acetamide has shown liver tumours in rats which were nearly completely suppressed by co-feeding of arginine glutamate (15). Aniline has shown mostly negative responses in other *in vitro* and *in vivo* assays (16); tumorigenic responses at high doses in the spleen of rats have been regarded as a sequel of methaemoglobin formation with iron overload of splenal tissues oxidative stress (17,18). Methyl carbamate has shown liver tumours in rats but not in mice which appear to hydrolyse the material at a faster rate than rats (19) and no mutagenic effects were observed in a number of *in vitro* experiments (20). This is a profile very different from ethylcarbamate (urethane) which is metabolized to vinylcarbamate and other DNA-reactive chemicals (21). Thiourea inhibits iodine uptake and showed equivocal evidence of carcinogenicity in a number of earlier studies for thyroidal tumours (22) and hepatoma (23,24). Several Ames tests have been mostly negative (25–28) and neither initiating nor promoting activities were detected in the rat liver foci bioassay (29).

Table V. Summation of performance agreements between the Ames and Ames II test procedures

Response	Number	%
Mutagenic in both procedures	31/62	50
Non-mutagenic in both procedures	21/62	34
Agreement on mutagenicity	**52/62**	**84**
Disagreement on mutagenicity	10/62	16
BPS and FS mutations induced in both procedures	17/31	55
Only BPS induced in both procedures	6/31	19
Agreement of mutation spectra	**23/31**	**74**
Disagreement of mutation spectra	8/31	26

BPS, base pair substitution mutations (NTP results: TA100 and/or TA1535; Ames II results: TAMix (TA7001–TA7006). FS, frameshift mutations (NTP results: TA98, TA97; Ames II results: TA98).
Note: Agreement summations are in bold.

Table VI. Comparison of the cancer predictivity of both *Salmonella* test procedures

	Ames		Ames II		Total chemicals
	+	−	+	−	
Cancer+	30	18	28	20	48
Cancer−	4	4	3	5	8
Total chemicals	34	22	31	25	56

Ames: NTP *Salmonella* pre-incubation test protocol. Ames II: fluctuation (micro-well) assay. Only clearly positive results in the Ames tests (+ or w+) were counted as positive; equivocal or possibly positive results (EQ or +?) were conservatively counted as negative for cancer predictivity.

Use of the tests and regulatory implications

Both the traditional *Salmonella* tester strains and the TA700x strains have shown themselves to be useful for identifying mutagens and classifying (to varying degrees) the types of molecular mechanisms responsible for a mutagenicity and also for identifying potential carcinogens. The Ames II procedure has several advantages over the (standard) Ames procedure: it is offered as a standardized kit with quality-controlled bacterial strains, it requires considerably less (up to 3×) test substance, S9 mix and plasticware, than the traditional Ames procedure, and needs a substantially shorter hands-on time. The micro-well

Table VII. Summary performance of the Ames and Ames II assays for predicting rodent carcinogenicity[a]

	Ames[b]		Ames II[b]		NTP 1998[c]
	No.	%	No.	%	%
Sensitivity	30/48	63[d]	28/48	58	54
Specificity	4/8	50	5/8	63	79
Positive predictivity	30/34	88[d]	28/31	90	77
Negative predictivity	4/22	18[d]	5/25	20	57
False positive	4/8	50	3/8	38	21
False negative	18/48	38[d]	20/48	42	46
Concordance	34/56	61[d]	33/56	59	65
Prevalence	48/56	86	48/56	86	56

[a]The various measures of performance are sensitivity, proportion of carcinogens correctly identified; specificity, proportion of non-carcinogens correctly identified; positive/negative predictivity; the proportion of positives/negatives that correctly predict cancer/non-cancer; false positive/negative, the proportion of non-carcinogens/carcinogens that are incorrectly predicted; concordance, overall agreement between *Salmonella* and carcinogenicity test results and prevalence, the proportion of carcinogens in the tested population.
[b]The same chemicals were tested in the Ames, Ames II and cancer tests.
[c]Data from Zeiger, 1998 (based on 363 chemicals). The 56 chemicals reported in this paper are included in the Zeiger, 1998, chemicals.
[d]These values were 67, 89, 20, 33 and 64%, respectively, before the results were adjusted to ignore the two positive responses dependent on hamster liver S9 and use the negative, rat liver S9 responses instead.

format and the colorimetric readout allow for automation of substantial parts of the assay.

Industrial organizations use the *Salmonella* test both for preliminary screening (triage) of candidate chemicals for subsequent development and to provide genetic toxicity information to regulatory authorities when seeking marketing approval of their product. The TA700x tester strains are not included among the recommended tester strains in the current, formal test guidelines (2,30,31), and therefore negative results in the test may not be accepted in lieu of the traditional strains by regulatory authorities. However, the results presented here and previous comparisons of the two test procedures (3,4) show that the Ames II assay provides equivalent positive predictivities for rodent cancer, and the incidences of false positives and false negatives are comparable in the two test procedures. The two procedures can therefore be considered interchangeable for screening to identify mutagens and potential carcinogens.

Recently, the Ames II format has been expanded to include the other tester strains suggested in the guidelines mentioned above. The *Salmonella* strains TA100, TA1535 and TA1537 as well as the *Escherichia coli* strains wp2 *uvrA* and wp2 [pKM101] are available in the same liquid microplate format as the Ames II test. This allows now to perform the bacterial fluctuation test in the liquid microplate format (Ames MPF™) in full accordance with the mentioned guidelines (2,30,31).

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Conflict of interest statement: MK and SF-I are employees of Xenometrix AG in Allschwil, Switzerland. Xenometrix produces and sells the Ames II mutagenicity kit.

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Research Articles

Comparison of the Salmonella/Microsome Microsuspension Assay with the new Microplate Fluctuation Protocol for Testing the Mutagenicity of Environmental Samples

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The objective of this study was to compare the responses of the Salmonella/microsome microsuspension assay with the new microplate fluctuation protocol (MPF) for the evaluation of the mutagenic activity of environmental samples. Organic extracts of total particulate atmospheric air samples, surface waters, and effluents were tested in dose–response experiments. The assays were performed with strain TA98 in the absence and presence of S9 mix. Both protocols produced similar results, despite the fact that the maximum score of the MPF is limited to 48 wells, whereas in the regular plate assay it is possible to count up to 1,500 colonies using an automatic counter. Similar sensitivities based on the lowest dose that resulted in a positive response were obtained for both assays. The MPF procedure is less laborious (e.g., all-liquid format, use of multichannel pipettors) and allows for automation of the pipetting and dispensing steps, thus, reducing time of the analysis which is particularly important in environmental quality monitoring programs or in effect-directed analysis. The results show that the MPF procedure is a promising tool to test environmental samples for mutagenic activity. Environ. Mol. Mutagen. 00:000–000, 2009. © 2009 Wiley-Liss, Inc.

Key words: mutagenicity; monitoring; Salmonella/microsome assay; ames MPF; microsuspension; microplate fluctuation test

INTRODUCTION

The Salmonella/microsome assay has been widely used for testing chemicals and environmental samples. A recent review of the mutagenicity of environmental samples showed that the assay is the most widely used for testing surface waters (37%) [Ohe, 2004], aquatic sediments (41%) [Chen and White, 2004], soil (38%) [White and Claxton, 2004], and atmospheric samples [Claxton et al., 2004]. In addition, the use of the assay for environmental regulatory purposes [CONSEMA, 2006], in water quality monitoring programs [Umbuzeiro et al., 2001; Arimoto-Kobayashi et al., 2007], and effect-directed analysis (EDA) [Marvin and Hewitt, 2007] are increasing. Simpler protocols and automation could provide important tools to the effective use of the Salmonella/microsome assay around the world. The microsuspension version of the Salmonella/microsome assay was developed by Kado et al. [1983] to test urine samples and has been frequently applied to test environmental samples because it requires less sample quantity when compared with the regular plate or preincubation assay.

The Ames microplate fluctuation protocol (MPF) assay kits from Xenometrix are a liquid microplate modification of the traditional Salmonella fluctuation method [Green et al., 1976; Gee at al., 1998; Flückiger-Isler et al., 2004]. The use of these kits reduces sample consumption and hands-on time, and increases the throughput as compared with the traditional plate test method. The kits contain

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ready-to-use media and performance-tested Salmonella tester strains that are phenotyped (*uvr*B, *rfa*, Δ*bio*, Ampicillin resistance) and sequenced to confirm their respective *his*$^-$ genotypes.

The aim of the present study was to compare the responses of the Salmonella/microsome microsuspension assay, which has been extensively used to test environmental samples, with the new MPF-microplate format protocol for the evaluation of the mutagenic activity of different environmental samples.

MATERIALS AND METHODS

Sampling and Sample Preparation Procedures

Two total particulate atmospheric air samples (Air 1 and Air 2) were collected in Sao Paulo city with glass fiber filters using a 24-hr high-volume sampler [Umbuzeiro et al., 2008]. Four samples of river surface water (Water 1–4) and three samples of different industrial effluents (Effluent 1–3) were collected, transported to the laboratory protected from light, and stored refrigerated for a maximum of 14 days before extraction. Effluent 1 was from a dye manufactory industry, Effluent 2, from a textile dyeing plant, and Effluent 3, from a petrochemical facility.

Atmospheric samples were extracted according to Sato et al. [1995]. Briefly, the sample filters or a clean filter (blank) were extracted three times by ultrasonication with methylene chloride. The extracts were filtered through Teflon membranes, the volume was reduced using a rotary evaporator, dried under a gentle stream of pure nitrogen gas, and resuspended in dimethylsulfoxide (DMSO) just before testing. The extractable organic matter (EOM) was obtained for each sample by gravimetric analysis.

Volumes of 10 L of the surface water samples were extracted according to Umbuzeiro et al. [2004]. Briefly, the samples were serially extracted with XAD4 resin at neutral and acidic pH using methanol and methylene chloride (1:4), and methanol and ethylacetate (1:4), respectively. Both extracts were combined, the volume reduced in a rotary evaporator, dried in a gentle stream of pure nitrogen gas, and resuspended in DMSO just before testing. A blank of the extraction procedure was performed using ultrapure water.

For the effluent samples, 1.5 L of each sample were extracted with methanol and methylene chloride in a proportion of 1:2.5 at neutral, basic and acidic pH as described by Umbuzeiro et al. [2004]. The different pH extracts were combined, the volume reduced in a rotary evaporator, dried in a gentle stream of pure nitrogen gas, and resuspended in DMSO just before testing. A blank of the extraction procedure was performed using ultrapure water.

Salmonella/Microsome Microsuspension Assay

Samples were tested in the microsuspension Salmonella/microsome assay using *Salmonella typhimurium* TA98 (*HisD*3052, *rfa*, Δ*bio*, *uvr*B, pKM101) kindly provided by Dr. Larry Claxton, from United States Environmental Protection Agency (USEPA). The assays were performed using five doses and triplicate plates/dose, both in the presence and absence of S9 using preincubation of 90 min at 37°C [Kado et al., 1983].

TABLE I. Comparison of the MPF and Microsuspension Protocols for Testing Atmospheric Sample Extracts with *S. typhimurium* Strain TA98 Without S9

	MPF protocol (–S9)				Microsuspension protocol (–S9)				
	Positive wells per microplate				Revertants per plate				
Sample	Concentration (μg EOM)	Mean	SD	FIB	Concentration (μg EOM)	Mean	SD	MR	*P*
Air 1	0	1.33	0.58		0	22.2	3.03	0	
					0.5	28.3	2.52	1.3	
	1	1.67	1.15	0.87	1	26.0	1.00	1.2	
	5	5.00	1.73	**2.62**	5	58.0	5.29	**2.6**	**
	10	17.33	4.04	**9.07**	10	113.7	4.04	**5.1**	**
	25	20.67	3.51	**10.82**	25	225.7	25.8	**10.2**	**
	50	25.67	3.06	**13.43**	50	217.0	15.0	**9.8**	**
Air 2	0	1.00	1.00		0	20.2	4.60		
					0.5	32.3	4.04	1.6	*
	1	2.67	1.53	1.33	1	50.3	7.02	**2.5**	**
	2.5	4.00	0.00	**2.00**					
	5	5.33	1.53	**2.67**	5	75.7	19.5	**3.8**	*
	10	8.67	5.69	**4.33**	10	105.7	4.51	**5.2**	**
	25	16.33	4.73	**8.17**	25	292.0	30.5	**14.5**	**
	50	23.33	2.31	**11.67**	50	664.7	87.7	**32.9**	**
Air	0	1.67	1.53		0	21.0	2.45		
blank	0.1	0.67	0.58	0.21	0.1	17.0	0.00	0.8	
	0.5	1.33	1.53	0.42	0.5	19.0	5.66	0.9	
	1	1.00	1.00	0.31	1	21.5	6.36	1.0	
	2.5	0.67	0.58	0.21	2.5	17.5	7.78	0.8	
	5	2.00	1.73	0.63	5	20.5	2.12	1.0	
	10	1.33	1.53	0.42	10	20.5	2.12	1.0	

Values in bold indicates FIB greater than 2.
*$P < 0.05$, **$P < 0.01$.
EOM = extractable organic material; FIB = fold induction over baseline (baseline = mean zero-dose control + 1 SD); SD = standard deviation; MR= mutagenic ratio.

TABLE II. Comparison of the MPF and Microsuspension Protocols for Testing Atmospheric Sample Extracts with *S. typhimurium* Strain TA98 With S9

	MPF protocol (+S9)				Microsuspension protocol (+S9)				
	Positive wells per microplate				Revertants per plate				
Sample	Concentration (μg EOM)	Mean	SD	FIB	Concentration (μg EOM)	Mean	SD	MR	*P*
Air 1	0	1.00	1.00		0	30.3	4.35		
					0.5	26.3	5.13	0.9	
	1	1.00	1.00	0.50	1	32.0	4.58	1.0	
	5	1.67	2.08	0.83	5	35.7	6.11	1.2	
	10	9.67	1.15	**4.83**	10	44.3	14.3	1.5	
	25	16.67	2.52	**8.33**	25	46.3	5.51	1.5	*
	50	21.33	2.52	**10.67**	50	210.7	49.2	**7.0**	**
Air 2	0	2.27	1.53		0	23.6	4.93		
					0.5	29.3	2.31	1.2	
	1	2.67	0.58	1.33	1	27.7	2.89	1.2	
	2.5	4.33	1.15	**2.17**					
	5	6.67	0.58	**3.33**	5	26.0	1.00	1.1	
	10	13.33	3.51	**6.67**	10	31.0	1.00	1.3	
	25	26.33	3.21	**13.17**	25	48.3	6.35	**2.1**	*
	50	35.33	4.04	**17.67**	50	41.0	8.50	**1.7**	**
Air blank	0	1.33	1.15		0	26.6	5.66		
	0.1	2.00	1.00	0.80	0.1	27.0	6.36	1.0	
	0.5	1.00	1.00	0.40	0.5	25.5	4.24	1.0	
	1	3.00	1.00	1.21	1.0	27.0	0.71	1.0	
	2.5	1.00	1.00	0.40	2.5	20.5	0.71	0.8	
	5	1.67	0.58	0.67	5	17.5	10.6	0.7	
	10	2.33	1.15	0.94	10	25.5	8.50	1.0	

Values in bold indicates FIB greater than 2.
$^{*}P < 0.05$, $^{**}P < 0.01$.
EOM = extractable organic material; FIB = fold induction over baseline (baseline = mean zero-dose control + 1 SD); SD = standard deviation; MR= mutagenic ratio.

The dose–response experiments were performed with maximum doses of 50 μg of EOM for air, 50 mL equivalent for surface water, and 5 mL equivalent for effluent samples. The S9 mix was freshly prepared according to Maron and Ames [1983] before each test using lyophilized Aroclor-1254-induced rat liver S9 fraction (Moltox, Boone, NC), resulting in 4% v/v of S9 fraction in the mixture. Colonies were counted using an automatic counter (AccuCount, Biologics, Manassas, VA). The results were analyzed with the Salanal program kindly provided by Dr John Mayers from Research Triangle Institute, Research Triangle Park, NC.

Toxicity was evaluated by careful inspection of the background using a stereomicroscope (10 × magnification). A sample was considered positive when there was a significant positive dose response, a significant statistical difference between the tested doses and the negative control (ANOVA), and the mutagenic ratio was >2. Mutagenic ratio was calculated by dividing the mean of the revertants obtained in each tested dose by the concurrent negative control.

The positive controls were 4-nitroquinoline-1-oxide (Sigma) at 0.125 μg per plate without metabolic activation and 2-aminoanthracene (Sigma) at 5 μg/plate with S9. DMSO was used as negative control.

Ames MPF Assay

The Ames MPF assay was performed in liquid media in 24-well plates during sample exposure and in 384-well plates for revertant growth and for scoring. Growth, Exposure and Indicator Media, as well as *S. typhimurium* strain TA98, were included in the kit from Xenometrix AG, Allschwil, Switzerland. The test procedure described in the 'Ames MPF Instructions for Use' was followed.

Briefly, bacteria were grown overnight, diluted in Exposure Medium and exposed to test samples in 24-microwell plates for 90 min at 37°C with agitation in the presence or absence of 4.5% Aroclor 1254-induced rat liver S9 (Moltox). The exposed cultures were then diluted in Indicator Medium and the contents of each 24-well culture were distributed into 48 wells of a 384-well plate (50 μL per well). The Indicator Medium contains a pH indicator dye which changes from purple to yellow on bacterial growth. After 48-hr incubation at 37°C, the plates were scored by eye for yellow wells. Positive and negative controls were included as for the microsuspension assay, and all doses were done in triplicate. Note that the Ames MPF (microplate format) limits the number of positive wells to a maximum of 48 wells per sample.

The criteria used to evaluate the MPF results were the fold increase in number of positive wells over the solvent control baseline (FIB), and the dose dependency. The fold increase of revertants relative to the solvent control was determined by dividing the mean number of positive wells at each dose by the solvent control baseline. The solvent control baseline was defined as the mean number of positive wells in the solvent control plus 1 SD. All solvent controls from an experiment with identical conditions (same day, same bacterial culture, solvent and incubation conditions) were combined.

An increase of >2-fold relative to the baseline was classified as positive for that dose. Positive responses of >2-fold relative to the baseline at more than one dose with a dose–response led to the test sample being classified as positive. A test sample was classified as negative where no response >2 times the baseline and no dose–response was observed.

Positive controls used for the MPF protocol were 2-nitrofluorene (Sigma) at 2 μg/mL without metabolic activation and 2-aminoanthracene (Sigma) at 5 μg/mL with S9. DMSO was used as the negative control.

TABLE III. Comparison of the MPF and Microsuspension Protocols for Testing Water Sample Extracts with *S. typhimurium* Strain TA98 Without S9

	MPF protocol (–S9)				Microsuspension protocol (–S9)				
	Positive wells per microplate				Revertants per plate				
Sample	Concentration (mL equiv.)	Mean	SD	FIB	Concentration (mL equiv.)	Mean	SD	MR	*P*
Water 1	0	0.92	0.67		0	23.2	3.11		
	1	0.33	0.58	0.21					
	5	4.00	2.00	**2.52**	5	23.7	2.52	1.0	
	10	2.33	1.15	1.47	10	24.7	2.31	1.1	
	25	2.67	2.08	1.68	25	28.0	1.73	1.2	
	50	3.33	1.53	**2.10**	50	33.3	4.04	1.4	*
Water 2	0	0.92	0.67		0	37.4	6.58		
	1	2.00	2.00	1.26					
	5	2.33	1.53	1.47	5	42.7	4.16	1.1	
	10	4.00	1.73	**2.52**	10	53.3	1.53	1.4	*
	25	5.67	4.73	**3.57**	25	82.0	7.00	**2.2**	**
	50	9.00	5.29	**5.68**	50	53.7	6.03	1.4	
Water 3	0	0.92	0.67		0	22.8	3.27		
	1	2.00	1.00	1.26					
	5	1.67	1.15	1.05	5	24.3	0.58	1.1	
	10	3.00	0.00	1.89	10	23.3	1.53	1.0	
	25	4.33	1.15	**2.73**	25	46.7	5.03	**2.1**	**
	50	7.00	2.65	**4.42**	50	50.7	6.11	**2.2**	**
Water 4	0	1.00	1.00		0	28.0	2.65		
	1	0.33	0.58	0.17					
	5	1.33	0.58	0.67	5	28.7	3.21	1.0	
	10	2.33	2.52	1.17	10	26.3	5.69	0.9	
	25	3.33	0.58	1.67	25	35.3	4.62	1.3	
	50	4.33	2.31	**2.17**	50	43.3	6.11	1.6	*
Water blank	0	1.00	1.00		0	28.0	2.65		
	1	0.67	0.58	0.33					
	5	1.33	0.58	0.67	5	28.6	4.16	1.0	
	10	4.33	1.53	2.17	10	27.0	6.08	1.0	
	25	2.33	2.08	1.17	25	23.0	3.61	0.8	
	50	5.00	0.00	**2.50**	50	27.0	6.00	1.0	

Values in bold indicates FIB greater than 2.
*$P < 0.05$, **$P < 0.01$.
EOM = extractable organic material; FIB = fold induction over baseline (baseline = mean zero-dose control + 1 SD); SD = standard deviation; MR= mutagenic ratio.

Calculation of Potencies

The potencies (slopes) for both procedures were expressed as the number of revertants per unit, depending on the sample tested; atmospheric samples were expressed as revertants per mg of EOM and liquid samples (surface water and effluents) as revertants per milliliter equivalent. For the microsuspension assay, the slopes were calculated from the revertants per plate using the Bernstein et al. [1982] model. For the MPF assay, the slope of the linear part of the dose–response curve from the number of positive wells was calculated using the linear regression function of Microsoft Excel. The slopes of each assay were log 10-transformed and compared using the same Microsoft Excel function.

RESULTS AND DISCUSSION

The negative control values (DMSO) obtained for both assays were within the expected ranges (Tables I–VI) with one exception, the negative control of the Ames MPF in Table V, which provided an unexpectedly high spontaneous rate. All the positive controls provided the expected responses (data not shown).

Air 1 and Air 2 were clearly mutagenic in the absence and presence of metabolic activation (Tables I and II). A comparison of the lowest positive dose obtained in each test is presented in Table VII. In the absence of S9, the lowest positive dose of Air 1 was identical (5 μg of EOM) in the microsuspension and MPF assays. For Air 2, the lowest positive dose was 1 μg of EOM in the microsuspension assay and 2.5 μg of EOM in the MPF assay. Sorensen et al. [1982] compared the mutagenicity results of air atmospheric samples tested without S9 in the standard plate incorporation Salmonella/microsome assay and the fluctuation test. They also observed a slight advantage in sensitivity for the standard Salmonella assay in the absence of S9. In the presence of S9, the MPF assay was

TABLE IV. Comparison of the MPF and Microsuspension Protocols for Testing Water Sample Extracts with *S. typhimurium* Strain TA98 With S9

	MPF protocol (+S9)				Microsuspension protocol (+S9)				
	Positive wells per microplate				Revertants per plate				
Sample	Concentration (mL equiv.)	Mean	SD	FIB	Concentration (mL equiv.)	Mean	SD	MR	*P*
Water 1	0	1.58	1.16		0	28.0	3.46		
	1	1.00	1.00	0.36					
	5	2.67	1.53	0.97	5	22.7	3.06	0.8	
	10	1.67	2.89	0.61	10	24.3	5.13	0.9	
	25	3.33	1.15	1.21	25	29.7	9.50	1.1	
	50	4.67	1.15	1.70	50	32.0	3.00	1.1	
Water 2	0	1.58	1.16		0	33.0	6.07		
	1	1.00	1.00	0.36					
	5	2.67	0.58	0.97	5	48.7	6.03	1.5	
	10	2.33	1.15	0.85	10	48.3	3.79	1.5	*
	25	6.00	1.00	**2.18**	25	69.3	3.06	**2.1**	**
	50	6.00	1.73	**2.18**	50	72.0	14.11	**5.1**	*
Water 3	0	1.58	1.16		0	28.6	6.80		
	1	4.00	1.00	1.46					
	5	7.33	1.53	**2.67**	5	29.0	7.55	1.0	
	10	17.00	3.61	**6.19**	10	56.0	4.58	**2.0**	**
	25	18.67	1.15	**6.79**	25	83.7	5.86	**2.9**	**
	50	13.00	3.00	**4.73**	50	61.7	6.11	**2.2**	**
Water 4	0	2.27	1.53		0	26.0	6.06		
	1	1.67	1.53	0.44					
	5	2.00	0.00	0.53	5	27.0	4.00	1.0	
	10	4.00	1.00	1.05	10	32.7	7.57	1.3	
	25	3.00	1.00	0.79	25	34.7	6.11	1.3	
	50	10.00	1.00	**2.63**	50	41.7	4.73	1.6	*
Water	0	2.27	1.53		0	26.0	6.06		
Blank	1	3.00	3.00	0.79					
	5	2.67	3.06	0.70	5	25.3	4.73	1.0	
	10	2.67	0.58	0.70	10	24.7	4.51	1.0	
	25	4.00	1.00	1.05	25	22.7	1.15	0.9	
	50	2.00	1.73	0.53	50	26.0	7.07	1.0	

Values in bold indicates FIB greater than 2.

*$P < 0.05$, **$P < 0.01$.

EOM = extractable organic material; FIB = fold induction over baseline (baseline = mean zero-dose control + 1 SD); SD = standard deviation; MR= mutagenic ratio.

more sensitive for the air samples when compared in terms of the lowest dose per plate that produced a positive response (Tables II and VII). Negative results were obtained with the blank filters using both assays (Tables I and II).

Water 1 without S9 seemed to show a weak positive response in the MPF assay, but it did not fulfill the criteria for a clear positive response: although there were two doses with a >2-fold induction over the baseline, a dose-dependent response was not observed (Table III). Such weak positive result can occur when very low spontaneous revertant levels occur. This illustrates the importance of requiring both a minimum of a >2-fold induction and a clear dose–response. Water 1 did not show >2-fold induction with metabolic activation at any dose. Therefore, this sample should be considered negative in the MPF assay (Table VII). This sample was clearly negative with and without S9 in the microsuspension assay (Tables III and IV).

Water 2 and Water 3 were positive with and without metabolic activation in both the MPF and the microsuspension assays. In both assays, Water 2 with S9 and Water 3 without S9 showed the same sensitivity in terms of the lowest dose that provided a positive response (Table VII). The MPF assay was more sensitive with Water 2 in the absence of S9 (10 vs. 25 mL equivalent) and with Water 3 with S9 (5 vs. 10 mL equivalent).

In the MPF assay, Water 4 showed a >2-fold increase over the baseline only at the highest concentration tested (Tables III and IV). Because the blank controls showed a similar response, Water 4 is likely to be negative and would need retesting at higher doses for confirmation of

TABLE V. Comparison of the MPF and Microsuspension Protocols for Testing Effluent Sample Extracts with *S. typhimurium* Strain TA98 Without S9

	MPF protocol (–S9)				Microsuspension protocol (–S9)				
	Positive wells per microplate				Revertants per plate				
Sample	Concentration (mL equiv.)	Mean	SD	FIB	Concentration (mL equiv.)	Mean	SD	MR	*P*
Effluent 1	0	9.17	4.43		0	22.7	3.10		
	0.05	17.33	4.93	1.28	0.05				
	0.1	17.33	4.04	1.28	0.1	38.0	3.61	1.7	*
	0.5	29.00	2.65	**2.13**	0.5	64.7	11.15	**2.8**	**
	1	37.33	3.06	**2.75**	1	98.7	2.52	**4.3**	**
	2.5	45.00	1.00	**3.31**	2.5	234.3	32.64	**10.3**	**
	5	48.00	0.00	**3.53**	5	339.0	32.51	**14.9**	**
Effluent 2	0	9.17	4.43		0	21.0	1.10		
	0.05	9.00	1.73	0.66	0.05	30.0	4.36	1.4	
	0.1	16.33	4.62	1.20	0.1	37.7	7.02	1.7	*
	0.5	41.00	1.73	**3.02**	0.5	107.0	3.61	**4.9**	**
	1	46.67	1.53	**3.43**	1	167.0	21.07	**7.7**	**
	2.5	47.67	0.58	**3.51**	2.5	540.3	68.25	**24.8**	**
	5	48.00	0.00	**3.53**	5	1292	119.15	**59.3**	**
Effluent 3	0	9.17	4.43		0	22.7	3.10		
	0.05	12.33	9.02	0.91	0.05				
	0.1	14.67	3.06	1.08	0.1	26.7	3.51	1.2	
	0.5	20.33	3.79	1.50	0.5	23.3	0.58	1.0	
	1	18.33	2.08	1.35	1	29.0	4.36	1.3	
	2.5	20.00	6.00	1.47	2.5	51.3	9.61	**2.3**	*
	5	26.33	2.52	1.94	5	87.0	11.14	**3.8**	**
Effluent	0	9.17	4.43		0	26.6	2.41		
blank	0.05	13.00	5.57	0.96	0.05	22.3	0.58	0.8	
	0.1	14.00	4.36	1.03	0.1				
	0.5	6.00	5.29	0.44	0.5	22.3	3.21	0.8	
	1	6.00	4.58	0.44	1				
	2.5	12.00	7.00	0.88	2.5	26.5	0.71	1.0	
	5	9.67	1.15	0.71	5	23.7	2.52	0.9	

Values in bold indicates FIB greater than 2.

*$P < 0.05$, **$P < 0.01$.

EOM = extractable organic material; FIB = Fold Induction over Baseline (baseline = mean zero-dose control + 1 SD); SD = standard deviation; MR= mutagenic ratio.

the results. Water 4 was judged negative in the microsuspension assay, although a significant ANOVA value was obtained for the highest dose tested.

Effluent 1 and Effluent 2 were clearly mutagenic in the MPF assay, both with and without metabolic activation (Tables V and VI). The lowest positive dose was 0.5 mL equivalent (Table VII). Effluent 3 did not fulfill the criteria for mutagenicity in the absence of S9 due to an unusually high spontaneous rate in this experiment. The results suggest a possible weak mutagenic activity but it would need to be confirmed. In the presence of S9, Effluent 3 was clearly mutagenic at doses >1 mL equivalent (Tables VI and VII). The responses in the microsuspension assay were very similar to those of the MPF assay: all effluents were positive including Effluent 3, which was clearly positive also in the absence of S9 (Tables V). Very similar lowest positive doses were obtained for the effluent samples in both assays (Table VII). The blank effluent control showed a clear negative response in both assays.

Potencies for all samples were calculated. The quantification of the mutagenic response (slopes of the linear part of the dose–response) is required for environmental sample testing, especially in monitoring programs, or EDA studies, where it is important to understand how the samples vary over time or within the fractions, respectively.

To compare the mutagenic potencies obtained in both assays a regression analysis was performed after potencies were log (10) transformed. A good correlation coefficient (0.84) was obtained (Fig. 1). The calculated linear equation ($y = 0.8386x - 0.1439$) allows an estimation of the potency in both assays. The potency values for the microsuspension assay were approximately 10-fold higher than in the MPF assay. This is a numerical difference that is related to the counts per plate that occur in each assay

TABLE VI. Comparison of the MPF and Microsuspension Protocols for Testing Effluent Sample Extracts with *S. typhimurium* Strain TA98 With S9

	MPF protocol (+S9) Positive wells per microplate				Microsuspension protocol (+S9) Revertants per plate				
Sample	Concentration (mL equiv.)	Mean	SD	FIB	Concentration (mL equiv.)	Mean	SD	MR	*P*
Effluent 1	0	6.42	2.11		0	25.8	4.92		
	0.05	8.00	3.46	0.94	0.05				
	0.1	7.67	2.08	0.90	0.1	28.3	2.52	1.1	
	0.5	19.67	4.04	**2.31**	0.5	38.0	4.00	1.5	
	1	28.67	2.31	**3.36**	1	53.0	7.94	**2.1**	*
	2.5	44.33	2.08	**5.20**	2.5	120.7	4.04	**4.7**	**
	5	47.67	0.58	**5.59**	5	187.7	4.16	**7.3**	**
Effluent 2	0	6.42	2.11		0	24.2	4.92		
	0.05	8.00	3.46	0.94	0.05	22.7	1.15	0.9	
	0.1	10.00	1.00	1.17	0.1	30.0	4.58	1.2	
	0.5	26.00	2.65	**3.05**	0.5	48.0	4.51	**2.0**	**
	1	34.33	2.52	**4.03**	1	65.0	2.65	**2.7**	**
	2.5	47.67	0.58	**5.59**	2.5	186.5	40.31	**7.7**	**
	5	48.00	0.00	**5.63**	5	457.7	29.14	**18.9**	**
Effluent 3	0	6.42	2.11		0	25.8	4.92		
	0.05	10.67	1.53	1.25	0.05				
	0.1	11.00	4.36	1.29	0.1	27.7	2.31	1.1	
	0.5	15.00	4.36	1.76	0.5	29.0	0.00	1.1	
	1	29.33	3.79	**3.44**	1	34.5	0.71	1.3	
	2.5	46.33	1.53	**5.43**	2.5	65.7	10.69	**2.5**	**
	5	48.00	0.00	**5.63**	5	126.7	7.23	**4.9**	**
Effluent blank	0	6.42	2.11		0	24.6	2.79		
	0.05	4.67	2.08	0.55	0.05	28.0	2.00	1.1	
	0.1	7.00	2.65	0.82	0.1				
	0.5	5.67	3.21	0.66	0.5	26.7	4.51	1.1	
	1	4.67	2.89	0.55	1				
	2.5	5.67	1.53	0.66	2.5	27.5	7.78	1.1	
	5	9.33	3.06	1.09	5	22.7	1.53	0.9	

Values in bold indicates FIB greater than 2.
*$P < 0.05$, **$P < 0.01$.
EOM = extractable organic material; FIB = fold induction over baseline (baseline = mean zero-dose control + 1 SD); SD = standard deviation; MR= mutagenic ratio.

TABLE VII. Lowest Dose Per Plate that Provided a Positive Response in Each Assay for the Tested Samples

	TA98-S9		TA98+S9	
Samples	MPF	Microsuspension	MPF	Microsuspension
Air 1	5	5	10	25
Air 2	2.5	1	2,5	25
Water 1	Negative	Negative	Negative	Negative
Water 2	10	25	25	25
Water 3	25	25	5	10
Water 4	Negative	Negative	Negative	Negative
Effluent 1	0.5	0.5	0.5	1
Effluent 2	0.5	0.5	0.5	0.5
Effluent 3	Negative[a]	2.5	1	2.5

For the air samples, the dose is expressed in μg of EOM per plate and for the liquid samples (water and effluent) in mL equivalent per plate.
[a]Sample was not classified as positive based on our evaluation criteria because it exhibited an elevated baseline.

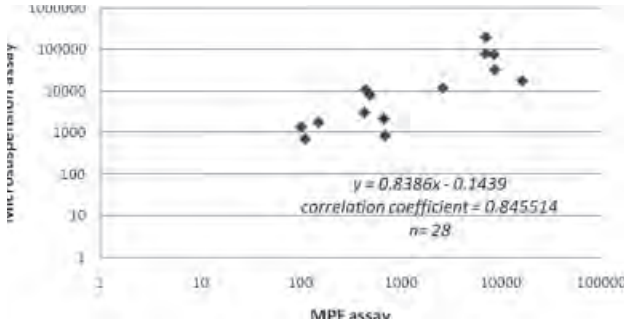

Fig. 1. Correlation of the potencies expressed in log of number of revertants per μg of EOM or mL equivalent obtained in the MPF assay and in the microsuspension Salmonella/microsome assay for the samples tested using TA98 with and without S9.

but is not related to the assay sensitivity (Table VII). The microsuspension assay counts vary from 20 to 1,500 colonies per plate, and in the MPF assay counts vary between 0 and 48 positive wells per plate. The equation shown in Figure 1 can be used to compare a result obtained with the MPF with historical results of the microsuspension assay.

CONCLUSIONS

The results from the Ames MPF and the microsuspension assays were in agreement with respect to their identification of environmental samples as positive, both in the absence or presence of metabolic activation. In the absence of S9, the Ames MPF was slightly less sensitive than the microsuspension assay with respect to the lowest mutagenic sample concentration. Conversely, in the presence of S9, the Ames MPF assay was slightly more sensitive.

The mutagenic potencies, i.e., revertants per sample unit, obtained for this set of samples correlated well when tested in both assays. Because the Ames MPF assay is easier to perform (e.g., all-liquid format, use of multichannel pipettors) and allows for automation of the pipetting and dispensing steps, it seems to be an interesting and valid alternative to the microsuspension assay especially when a large number of samples have to be tested, such as in monitoring programs and EDA studies.

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Accepted by—
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Evaluation of a Battery of Early Genotoxicity Assays to Predict Regulatory Testing

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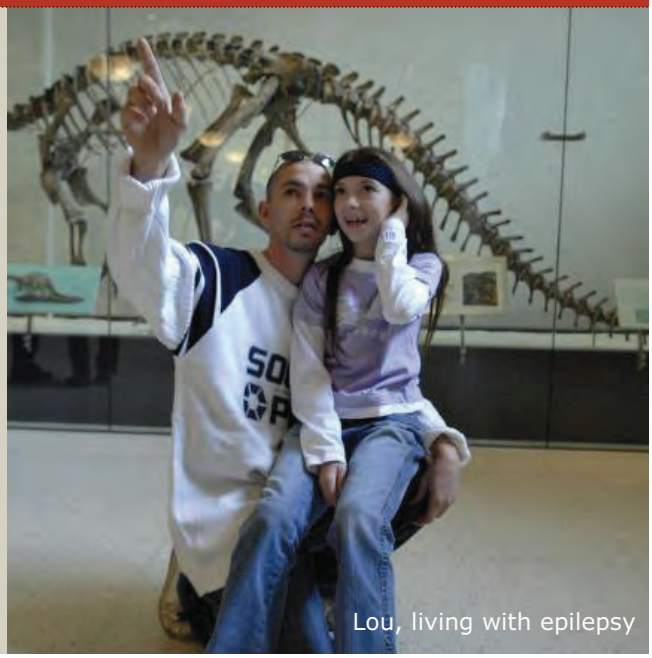
Lou, living with epilepsy

19/1/09

ADMET meeting, Brussels 22-23 Jan 2009

Plan

- Screening strategy: Overview of our activities
- Genotoxicity: current problem
- Improvement of our battery
- Other relevant approaches?
- Conclusion

Screening strategy: overview of the activities

In silico Toxicology

DEREK

In vitro Toxicology

Cytotoxicity Screen	***Genotoxicity Screen***			***PLD Screen***
HepG2	Vitotox	GreenScreen	Ames II	HepG2, Cell imaging

In vivo Toxicology

Toxicogenomics

Genotoxicity: background information

Assays required by authorities:

- **Before phase I clinical study**:
  - **Ames test** (in vitro): detection of mutations
  - **In vitro Mammalian test**: e.g mouse lymphoma assay: detection of mutations & structural chromosomal damage
- **Before phase II clinical study**:
  - **In vivo Mammalian assay**: e.g. rodent bone marrow micronucleus assay measurement of chromosomal damage and numerical chromosomal damage

Major Drawbacks:
- Amount of cpd required
- Time consuming
- Not possible to use it for early screening

→ Need to use a battery of assays predictive of the regulatory testings

Genotoxicity Screening Strategy at UCB Pharma

	1) In-house			2) CRO
	Vitotox (VT)	GreenScreen (GS)	Ames II (AII)	Mini Ames
Amount	10 mg			250 mg
Advantages	Simple, low amount of cpd			Predictivity (Ames)
Test systems	bacteria	yeast	bacteria	bacteria
Principle:	DNA repair	DNA repair	Mutations	Mutations
Correlation:	genotox prokaryotes	genotox eukaryotes	genotox prokaryotes	genotox prokaryotes

Are Vitotox, GS and Ames II predictive of mini Ames?

Conclusion:

- Negative cpds are correctly classified
- Cpds with medium/strong mutagenic potential are detected
- Cpds with low mutagenic potential are not detected

Evaluation of new assays to improve predictivity

Bacterial Assay:

- **Ames MPF** (Xenometrix):
  - Same principle as the Ames II assay (detection of mutation)
  - Strains used: TA98, TA100, TA1535 and TA1537 (= Ames test)

  NB: E. coli (uvrA et pKM101) available in 2009

Yeast and Mammalian Assays:

- **RadarScreen** (Remynd):
  - Yeast Genetically modified yeasts (MDR pumps deleted)
  - Measurement of induction of DNA repair gene (pRAD54)
  - pRAD54 (yeast) is equivalent to GADD45a (human cells)

- **Human GreenScreen** (Gentronix):
  - Human cells (TK6) Genetically modified
  - p53 competent
  - Measurement of induction of DNA repair gene (GADD45a)

Objectives

1) Predict regulatory tests (mini Ames and micronucleus assays)

2) Select the best bacterial and mammalian assays

3) Use a bacterial and mammalian assay for screening

Prediction of mini Ames data (mutations in bacteria)

	Vitotox	Ames II	Ames MPF
SPECIFICITY	82.6	81.8	72.7
n=	23	22	22

Specificity: nb of cpds negative in Mini Ames/nb of cpds negative in assay under evaluation

	Vitotox	Ames II	Ames MPF
SENSITIVITY	18.8	37.5	68.8
n=	16	16	16

Sensitivity: nb of cpds positive in Mini Ames/nb of cpds positive in assay under evaluation

Conclusion (prediction of mutation in bacteria)

- **AMES MPF**: best assay to predict mini Ames results under evaluation
- Prediction of weak mutagenic cpds
  - 10 cpds weakly mutagenic in mini Ames
  - 5 cpds detected in Ames MPF
  - 5 cpds not detected in Ames MPF
- **Main differences between mini Ames assay and Ames MPF** :
  - Solid (mini Ames) versus liquid (Ames MPF) format
  - Top concentration: 5000 µg/plate or 10 mM (miniA) vs 2mM (MPF)

  NB: 3 cpds (weak positive in mini Ames, negative in Ames MPF 2mM):

  Retested in Ames MPF at 10 mM: 1: +, 2: -