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## Ames II<sup>™</sup> Mutagenicity Assay



XENOMETRIX

- Quality controlled S. typhimurium strains
- Ready to use reagents
- 30 mg of test compound requested only
- Less hands-on time
- Microplate format suitable for automation

## Ames II Mutagenicity Assay Technical Documentation

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### 1. Introduction

The traditional Ames plate incorporation test is one of the most commonly performed safety 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 II 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. When these bacteria are exposed to mutagenic agents, under certain conditions reverse mutation from histidine auxotrophy to histidine 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 traditional Ames test.

The Ames II Mutagenicity Assay, based on the same principle as the traditional test, sets a new standard for this type of testing, offering several advantages over the traditional Ames test.

The Ames II Mutagenicity Assay, available through OB and a liquid microplate modification of the Ames test which offers a higher speed format, new strains, 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, shows good correlation with the traditional assay and was developed in the Bruce Ames laboratory at U.C. Berkeley (1). Due to the possibility of automatization, hundreds of substances can be run within a month.

### 3. Strains used in the Ames II Mutagenicity Assay

The two strains provided in the Ames II test kit are the Ames II TAMix and TA98. The Ames II TAMix contains an equimolar mixture of the Ames II TA7001-TA7006 strains. Like the traditional strains, the genetic background of the TA700X series strains have been modified to improve the sensitivity of their reversion. Individually, these strains are designed to revert by only one specific base-pair substitution out of all possible changes. Thus, when mixed, all base pair substitution mutations can be represented in one culture. The TA98 strain is used for the detection of frameshift mutations (1).

### 4. Ames II Mutagenicity Assay Description

Approximately 10<sup>7</sup> 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 to support approximately two cell divisions. After 90 minutes, the exposure cultures are diluted in pH indicator medium lacking histidine, and aliquoted into 48 wells of a 384-well plate. Within two days, cells which have undergone the reversion to *His* will grow into colonies. Metabolism by the bacterial colonies reduces the pH of the medium, changing the colour of that well. This colour 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 control. It is recommended to test each dose in triplicate.

An 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 II Mutagenicity Assay.

<u>Strain</u>	<u>Mutation</u>	<u>Type</u>	<u>Target</u>	<u>Cell Wall<sup>1</sup></u>	<u>Repair<sup>2</sup></u>	<u>pKM101</u> <sup>3</sup>
TA98	hisD3052	Frameshift	GC	rfa	uvrB	yes
TAMix contain	IS:					
TA7001	hisG1775	b.p. sub.	A:T>G:C	rfa	uvrB	yes
TA7002	hisC9138	b.p. sub.	T:A>A:T	rfa	uvrB	yes
TA7003	hisG9074	b.p. sub.	T:A>G:C	rfa	<i>uv</i> rB	yes
TA7004	hisG9133	b.p. sub.	G:C>A:T	rfa	uvrB	yes
TA7005	hisG9130	b.p. sub.	C:G>A:T	rfa	uvrB	yes
TA7006	hisC9070	b.p. sub.	C:G>G:C	rfa	uvrB	yes

Genotypes of the Ames II TAMix & TA98 Salmonella typhimurium strains provided

<sup>1</sup> These mutations affect the lipopolysaccharide (LPS) component of the cell envelope. These strains have increased permeability for bulky molecules.

<sup>2</sup> Strains carrying the *uvr*B mutation are deficient in excision repair of bulky lesions as measured by their lack of survival following UV<sub>254</sub> irradiation.

<sup>3</sup> This R factor plasmid carries the *mucA* and *mucB* genes to compensate for the weak mutagenic activities of the *umu* operon in *Salmonella*.

### 5. Ames II Mutagenicity Assay Test Kits

### a. 1 Sample Kit

This Manual System Kit or starter kit, contains all the consumable components ready-to-use and step-by-step "Instructions for Use". It allows to test manually 1 sample in triplicate, 6 concentrations, positive and negative controls, with and without metabolic activation or 3 samples without replicate, 6 concentrations, positive and negative controls, in the absence and presence of metabolic activation.

#### b. 10 Samples Kit

This kit is used for testing at least 10 compounds in triplicate, 6 concentrations, positive and negative controls, in the absence and presence of metabolic activation.

#### c. Design your own kit

Any components and media necessary for the assay are available separately in any number.

### 6. Automation of the Ames II Mutagenicity Assay

The Ames II Mutagenicity Assay has been validated to combine the reliable experience of the standard Ames test with the high-throughput potential of the Ames II Mutagenicity Assay. A pipetting robot system with a needle and a disposable tip arm was used. It was concluded that the Ames II Mutagenicity Assay is a practicable test system for the purposes of lead optimization (2) (3).

### 7. Validation Studies

### a. Internal Validation Study of the Ames II Mutagenicity Assay

An Internal Validation Study has been performed with the goal to evaluate the ability of the Ames II Mutagenicity Assay and its TA700X series of *Salmonella his*<sup>-</sup> mutant tester strains to detect mutagens as classified by the National Toxicology Program (NTP) by the traditional tester strains (TA100, TA98, TA1535, TA1537, TA97, TA102).

Experts at the National Institute of Environmental Health Science (NIEHS) chose thirty coded chemicals which would serve as test articles in this study. At least 5 doses of each chemical were tested in triplicate, and repeat experiments were performed at least one week following the initial trial. The assay has been performed manually as well as in a high throughput system, using the a pipetting station. The high concordance with the standard plate incorporation test, and the reproducibility among the cultures and replicates demonstrated that the Ames II tester strains is an effective screen for identifying Salmonella mutagens (4).

One important component of this validation study was the proper recognition and adjustment for the various sources of statistical variability of the Ames II Mutagenicity Assay data. The statistical modeling of data obtained in the previous study are described in this report (5).

Further studies confirmed the conclusion, that the Ames II Mutagenicity Assay is a very effective alternative screening method to the standard plate incorporation test (6) (7).

## b. Multi Center Study

The study had two goals, to corroborate the use of the Ames II Mutagenicity Assay as a suitable alternative screening assay to the traditional Ames plate incorporation method, and to test the Ames II Mutagenicity Assay system for its reproducibility among different laboratories. The following companies participated in this study: Aventis Pharma Deutschland GmbH, BASF AG, Boehringer Ingelheim, Johnson & Johnson Pharmaceutical Research & Development, Novartis Consumer Health, Schering AG, Servier Group, Federal Environmental Agency and Xenometrix.

Nineteen coded chemicals were tested in these nine independent laboratories, for their mutagenic activity using the Ames II Mutagenicity Assay. The test compounds were selected from a published study with a large data set from the standard Ames plate incorporation test. The results of both assay systems were compared. The Ames II test results were all well reproducible among the different laboratories with a consistency of 89,5% and the sensitivity of both tests assays were comparable.

The conclusion is, that the Ames II Mutagenicity Assay is an effective screening alternative to the standard Ames test, requiring less material and labor (8).

### c. Comparison with DEREK

In a study performed by Aventis Pharma Germany, the results of the Ames II assay and the standard Ames test were compared with results predicted by DEREK. It could be shown, that the major number of compounds with DEREK alerts was identified with the Ames II assay (9).

### 8. Advantages of the Ames II Mutagenicity Assay

- Substantially lower amount of test chemicals than the traditional Ames test
- Quality controlled *S. typhimurium* strains no genotype analysis necessary
- Ready to use reagents, no media preparation, no autoclaving and sterility testing necessary
- Significantly reduced spontaneous reversion rates
- Easy handling, less hands-on-time than with the traditional Ames test
- Microplate format
- Automatable for high throughput screening
- Six-fold less plasticware, reduced disposal costs

#### 9. Xenometrix Services

- 1) Training Program Training how to perform the Ames II Mutagenicity Assay in our/your facility, support of the evaluation setup in your own laboratory.
- 2) Client Research Laboratory

Xenometrix has, at its Allschwil facility, a fully staffed and equipped laboratory for the purpose of performing an optimal Ames II Mutagenicity 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 II Mutagenicity Assay, making for a very efficient and cost-effective process. Depending upon answers to a clients questionnaire, detailed reports are likely to be completed within 7 days. Data can also be communicated immediately upon assay completion (3 days).

#### 10. Literature

- (1) P Gee, DM Maron, and BN Ames; Detection and classification of mutagens: A set of base-specific Salmonella tester strains; Proc Nat Acad Sci USA (1994) 91, 11606-11610.
- (2) 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).
- (3) M Crook; Automation of the Ames II toxicology test; MipTec-ICAR (2000) P07.
- (4) P Gee, CH Sommers, AS Melick, XM Gidrol, MD Todd, RB Burris, ME Nelson, RC Klemm, E Zeiger; Comparison of responses of base-specific Salmonella tester strains with the traditional strains for identifying mutagens: The results of a validation study; Mutation Res (1998) 412, 115-130.
- (5) WW Piegorsch, SJ Simmons, BH Margolin, E Zeiger, XM Gidrol, P Gee; Statistical modeling and analyses of a base-specific Salmonella mutagenicity assay; Mutation Res (2000) 467, 11-19.
- (6) G Engelhardt, E Jacob, R Jäckh; Ames II assay: results of a validation study; Naunyn-Schmiedeberg's Arch Pharmacol (1999) 359, 179.
- (7) V Gervais, D Bijot, N Claude; Assessment of a screening experience with the Ames II test and future prospects; European Environmental Mutagen Society Annual Meeting: From Hazard to risk, Aberdeen Scotland (UK) (2003) P120.
- (8) 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.
- (9) K. Braun, H.-P. Spirkl, A. Czich, I. Stammberger, A. Amberg, Comparison of DEREK, Ames and Ames II, Gesellschaft f
  ür Umwelt-Mutationsforschung e.V., 21. GUM-Tagung, W
  ürzburg (D), 2004.

Product	Art. No.	Content
AMES II MUTAGENICITY ASSAY SYSTEMS		
Ames II TA225 Manual System Kit (Liquid/Frozen)	AE01-213	1 sample kit
Ames II TA225 - 1 Sample Kit-Incl. Pos. Controls (Liquid/Frozen)	AE01-213-S1-P	1 sample kit
Ames II TA225 - 1 Sample Kit - Pos.Contr. S2 (Liquid/Frozen)	AE01-213-S2-P	1 sample kit
Ames II TA220 Automated System for High Throughput (Liquid/Frozen)	AE10-213	10 samples kit
Ames II TA220 - 10 Sample Kit-Incl. Pos. Controls S1 (Liquid/Frozen)	AE10-213-S1-P	10 samples kit
Ames II TA220 - 10 Sample Kit - Pos.Contr. S2 (Liquid/Frozen)	AE10-213-S2-P	10 samples kit
AMES II AQUA TESTING		
Ames II Aqua - 1 Sample Kit - 48 Measuring Points/Strain	AL01-213	1 sample kit
Ames II Aqua - 1 Sample Kit - 48 Measuring Points/Strain-Rat Liver S9-Pos. Controls	AL01-213-S1-P	1 sample kit
Ames II Aqua - 1 Sample Kit - 48 Measuring Points - Lyophilized Rat Liver S9 - Pos. Controls S2	AL01-213-S2-P	1 sample kit
Ames II Aqua - 5 Sample Kit - 240 Measuring Points/Strain	AL05-213	5 samples kit
Ames II Aqua - 5 Sample Kit - 240 Measuring Points/Strain-Rat Liver S9-Pos. Controls	AL05-213-S1-P	5 samples kit
Ames II Aqua - 5 Sample Kit - 240 Measuring Points - Lyophilized Rat Liver S9 - Pos. Controls S2	AL05-213-S2-P	5 samples kit
AMES II MEDIA		
Ames II Salmonella Exposure Medium (RT) (Liquid/Frozen) (100 ml)	APMA-EM41	100 ml
Ames II Salmonella Exposure Medium (RT) (Liquid/Frozen) (50 ml)	APMA-EM42	50 ml
Ames MPF, Ames II & E.coli 50 ml Growth Medium (RT) (Liquid/Frozen)	APMM-GM00	50 ml
Ames II Salmonella Reversion Indicator Medium (RT) (Liquid/Frozen) (550 ml)	APMA-IM51	550 ml
Ames II Salmonella Reversion Indicator Medium (RT) (Liquid/Frozen) (100 ml)	APMA-IM52	100 ml
Ames II Strains		
Ames Ampicillin	APAM-0001	1 vial
Ames TA98 S. typhimurium Strain (Liquid/Frozen)	APLI-0110	50 μl
Ames TAMix S. typhimurium Strain (Liquid/Frozen)	APLI-0114	50 µl

## Additional Xenometrix Products Cytotoxicity Test Kits

PAC Acid Phosphatase (300)

#### **Product Size** Art. No. Product **One Parameter** CVDE Crystal Violet Dye Elution (1200) 1200 tests without microplates AKCV96-1200 CVDE Crystal Violet Dye Elution (1210) 1200 tests with 16 microplates. 40 reservoirs AKCV96-1210 CVDE Crystal Violet Dye Elution (300) 300 tests without microplates AKCV96-300 CVDE Crystal Violet Dye Elution (310) 300 tests with 4 microplates, 10 reservoirs AKCV96-310 CVDE Crystal Violet Dye Elution (9600) 9600 tests without microplates (2.5 g) AKCV96-9600 GLU Glucose (1200) 1200 tests without microplates AKGLU96-1200 GLU Glucose (1210) 1200 tests with 32 microplates, 24 reservoirs AKGLU96-1210 GLU Glucose (400) 400 tests without microplates AKGLU96-400 GLU Glucose (410) 400 tests with 10 microplates, 9 reservoirs AKGLU96-410 LDHe Extracellular Lactate Dehydrogenase (310) 300 tests with 8 microplates, 4 reservoirs AKLE96-310 LDHe Extracellular Lactate Dehydrogenase (1210) 1200 tests with 32 microplates, 16 reservoirs AKLE96-1210 LDHe Extracellular Lactate Dehydrogenase (1200) 1200 tests without microplates AKLE96-1200 LDHe Extracellular Lactate Dehydrogenase (2400) 2400 tests without microplates AKLE96-2400 LDHe Extracellular Lactate Dehvdrogenase (300) 300 tests without microplates AKLE96-300 MTT Diphenyltetrazolium Bromide (1200) 1200 tests without microplates AKMT96-1200 MTT Diphenyltetrazolium Bromide (1210) 1200 tests with 16 microplates, 40 reservoirs AKMT96-1210 MTT Diphenyltetrazolium Bromide (300) 300 tests without microplates AKMT96-300 MTT Diphenyltetrazolium Bromide (310) 300 tests with 4 microplates, 10 reservoirs AKMT96-310 MTT Diphenyltetrazolium Bromide (9600) 9600 tests without microplates (500 mg) AKMT96-9600 NR Neutral Red (1200) 1200 tests without microplates AKRN96-1200 NR Neutral Red (1210) AKRN96-1210 1200 tests with 16 microplates, 40 reservoirs NR Neutral Red (2400) 2400 tests without microplates AKRN96-2400 NR Neutral Red (300) 300 tests without microplates AKRN96-300 NR Neutral Red (310) 300 tests with 4 microplates, 10 reservoirs AKRN96-310 NR Neutral Red (9600) 9600 tests without microplates (400 ml, 1:300 solution) AKRN96-9600 PAC Acid Phosphatase (1200) 1200 tests without microplates AKPA96-1200 PAC Acid Phosphatase (1210) 1200 tests with 16 microplates, 32 reservoirs AKPA96-1210

300 tests without microplates

AKPA96-300

Product	Product Size	Art. No.
DAC Asid Dharshataas (210)		
PAC Acid Phosphatase (310)	300 tests with 4 microplates, 8 reservoirs	AKPA96-310
SRB Sulfornodamine B (1200)	1200 tests with 16 microplates	AKSR96-1200
SRB Sulfornodamine 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 - CVDF (1210)	2 x 1200 tests with 16 microplates, 88 reservoirs	AKSRCV96-1210
SRB - CVDE (300)	2 x 300 tests without microplates	AKSRCV96-300
SRB - CVDF (310)	2 x 300 tests with 4 microplates 22 reservoirs	AKSRCV96-310
XTT - CVDF (300)	2 x 300 tests without microplates	AKXCV96-300
XTT - CVDF (1200)	2 x 1200 tests without microplates	AKXCV96-1200

Product	Product Size	Art. No.
XTT CV/DE (1210)	2 x 1200 tasts with 16 microplatos 64 reservoirs	AKYCV06 1210
XTT - CVDE (1210) XTT - CVDE (310)	2 x 1200 tests with 10 microplates, 04 reservoirs	AKXCV90-1210
XTT = PAC (1200)	2 x 1200 tests without microplates	
XTT = PAC (1200)	2 x 1200 tests with 16 microplates 56 reservoirs	AKXI AC90 1200
XTT = PAC (300)	2 x 300 tests without microplates	AKXPAC96-300
XTT - PAC (300)	2 x 300 tests with 4 microplates 14 reservoirs	
XTT - SRB (1200)	2 x 1200 tests without microplates	AKXSR96-1200
XTT - SRB (1200)	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 with a 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

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Product Size	Art. No.
3x300 tests with 4 microplates, 24 reservoirs	AKXTRCV96-310
3 x 1200 tests without microplates	AKXTRS96-1200
3 x 1200 tests with 32 microplates, 112 reservoirs	AKXTRS96-1210
3 x 300 tests without microplates	AKXTRS96-300
3 x 300 tests with 8 microplates, 28 reservoirs	AKXTRS96-310
3 x 1200 tests without microplates	AKXTSCV96-1200
3 x 1200 tests with 32 microplates, 112 reservoirs	AKXTSCV96-1210
3 x 300 tests without microplates	AKXTSCV96-300
3 x 300 tests with 8 microplates, 28 reservoirs	AKXTSCV96-310
4 x 1200 tests without microplates	AKLGXP96-1200
4 x 1200 tests with 48 microplates, 80 reservoirs	AKLGXP96-1210
4 x 300 tests without microplates	AKLGXP96-300
4 x 300 tests with 12 microplates, 20 reservoirs	AKLGXP96-310
4 x 1200 tests without microplates	AKLGXS96-1200
4 x 1200 tests with 48 microplates, 104 reservoirs	AKLGXS96-1210
4 x 300 tests without microplates	AKLGXS96-300
4 x 300 tests with 12 microplates, 26 reservoirs	AKLGXS96-310
4 x 1200 tests without microplates	APANI96-1200
4 x 1200 tests with 48 microplates, 120 reservoirs	APANI96-1210
4 x 300 tests without microplates	APANI96-300
4 x 300 tests with 12 microplates, 30 reservoirs	APANI96-310
	<ul> <li>Product Size</li> <li>3x300 tests with 4 microplates, 24 reservoirs</li> <li>3 x 1200 tests with 01 microplates</li> <li>3 x 1200 tests with 32 microplates, 112 reservoirs</li> <li>3 x 300 tests with 2 microplates, 28 reservoirs</li> <li>3 x 1200 tests with 2 microplates, 112 reservoirs</li> <li>3 x 1200 tests with 01 microplates</li> <li>3 x 1200 tests with 02 microplates, 112 reservoirs</li> <li>3 x 300 tests with 01 microplates</li> <li>3 x 300 tests with 01 microplates</li> <li>3 x 300 tests with 01 microplates, 28 reservoirs</li> <li>3 x 300 tests with 8 microplates, 28 reservoirs</li> <li>3 x 300 tests with 8 microplates, 28 reservoirs</li> <li>4 x 1200 tests with 9 microplates, 20 reservoirs</li> <li>4 x 300 tests with 12 microplates, 20 reservoirs</li> <li>4 x 1200 tests with 48 microplates, 104 reservoirs</li> <li>4 x 300 tests with 12 microplates, 20 reservoirs</li> <li>4 x 1200 tests with 12 microplates, 104 reservoirs</li> <li>4 x 300 tests with 12 microplates, 20 reservoirs</li> <li>4 x 1200 tests with 48 microplates, 104 reservoirs</li> <li>4 x 300 tests with 12 microplates, 20 reservoirs</li> <li>4 x 300 tests with 12 microplates, 20 reservoirs</li> <li>4 x 300 tests with 12 microplates, 104 reservoirs</li> <li>4 x 300 tests with 12 microplates, 20 reservoirs</li> <li>4 x 1200 tests with 48 microplates, 104 reservoirs</li> <li>4 x 300 tests with 12 microplates, 104 reservoirs</li> <li>4 x 300 tests with 12 microplates, 104 reservoirs</li> <li>4 x 300 tests with 12 microplates, 104 reservoirs</li> <li>4 x 300 tests with 12 microplates, 120 reservoirs</li> <li>4 x 300 tests with 12 microplates, 120 reservoirs</li> <li>4 x 300 tests with 12 microplates, 30 reservoirs</li> <li>4 x 300 tests with 12 microplates, 30 reservoirs</li> </ul>

# **Ames II Assay**



Form AA08 01-2012



# What are the Advantages

- 30 mg of test compound requested only
- Quality controlled S. typhimurium strains no genotype analysis necessary
- Ready to use reagents
- Less hands-on time
- Microplate format
- Automatable for high throughput screening
- Six times less plastic ware, reduced disposal costs

## Detection and classification of mutagens: A set of base-specific Salmonella tester strains

(histidine operon mutations/carcinogens)

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Contributed by Bruce N. Ames, August 19, 1994

A detection and classification system for mu-ABSTRACT tagens has been developed that identifies the six possible base-pair substitution mutations. A set of six Salmonella typhimurium (TA7001 to TA7006) strains has been constructed, each of which carries a unique missense mutation in the histidine blosynthetic operon. In addition to the his mutation, these strains carry different auxiliary features that enhance the mutability of the target his mutation. These include the R factor pKM101, which has the SOS-inducible mucAB system; a deletion of the uvrB component of excision repair; and rfa mutations to increase the accessibility of bulky chemicals to the bacteria. Another set of strains (TA7041 to TA7046) contain a wild-type rfa gene. Reversion via the base substitution unique to each strain was verified by sequence analyses of >800 revertants obtained from different types of mutagens. The strains have considerably lower spontaneous reversion frequencies and detect a variety of mutagens at a sensitivity comparable to the Salmonella tester strains TA100, TA102, and TA104. The low spontaneous frequency of reversion of a mixture of the six tester strains (~10 revertants per plate) enables a single mutation assay with the mixture that is followed by classification of the type of mutation with the individual strains.

The detection of mutagens and determination of the types of mutation induced are of importance to the understanding of the etiology of cancer and other degenerative diseases that involve mutations. Point mutations in human oncogenes or tumor suppressor genes (e.g., p53) may lead to cancer, and the pattern of missense mutations can give clues as to the mutational events involved (1). Thus a simple test that detects mutagens and determines the pattern of the six possible base substitutions induced by each would be useful for providing information on mutagenic mechanisms.

The previous Salmonella mutagenicity test (2) has been used extensively over the past two decades to measure the mutagenic potential of many compounds. These strains have point mutations in the histidine biosynthetic operon that render them unable to grow in the absence of histidine; however, they are not diagnostic for the type of base-pair substitution caused by the mutagen.

Each of the six strains described here, either with (TA7001-TA7006) or without (TA7041-TA7046) the *rfa* mutation, reverts by only one specific base-pair substitution out of the six possible changes. Reversion of the target mutation in a gene for histidine biosynthesis restores the mutant *his* gene to the wild type so that the cell can grow and form a colony without histidine. The number of colonies formed is a direct measure of the mutagenic potential of the test compound. The spontaneous reversion rates of the strains described here are considerably lower than that of the

previous Salmonella tester strains (2), and their sensitivity to reversion by mutagens is comparable. In addition, the strains described here have added the ability to determine the spectrum of base substitutions.

Two other systems that detect all six possible base substitutions without further genetic or biochemical analysis have been reported. The Saccharomyces cerevisiae system (3) is based on an essential Cys-22 residue in iso-1cytochrome c encoded by the CYC1 gene. The Escherichia coli system (4) has a mutational target on a plasmid in an active site glutamate residue in the  $\beta$ -galactosidase gene. Since the point mutation is extrachromosomal, it can be transferred into various backgrounds such as those differing in mismatch repair.

#### MATERIALS AND METHODS

Target Mutation (A·T  $\rightarrow$  G-C) in Set 1. The mutation hisG1775 (5) was recombined with a bacteriophage clone, M13mp9::his4 (6), deleted for part of the hisG gene (covering the hisG1775 mutation) and the hisD gene. Recombinant M13 phage were selected with an active hisD gene product that allowed growth on histidinol and were plaque-purified to prepare single-stranded DNA templates for dideoxynucleotide sequencing. The hisG1775 mutation was identified as a G-C  $\rightarrow$  A·T transition in which the wild-type Gly-153 (GGT) was replaced by the mutant Asp-153 (GAT). No other mutations were found in hisG. The hisG1775 mutation is the basis of the TA7041 and TA7001 strains in set 1 (see Table 1).

In Vitro Mutagenesis. Target mutations for sets 2-5 were synthesized in DNA oligomers. These oligomers were used as primers to extend single-stranded M13 DNA templates (7) or were used in symmetrical polymerase chain reactions (PCRs) to fix the mutations in double-stranded DNA fragments (8), which were subcloned into appropriate M13 clones. These M13 mutant clones were used to transform competent DH5 $\alpha$ F'IQ cells (GIBCO/BRL) (9) and mutant plaques were screened by dideoxynucleotide sequencing using deoxyadenosine 5'-[ $\alpha$ -[<sup>35</sup>S]thio]triphosphate. Target Mutation (T·A  $\rightarrow$  A·T) for Set 2. The codon for

Target Mutation (T·A  $\rightarrow$  A·T) for Set 2. The codon for Lys-217 (AAA) of *hisC* was changed to Ile-217 (ATA) by using PCR to generate a DNA fragment that was subcloned into M13mp8::hisDC1. The *ile* mutation was transferred to the chromosome in TA4302 ( $\Delta his129/F42finP301$  lac<sup>+</sup>) by M13 transduction using histidinol selection (10). Transductants carrying the designated mutant allele, *hisC9138*, formed the basis of the TA7042 and TA7002 strains in set 2 (see Table 1).

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Abbreviations: STN, streptonigrin; NQNO, 4-nitroquinoline-1oxide; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; MMS, methyl methanesulfonate; N4AC, N<sup>4</sup>-aminocytidine; 5azaC, 5-azacytidine; ANG, angelicin.

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Target Mutations for Sets 3 (T·A  $\rightarrow$  G·C), 4 (G·C  $\rightarrow$  A·T), and 5 (C·G  $\rightarrow$  A·T). For set 3, the site of the mutation identified for hisG1775, Gly-153 (GGT wild type), was changed to Val-153 (GTT) by in vitro site-directed mutagenesis (7). The target mutations for sets 4 and 5 were designed to substitute Gly-169 (GGG) and Ala-169 (GCG), respectively, for the wild-type Asp-169 (GAT). Both mutants revert to Glu-169 (GAG) because the wobble base was changed from thymidine to guanosine to obtain the specificity of reversion. There was no difference in growth rates of Glu-169 and Asp-169 strains. PCR was used to fix the Gly-169 and Ala-169 mutations into double-stranded DNA fragments, which were subcloned into M13mp9::his1 (6). Each of these mutations in hisG was transferred to AZ1549 by M13 transduction (10). The Val-153 (GTT), Gly-169 (GGG), and Ala-169 (GCG) mutations were designated hisG9074 (TA7043 and TA7003 of set 3), hisG9133 (TA7044 and TA7004 of set 4), and hisG9130 (TA7045 and TA7005 of set 5), respectively (see Table 1).

Target Mutation (C·G  $\rightarrow$  G·C) in Set 6. The mutation in *hisC*9070 (11) was found by direct PCR sequence analyses to be a G-C to C·G transversion in which the wild-type Gly-163 (GGA) was replaced by Arg-163 (CGA) in the *hisC* gene. No other mutations were found in *hisC* and this mutation became the basis for the TA7046 and TA7006 strains in set 6 (see Table 1).

Intermediate Bacterial Strains. To obtain a common genetic background for the tester strains, P22int4HT (12, 13) carrying each target mutation was used to transduce SB8052 to growth on histidinol. The hisD3052 mutation of SB8052 was made in a stable deletion mutant, ara-9 (14), which is present in the current Salmonella tester strains and the tester strains described here designated TA70xx. The R factor donor strain, TA4593 (argB69/pKM101), was constructed by transferring pKM101 (15) from TA94 (16) to SA4757 (argB69) by conjugation. TA4490 [hisD3052 Dara9 Dchl1004 (bio ch1D uvrB chlA)galE503/(pKM101)] was made by mating TA2684 (11) with TA4593 and was used as the recipient for strains carrying TA704x backgrounds. These strains carry the R factor and are deficient in excision repair. In addition, the complete tester strains TA700x carry rfa mutations (rfa1041-1046) that were isolated by resistance to lysis by the bacteriophage C21 (17). Strains that carry the his mutation in SB8052 background (TA701x), pKM101 (TA702x), and the uvrB deletion (TA703x) will be discussed elsewhere.

Mutagenicity Testing. The protocols detailed in Maron and Ames (2) were used, except that the glucose in the agar plates was reduced from 2.0% (wt/vol) to 0.4% to optimize cell growth. High concentrations of glucose have been shown to reduce the reversion response for some mutagens (18). Enhanced sensitivity of the strains to some mutagens was obtained by a preincubation of bacteria in liquid medium containing the mutagen at 37°C before top agar was poured on the plates (2).

The concentrations of mutagens used for testing were chosen from doses previously reported for *Salmonella* tester strains (2, 11, 16, 19) to compare the sensitivity of the base-specific strains described here directly to that of TA100, TA102, and TA104.

Mutagenic Agents. Streptonigrin (STN, Flow Laboratories), 4-nitroquinoline-1-oxide (NQNO, Sigma), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG, Aldrich), and methyl methanesulfonate (MMS, Aldrich) were dissolved in dimethyl sulfoxide (Sigma). N<sup>4</sup>-Aminocytidine (N4AC, Sigma) and 5-azacytidine (5azaC, Sigma) were dissolved in H<sub>2</sub>O. Angelicin (ANG, HRI Associates, Concord, CA) was dissolved in 95% ethanol.

Before testing ANG with UVA (320-400 nm) irradiation, the cells were removed from Oxoid broth. A 12-hr overnight culture (5.5 ml) was centrifuged at  $1080 \times g$  for 10 min. The cell pellet was washed and resuspended in  $1 \times VBC$  medium

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(2) to the same density as the original overnight culture as measured by absorbance at 650 nm. ANG (7.5  $\mu$ l of 200  $\mu$ g/ml) plus 1.5 ml of cell suspension was shaken at 37°C for 30 min, transferred to a Petri dish (60 × 15 mm), and rotated gently during the 7.5-min irradiation, 10 cm from a near-UV source (5.9 ± 0.1 J) (Gates-Raymaster from Thomas). A 100- $\mu$ l aliquot of this mixture was plated according to the standard plate incorporation assay (2). Control plates contained cells exposed to UVA only or to ANG in the absence of UVA activation.

Reversion Analyses. Results from the reference mutagens are summarized in Table 1. Data are reported as revertants per plate—i.e., the difference between the number of revertants on plates with and without the mutagen (20). The mutagenic potency of a compound is also expressed as a fold mutagenicity (the ratio of induced reversion frequency to spontaneous frequency).

Sequence Analyses of Revertants. At least 10 revertants induced by the reference compounds listed in Table 1 were sequenced for each strain. In addition, >100 revertant colonies induced (48 hr at 37°C) by each of 16 mutagens (data not shown) other than those used as reference testing agents were sequenced from each set of strains. These mutagens included 2-aminopurine, bromodeoxyuridine, *t*-butyl hydroperoxide, chlorambucil, cumene hydroperoxide, 2,3-epoxy-1-propanol, ethylmethanesulfonate, formaldehyde,  $N^4$ -hydroxycytidine, hydrogen peroxide, ICR-191, methyl glyoxal, mitomycin C, 4-nitrobiphenyl, 2-nitrofluorene, and styrene oxide. Another 10-20 revertants from each strain that arose after 72 and 96 hr were also analyzed.

One or two colonies were dispersed in 30  $\mu$ l of deionized H<sub>2</sub>O. A 2- $\mu$ l aliquot was used as template in a total reaction volume of 50  $\mu$ l for asymmetrical amplification by PCR (8) using primer ratios of 100:1 to generate single-stranded DNA templates for sequencing. Excess primers and PCR buffer salts were removed by Sephadex spin columns (G-50 fine, Boehringer Mannheim). The sequencing primer was 5'-end-labeled with adenosine 5'-[ $\gamma$ -<sup>32</sup>P]triphosphate by using T4 polynucleotide kinase (United States Biochemical). This primer was used to sequence 7  $\mu$  of the PCR product by Sequenase version 2.0 using dITP mixes (United States Biochemical).

#### RESULTS

Specificity of Reversion in Differing Genetic Backgrounds. Strains of each set reverted only by the base change indicated in Table 1 when induced by >20 mutagenic agents. Components such as the R factor pKM101 did not change the specificity of reversion of any of the target mutations in spite of its error-prone repair characteristics nor did the uvrB or rfa genes. The uvrB mutation causes a deficiency in excision repair of bulky lesions as measured by lack of survival after UV irradiation at 254 nm. The R factor plasmid pKM101 carries the mucAB genes, which compensate for the weak SOS-mediated mutagenic activities of the two umu-like operons in Salmonella (21). Several mutations affect the lipopolysaccharide component of the cell envelope. All strains carry the galE503 mutation (22) and cannot synthesize galactose residues for the outer core of the lipopolysaccharide component. The primary tester strains (TA7001-TA7006) carry rfa mutations and have alterations in the core structure of the lipopolysaccharide component that increase cell permeability.

Specificity of Reversion with Various Mutagens. The sequence of revertants obtained from mutagenesis by >20mutagens was found to change to the wild-type base (Table 1). Missense suppression may result in revertants that grow more slowly and have mutations at an alternate site. The incubation period was extended from 48 hr to 72 hr and 96 hr

			Revertants, r	no. per plate	Mutagenicity
Base change	Strain Mutagen	Mutagen	Spontaneous	Induced	ratio
Set 1: A·T $\rightarrow$ G·C	TA7001	STN	2.5 ± 1.5	103 ± 19	41 ± 8
	TA7001	N4AC	$2.5 \pm 1.5$	990 ± 54	396 ± 22
	TA7041	N4AC	$2.6 \pm 1.6$	2120 ± 35	815 ± 13
Set 2: T·A $\rightarrow$ A·T	TA7002	STN	$9.3 \pm 3.0$	$180 \pm 7$	$19 \pm 0.7$
	TA7002	MMS	$9.3 \pm 3.0$	110 ± 22	$12 \pm 2.4$
	TA7042	MMS	$6.8 \pm 2.9$	$110 \pm 23$	$16 \pm 3.3$
Set 3: T·A $\rightarrow$ G·C	TA7003	STN	$0.1 \pm 0.05$	63 ± 7	630 ± 70
	TA7003	ANG/UVA	$0.1 \pm 0.05$	195 ± 12	$1950 \pm 120$
	TA7043	ANG/UVA	$0.7 \pm 0.3$	$153 \pm 12$	$214 \pm 14$
Set 4: G-C $\rightarrow$ A·T	TA7004	NQNO	17.7 ± 3.5	617 ± 97	35 ± 5.5
	TA7004	MNNG	$17.7 \pm 3.5$	$1767 \pm 173$	$100 \pm 9.8$
	TA7044	MNNG	$20.0 \pm 4.4$	4904 ± 770	$245 \pm 38$
Set 5: C·G $\rightarrow$ A·T	TA7005	NQNO	$22.7 \pm 2.3$	4690 ± 126	$207 \pm 5.6$
	TA7045	NQNO	$20.0 \pm 4.6$	5888 ± 420	$294 \pm 21$
	TA7005	MMS	$22.7 \pm 2.3$	$286 \pm 31$	$13 \pm 1.5$
Set 6: C·G $\rightarrow$ G·C	TA7006	NQNO	$4.2 \pm 2.0$	823 ± 35	196 ± 8.3
	TA7006	5azaC	$4.2 \pm 2.0$	$253 \pm 30$	$60 \pm 7.1$
	TA7046	SazaC	5.3 ± 2.3	$270 \pm 14$	51 ± 2.6

Mutagens: STN, 0.1  $\mu$ g per plate; N4AC, 10  $\mu$ g per plate; MMS, 650  $\mu$ g per plate; ANG, 0.1  $\mu$ g per plate (activated by UVA); NQNO, 1.0  $\mu$ g per plate; MNNG, 4.0  $\mu$ g per plate; 5azaC, 25  $\mu$ g per plate. The spontaneous revertants have not been subtracted from the induced numbers. The number of cells plated in 0.1-ml aliquots of an overnight culture was 2 × 10<sup>8</sup> cells. The histidine in the initial plating gives 1–20 cell doublings for 6 × 10<sup>8</sup> cells per plate in each experiment. Experiments (37°C for 48 hr) were done at least in triplicate for each condition. Data are the mean ± SEM.

to look for possible slow-growing revertant colonies due to missense suppression though <1% additional colonies were found. Of the 20 revertant colonies of TA7045 obtained after 72 hr that were sequenced, one revertant (from 5azaC) retained the original mutant sequence, presumably due to missense suppression. This revertant colony was not visible at 48 h and, therefore, would not contribute to the base substitution analyses.

Table 1. Reversion of strains

**Reference Mutagens.** To verify the mutability of complete tester strains from all six sets, only two mutagens, STN and NQNO, are necessary since they included reversion frequencies of at least 15-fold over spontaneous frequencies (Table 1). STN induced reversions at T·A or A·T base pairs, which are target mutations in sets 1–3 (e.g., TA7001, TA7002, and TA7003, respectively) but not at G·C or C·G base pairs (Fig. 1). On the other hand, the strains of sets 4–6 (e.g., TA7004, TA7005, and TA7006, respectively) have G·C or C·G base pairs as their target mutations and respond to NQNO at least 30-fold over spontaneous (Fig. 2). Similarly, reversion of T·A or A·T targets by NQNO was not detected.

A second positive control mutagen can establish the target mutation unambiguously. Set 1 strains are reverted strongly by N4AC (Fig. 3). While set 4 strains were reverted weakly by N4AC, these strains were not reverted to any significant extent by STN (Fig. 1). Set 2 strains can be distinguished from those of set 3 although both sets were induced by MMS and ANG/UVA, because TA7002 and TA7042 are reverted



more strongly by MMS ( $\approx$ 100 revertants per plate) than TA7003 and TA7043 ( $\approx$ 10 revertants per plate) (Fig. 4). Set 3 strains responded to ANG/UVA at 150- to 200-fold over spontaneous frequencies, while TA7002 and TA7042 were at  $\approx$ 43- and 36-fold, respectively (Fig. 5).

Of the strains that respond to NQNO, set 4 strains are reverted weakly by N4AC and strongly by MNNG ( $\approx$ 100fold), whereas strains of other sets showed <10-fold by MNNG (Fig. 6). Set 5 strains were reverted by MMS at 13-fold over spontaneous compared to <3-fold for sets 4 and 6 (Fig. 4). 5azaC reverted only TA7006 and TA7046 of set 6 (Fig. 7). Eventually, a set of six mutagens, one specific for each base-pair substitution would be desirable.

Suggested Testing Strategies. To enhance detection of reversion of the strains at lower concentrations of compounds, a period of preincubation of 10–30 min in liquid medium was used. This may become a preferred procedure in the initial screening of compounds as a marked increase in sensitivity was observed for a number of reactive chemicals. For example, formaldehyde induced TA7005 ~20-fold over spontaneous reversion frequencies when this strain was preincubated with 10  $\mu$ g per plate for 20 min at 37°C. Without preincubation, 50  $\mu$ g per plate reverted TA7005 5-fold and 100  $\mu$ g per plate was needed to revert TA102 2.5-fold (19).

For the initial screening of compounds, the six strains of similar genetic background may be mixed and the mixture may be used in testing. This is possible due to the minimal complementation and low spontaneous reversion frequencies



FIG. 2. NQNO (1 µg)-induced base substitutions.



FIG. 3. N4AC (10 µg)-induced base substitutions.

of the mixture (7-13 revertants per plate). Cells from overnight cultures of TA7001-TA7006 were mixed in equal proportions to approximate the density of an overnight culture of a single strain and used to test MNNG. The number of revertants induced in this mixture was 2062 revertants per plate. Thus the number of revertants obtained by the mixture was similar to the arithmetic average of 1784 revertants per plate when tested by each individual strain (90 + 121 + 63 + 1108 + 8163 + 1156 divided by 6).

#### DISCUSSION

The six strains (Table 1) can not only indicate the mutagenic potential of a compound but also identify the base substitutions induced by a mutagen. Mutagenic potency is determined from the linear part of the dose-response curve and is expressed in two ways. The frequency of reversion per amount of mutagen is calculated by subtracting the spontaneous reversion frequency (20). The extent of the induced response per amount of mutagen also is expressed as a fold increase of the spontaneous level. The spectrum of missense mutations was obtained for seven reference mutagens: STN, NQNO, N4AC, MMS, ANG/UVA, MNNG, and 5azaC (Figs. 1-7).

Though some mutagens induce only one type of base substitution, most induce several types. For example, 5azaC induced only strains of set 6 to revert by a C·G  $\rightarrow$  G·C transversion (Fig. 7). This specificity was in agreement with the results reported in the yeast CYCI system (3), the E. coli lacZ system (4), and a previous Salmonella tester strain, TA4016 (11), although TA7006 is the most sensitive detection system. 5azaC replaces cytidine, preventing its methylation, and is bound to the DNA cytosine methylase irreversibly, thereby crosslinking the enzyme to the DNA strand and interfering in mismatch repair (23).

Another base analog, N4AC, induces  $A \cdot T \rightarrow G \cdot C$  transitions as indicated by reversion of set 1 strains and, to a lesser extent,  $G \cdot C \rightarrow A \cdot T$  transitions, as indicated by set 4 strains (Fig. 3). N4AC is metabolized via cytidine deaminase and/or uridine-cytidine kinase to N<sup>4</sup>-aminodeoxycytidine 5'triphosphate, which is incorporated into DNA (24). N<sup>4</sup>-Aminodeoxycytidine (N4AdC) in the imino form (25) can be mispaired with adenosine (26). If it is not removed, a gua-





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FIG. 5. ANG (0.1 µg) after UVA (0.39 J)-induced base substitutions.

nosine is inserted opposite the amino form of N4AdC in the next round of replication such that the original A·T base pair is replaced by a G·C base pair. This may be a simple explanation for the transitions detected by TA7001 and TA7041 of set 1. DNA polymerases also incorporate the major amino form of N4AdC opposite guanosine (24). Upon replication, an adenosine may be inserted opposite, and the original guanosine is replaced by adenosine. This results in a G·C  $\rightarrow$  A·T transition as detected by set 4 strains (Fig. 3).

Reactive mutagens often have complex patterns of mutagenesis. STN, a bulky quinone preferentially intercalates into the DNA helix at A·T base pairs since it induced reversion in strains of sets 1-3. When it is associated with DNA, STN has the capacity to generate superoxide radicals by redox cycling with molecular oxygen (27). STN also has been found to be a potent inhibitor of topisomerase I (28). There was a substantial increase in the mutagenicity induced by STN in all strains that carry *rfa* mutations, which enhances permeability. STN induces TA102  $\approx$ 6-fold above spontaneous (19) compared to a ratio of  $\approx$ 600-fold for set 3 (Table 1); however, the net number of revertants for TA102 (19) is somewhat greater than for sets 1-3.

NQNO reacts with guanosine at G-C or C-G base pairs (Fig. 3) giving two major DNA adducts at the N2 and C8 positions (29). The relative proportions of these adducts appear to be dependent on the context of the guanosine. The adducts formed by NQNO can induce all three base changes as illustrated by the reversion profiles of strains from sets 4-6 (Fig. 2). The effect of a *rfa* mutation is minimal with NQNO (Fig. 2), which is consistent with its small size. The target guanosine in sets 4 and 5 do not share the same context since set 4 is between guanosines and set 5, by far the main target, is between cytidines on the opposite strand. The specificity for G-C base pairs is similar to that found in the yeast system (3), though the set 5 strains are more sensitive.

ANG and other monofunctional psoralens have been studied because of their antiviral properties and their activity against psoriasis. The major products of the photochemical reactions of ANG with DNA are adducts linked by a cyclobutane ring to the 5-6 position of the pyrimidines. Their preference for thymine over cytosine is reflected by the response at T-A or A-T base pairs as compared to C-G or G-C



FIG. 6. MNNG (4  $\mu$ g)-induced base substitutions.

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base pairs (Fig. 5). The difference in response for sets 2 and 3, or sets 1 and 3, is presumably due to the difference in context of the target thymidine. While adducts are formed at cytidine less efficiently than at thymidine, ANG/UVA clearly induced transversions at C-G base pairs, as indicated by the response of set 5 and 6 (Fig. 5). The previous strains, TA102 and TA100, have a mutagenic ratio of <10-fold compared to ≈200-fold for set 3, though the absolute number of revertants is somewhat less for set 3.

Both MMS and MNNG induce a wide spectrum of mutations by alkylating purines and pyrimidines; however, the major MNNG adduct is O<sup>6</sup>-methylguanine, which mispairs with thymidine instead of cytidine. In the next round of replication, thymidine is correctly paired with adenosine replacing the original guanosine. The resulting  $G \cdot C \rightarrow A \cdot T$ transition can be detected by set 4 strains (Fig. 5). Similarly, the thymine adduct, O4-methylthymine causes misincorporation of guanosine on the opposite strand of DNA, resulting in a A·T -> G-C transition as detected by TA7001 of set 1. The mutagenicity ratios for strains of sets 1 and 4 were ≈10 times higher than for TA100.

MMS induced four out of the six possible base substitution to about the same extent (between 6- to 16-fold over spontaneous frequencies) as indicated in Fig. 4. Since this alkylating agent was much less specific, the absolute ratios of induction for any single type of base substitution were much lower than those found for other mutagens discussed here. The sensitivity is comparable to TA100.

The six sets of strains described here have low spontaneous reversion frequencies and high sensitivity to mutagens. Results obtained can be compared to the very large database on the previous Salmonella tester strains. The assay indicates the mutagenic potential and identifies the types of base substitution induced. Identification does not require further analysis such as DNA sequencing or hybridization, because the specificity of reversion has been verified by sequence analyses of >800 revertants. However, as the results above show, the context to the target base can affect the formation of the premutagenic lesion and also may modulate the mutagenic effectiveness of the lesion. Thus the context in which the target bases reside in these strains may influence the results obtained. With this possible caveat this system offers the potential to compare the patterns of base substitutions induced by particular environmental mutagens with patterns observed in genes such as p53.

These strains may be useful in increasing the understanding of the mechanisms involved in mutagenesis and structureactivity relationships. For a compound like ANG, which forms a stable adduct with pyrimidines in DNA upon irradiation with UVA, those strains that carried pyrimidines as the target base reverted and the reversion pattern correlated with

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the expected chemical yield of each type of premutagenic lesion. Thus, the system has the potential to demonstrate types of base substitutions in response to DNA lesions. Conversely, if a compound was found to revert a subset of the strains, the lesion(s) may be postulated and the significant DNA adduct might be characterized more easily.

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## Automation of the AMES II-Assay: High-Throughput Screening of Mutagenic Substances

### Background

A new version of the AMES Assay has been developed to identify base-repair substitution mutations upon detection of mutagens. Six Salmonella typhimurium strains have been constructed, each of which carries a different missense mutation in the histidine operon that is designed to revert uniquely to one of the six possible base substitutions. Reversion via the base substitution unique to each strain was verified by sequence analyses of more that 800 independent revertants induced by a variety of mutagens. AMES II permits identification of missense mutations caused by mutagens without the need to sequence by spectrophotometric analysis. AMES II strains can be combined and used as a single mixture for rapid screening due to minimal complementation among the 6 strains. Lower spontaneous reversion frequencies allow the detection of mutagens at lower concentrations without loss of sensitivity to a large range of doses. Liquid format in microtiter plates leads to increased sensitivity and easy automation.

The original Ames assay is a well established system in Aventis Pharma for mutagenicity testing during the development of compounds; the Ames II assay has been be validated also in Aventis Pharma to combine the reliable experience of the original test with the high-throughput potential of the Ames II assay. Ames II mutagenicity Assays is available in suspension culture form with combined strains for HTS configuration. This kit is sold by Aniara (AMAX automated system). It can be used to replace or complement classical Ames test approaches. A workstation would provide the throughput needed.

### **Technical Requirements**

- Expertise in AMES test analysis.
- An automated workstation with format versatility to provide 384-well configuration of plate formats.
- Test kits are on sale by Aniara.

## Impact

- The assay detects the pointmutagenic endpoint of a substance which should be considered as a "red flag" for the mutagenic and carcinogenic properties of a compound.
- The objective for these assays is to rank about 100 compounds per week.
- The approach would help in the early selection of compound for progression in the critical path and impact the design of new compound libraries as well.
- The compound consumption for one test is only five milligrams.

### Conclusion

The AMES II-Test makes it possible to make a rapid statement about the mutagenicity of a substance, using a very small amount of substance. Comparability of studies as against the standard Ames-Test lies around 90 %. The AMES II-Test is thus a practicable test system for the purposes of lead optimization.

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## Ames II assay

Automated High-Throughput-Screening for the detection of the mutagenic potential of drugs



## Principles

- detects relevant genetic changes (point mutations) and majority of genotoxic rodent carcinogens
- · Salmonella his' mutant tester strains
- TA 7000 series / base-pair substitution (Mixed Strains)
- TA 98 / frameshift

## Study design

performance and evaluation are in microtiter format

· reverse mutation indicated by purple to yellow color change in



(indicator = Bromocresol purple)

Automated robot system



wells

hotel with 24 MTP's



transfer of bacteria indicator mixture from 24 MTP to 384 MTP



automatic incubator



registration/identification of plates with barcode

Advantages

throughput:
 • reqd. 5 mg compound
 50 -100 compounds/wk

ound • no medium preparation necessary, testkit available from XENOMETRIX<sup>®</sup>

## Conclusion

- accordance between standard Ames and Ames II: approx. 90%
- important tool for drug lead identification

Knut Braun, Aventis Pharma

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## AUTOMATION OF THE AMES II TOXICOLOGY TEST

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The Ames test has been the standard method of determining toxicity of new compounds in the pharmaceutical industry. Developed by Dr Bruce Ames (University of California at Berkeley), the test is widely used. However the basic test cannot be automated. With the increasing numbers of compounds to be screened in the pharmaceutical industry, automation is a required development of this technique. A modified test has been developed by Xenometrix, Inc. (Boulder, Colorado) and this poster describes how this can be automated.

The automated procedure is a four-stage process: Stage 1: CULTURE Stage 2: EXPOSURE Stage 3: PLATING Stage 4: SCORING

To make full use of the investment in an automated system, the system has to function every day. The Ames II test adds a level of complexity, because a sample started on day 1 will be unloaded for the final plate reading on day 3. The control system has to be able to start new sample batches on each day, whilst still keeping track of batches that were started on previous days. This may be automated by using high quality equipment with a flexible control language.

Three Hamilton Microlab S series pipetting stations are used in the system, one to undertake the EXPOSURE plate preparation stage, and two others to undertake the PLATING stage. Two systems are required to keep the throughput through the system to a maximum throughout the 5-day process.

A Hamilton Microlab R3-5 robot is used to transport the plates around the system, a Labsystems Multiskan Ascent is used to scan the plates at the end of the procedure and Kendro incubator (capacity 189 microplates) is used to incubate the 24 well and 384 well microplates. A barcode reader is incorporated to track the microplates throughout the procedure and collated the final results with the starting sample microplate.

The overall control system is OVERLORD, which controls the step-by-step aspect of each stage of the process, as well as keeping track of running batches and collating the data for output at the end of the run. Data can be presented as a paper output, text file, Microsoft EXCEL spreadsheet or Microsoft Access database. An SQL database option will be available later in 2000.

This fully integrated system has been running for 1 year at a major pharmaceutical company in Germany and has allowed fast, accurate and reliable toxicological screening.



Mutation Research 412 (1998) 115-130



## Comparison of responses of base-specific Salmonella tester strains with the traditional strains for identifying mutagens: the results of a validation study

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#### Abstract

The ability of a TA7000 series of Salmonella his<sup>-</sup> mutant tester strains to detect mutagens as classified by the traditional tester strains (TA100, TA98, TA1535, TA1537, TA97, TA102 and TA104) was evaluated using 30 coded chemicals, 5 of which were duplicates with different code numbers. The TA7000 series of tester strains were TA7001, TA7002, TA7003, TA7004, TA7005 and TA7006, each of which reverts by a specific base substitution. In addition, each chemical was tested in a mixture of the base-specific strains (the Mix), plus the traditional strains, TA98 and TA1537. A liquid version of the Salmonella mutagenicity assay was performed in microtiter plates to allow partial automation for increased throughput. The results were compared to those in the National Toxicology Program (NTP) database, which were obtained from the traditional strains in the preincubation assay. In the two strains common to both protocols, TA98 and TA1537, the agreement was 80% and 85%, respectively. When compared to the NTP results for TA100, the Mix gave a 72% concordance, while the addition of the frameshift tester strain, TA98, increased the agreement to 76%. The overall agreement on positive or negative classifications of mutagenicity was 88% for the 25 chemicals tested. There were three notable exceptions to the overall agreement. Benzaldehyde was detected as a mutagen in TA7005 in contrast to its classification as a non-mutagen in the NTP database. This does not necessarily contradict the NTP results because the base-specific strains may respond to different mutagens. Two weak mutagens in the NTP database, 1-chloro-2-propanol and isobutyl nitrite, were not detected as mutagens in the base-specific new strains in the liquid protocol. While there are a number of major differences in the two assays, it was concluded that the results from each procedure are comparable. D 1998 Elsevier Science B.V.

Keywords: Salmonella mutagenicity test: Validation study; Fluctuation test; High throughput screening; Ames II

#### 1. Introduction

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The Salmonella/microsome reversion assay has been used extensively in genetic toxicology testing [1-3]. The tester strains that are used traditionally to

1383-5718/98/\$19.00 © 1998 Elsevier Science B.V. All rights reserved. PH \$1383-5718(97)00172-1 identify mutagens that induce reversion of specific base-pair substitution and small frameshift mutations in the *his* operon. In order to be sensitive to a broad range of chemicals, the *his*<sup>-</sup> strains chosen for routine use carry target sites that revert by numerous pathways of mutagenesis. As a result of this selection of strains, one can conclude that a mutagenic event such as a point mutation at the target site or suppression of point mutations was caused by a chemical [4]. However, the specific base mutation (in the case of base-pair substitutions) can not be discerned without molecular analyses of the reverted and mutant cells [5–8].

To identify base substitutions easily, a set of 6 mutant strains (TA7001, TA7002, TA7003, his TA7004, TA7005, and TA7006) was developed to revert by unique base-pair substitutions [9]. Each strain detects one, and only one, of 6 possible base substitutions and therefore results from testing with this set of strains can be used to determine the mutational spectrum caused by the mutagenic agent. Strains, TA7001, TA7002, and TA7003 detect base changes at A:T base pairs, while TA7004, TA7005, and TA7006, detect base changes at G:C base pairs. Because the reversion pathway for each strain is restricted to a specific base change, the spontaneous reversion frequencies are low (from less than 1 to about 25 revertants per plate).

There are many different procedures for performing bacterial mutagenicity tests. Several protocols deviate from the 'standard' agar plate incorporation or preincubation assay [10]. The use of solid agar plating was retained in an automated spiral application of test agent and bacterial culture to the standard agar plates [11,12] and in an automated liquid preincubation exposure protocol [13] to increase throughput. Attempts have been made to automate parts or all of these procedures for reductions in the time and cost/chemical, and to allow the test to be adapted to high throughput screening. We have modified the fluctuation assay [14-16] to allow automation of plating the exposed cells in selective media using the TA7000 series of tester strains which have been designated 'Ames II™' (Xenometrix, Boulder, CO, USA).

A validation study was performed to compare the performance of the TA7000-series tester strains with respect to the detection of mutagens identified by the traditional tester strains. In this study, 30 coded chemicals (5 of which were duplicates with different code numbers) were tested in the individual strains TA7001-TA7006, to identify mutagens that produced base-pair substitutions, in a mixture of these 6 strains (the Mix), and in strains TA98 and TA1537 to detect frameshift mutagens.

The study reported here addressed several aspects of these new tester strains and their proposed uses.

- The effectiveness of the individual TA7000 strains, or in the Mix, for identifying mutagens, as compared to different combinations of the traditional Salmonella tester strains.
- The usefulness of these base-specific strains, which measure only base-pair substitution mutations, in combination with frameshift-responding strains, to identify mutagens.
- A comparison of the individual strains and the Mix for identifying mutagens.
- The Mix was evaluated for its the degree of sensitivity in detecting mutagens when used to enhance the efficiency of screening.
- Comparisons of the various combinations of these new strains with the standard Salmonella strains currently used by the NTP for identifying mutagens.

The results from this study were compared with the results of testing these same chemicals using a preincubation procedure by the US National Toxicology Program (NTP).

#### 2. Methods and materials

#### 2.1. Bacterial strains

Eight individual Salmonella typhimurium strains and a mixture of the base-specific strains (Mix) were treated with the test chemicals. The strains and their genotypes are described in Table 1. Each of the 6 base-specific strains (TA7001–7006) carry a target missense mutation in the histidine biosynthetic operon that reverts to prototrophy by base-substitution events unique to each strain. Strains TA7001, TA7002, and TA7003 were developed to detect point substitutions at A:T base pairs while TA7004, TA7005, and TA7006 detect base changes at G:C base pairs. Both TA98 and TA1537 were used to

Table 1						
Bacterial	strains	used.	and	the	mixture	

Strains	Genotypes	Mutation detected	Reference
TA7001	hisG1775 Aara9 Ach11004 (bio ch1D uvrB ch1A) galE503 rfa1041 / pKM101	$T:A \rightarrow C:G$	[9]
TA7002	hisC9138 Dara9 Dehl1004 (bio chlD ucrB chlA) galE503 rfa1042/pKM101	$T:A \rightarrow A:T$	[9]
TA7003	hisG9074 Jara9 Achl1004 (bio chlD uvrB chlA) galE503 rfa1043/pKM101	$T:A \rightarrow G:C$	[9]
TA7004	hisG9133 Dara9 Dehl1004 (bio chlD uvrB chlA) galE503 rfa1044 /pKM101	$C:G \rightarrow T:A$	[9]
TA7005	hisG9130 Dara9 Dehl1004 (bio chID uvrB chIA) galE503 rfa1045/pKM101	$C:G \rightarrow A:T$	[9]
TA7006	hisC9070 Dara9 Dehl1004 (bio chlD urrB chlA) galE503 rfa1046/pKM101	$C:G \rightarrow G:C$	[9]
Mix	TA7001, TA7002, TA7003, TA7004, TA7005, TA7006	Missense	[9]
TA1537	hisC3076 Dara9 Dehl1007 (bio chl ucrB gal)rfa1003 (+1 G)	Frameshifts	[41]
TA98	hisD3052 Darag Achl1008 (bio chl urrB gal) rfa1004/pKM101 (-1 C)	Frameshifts	[41]

detect frameshift mutations. The Mix consisted of TA7001, TA7002, TA7003, TA7004, TA7005 and TA7006 in equal proportions and was stored as a mixture at  $-70^{\circ}$ C. This was used as the source inoculum and treated as if it were an individual strain throughout this study. During overnight growth of the Mix(ed) culture each strain was present, however each may not be represented in equal proportions due small differences in growth rates. Two to 8 µl of a freshly thawed frozen stock were inoculated into 5 to 15 ml, respectively, of Growth Media (Oxoid Broth, UnipathOxoid, Basingstoke, UK made for Xenometrix). These cultures were grown overnight (12 to 15 h) at 37°C in an environmental shaker at 250 rpm (New Brunswick Scientific, Edison, NJ) in the presence of 25 µg/ml ampicillin (Xenometrix). TA1537 cultures were grown in the absence of ampicillin because this strain does not carry pKM101 which contains an ampicillin resistance gene.

#### 2.2. Chemicals

Twenty-five chemicals were selected for this study from the NTP database of more than 1700 chemicals. Although all test results are, to some extent protocol-dependent, an attempt was made to remove all those chemicals where the positive response was highly dependent on methodology (i.e. those requiring reductive metabolism; gases and highly volatile; metal salts; etc.). Also excluded were chemicals not easily obtainable (or expensive), controlled substances, undefined substances or mixtures, mixtures of positional isomers, insolubles, inorganics, organometallics and chemicals with conflicting responses in different tests using the same protocol. Only chemicals with published results were considered [2,17-28] (see Table 2).

The 25 chemicals selected included 18 mutagens and 7 non-mutagens as classified by results from the traditional strains. Factors in the selection of the chemicals included chemical structure, responses in the standard Salmonella strains, and consistency of responses in replicate experiments, especially where weak. Preference was given to chemicals among the 114 evaluated by Tennant et al. [23] and Zeiger et al. [18]. The chemicals selected were the same salt and isomer as was tested by the NTP.

The purities, sources, and the dose ranges tested are listed in Table 2. Approximately 2-5 g, or 2-5 ml of each sample were distributed into vials, and coded with a 4-digit random number at NIEHS, prior to shipping to Xenometrix. Five chemicals were aliquoted in two different vials and treated as two independent samples for coding and shipping purposes. Sealed envelopes containing the chemical name, CAS no., and available safety information were sent with each shipment of chemicals to be opened only in the event of a spill or personnel exposure. All coded, 'unknown' chemicals were handled by the experimentalist as if they were carcinogenic and mutagenic, and since there were no incidents regarding safety these envelopes were not opened until after the chemicals were decoded.

#### 2.3. Control chemicals

Positive control chemicals including:  $N^4$ -aminocytidine (N4AC), methyl methanesulfonate (MMS), streptonigrin (STN), 4-nitroquinoline-N-oxide P. Gev et al. / Mutation Research 412 (1998) 115-130

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Table 2		
Chemicals	tested	

Chemical	CAS No.	Supplier	Purity 4 (%)	Solvent used	Dose range (per ml)	References <sup>n</sup>
9-Aminoacridine · HCl · H 10	52417-22-8	Aldrich	98	DMSO	5-100 µg	[17]
2-Amino-5-nitrophenol	121-88-0	Aldrich	90 (tech.)	DMSO	1-100 µg	[18,19]
5-Azacytidine	320-67-2	N.I.E.H.S.	Unknown	DMSO	5-100 µg	[2]
Benzaldehyde	100-52-7	Aldrich	99 +	DMSO	50-1000 µg	[20]
Benzo[a]pyrene	50-32-8	Sigma	Practical	DMSO	5-100 µg	[20,21]
Benzyl chloride	100-44-7	Aldrich	99%	DMSO	50-5000 µg	[19]
1-Chloro-2-propanol	127-00-4	Fluka	97 +	Water	50-1000 µg	[19]
Coumarin	91-64-5	Rhone-Poulenc	Unknown	DMSO	5-1000 µg	[20]
Crotonaldehyde	4170-30-3	Aldrich	99 +	DMSO	50-1000 µg	[20]
Cumene hydroperoxide	80-15-9	Aldrich	80 (tech.)	DMSO	1-100 µ.g	[17]
Dicumyl peroxide	80-43-3	Aldrich	98	DMSO	50-5000 µg	[22]
Di(2-ethylhexyl)phthalate	117-81-7	Aldrich	99.5	DMSO	0.5-1000 µ.g	[23.24]
Dimethyl sulfoxide	67-68-5	Fisher	Certified	Water	50-5000 µg	[2]
1.2-Epoxybutane	106-88-7	TCI America	99 +	DMSO	50-5000 µg	[21,25,26]
Ethylenediamine	107-15-3	Aldrich	Unknown	Water	50-1000 µg	[20]
8-Hydroxyquinoline	148-24-3	Aldrich	99.5	DMSO	1-50 µg	[22]
Isobutyl nitrite	542-56-3	King's Labs.	Unknown	Ethanol	1-1000 µ.c	[17]
Nitrofurantoin	67-20-9	Norwich/Eaton	Unknown	DMSO	0.1-5 µ2	[20]
4,4'-Oxydianiline	101-80-4	Aldrich	97	DMSO	10-500 µg	[22]
Phenol	108-95-2	Aldrich	99 -	DMSO	50-5000 µg	[20]
Proflavin HCl+1/2 H <sub>2</sub> O	952-23-8	Aldrich	~ 95	Water	0.5-10 µg	[27]
Quercetin	117-39-5	Freeman Industries	97	DMSO	5-100 µg	[2]
Tetracycline HCI	64-75-5	Aldrich	98	Water	0.5-10 µg	[28]
Trichloroacetonitrile	545-06-2	Aldrich	98	DMSO	50-1000 µg	[17]
Tricresyl phosphate	1330-78-5	Stauffer	Unknown	DMSO	10-1000 µg	[20]

<sup>a</sup> Purity (%): suppliers' stated purity; where there is no entry, purity is not known or reported.

<sup>b</sup> Publication containing the original NTP Salmonella mutagenicity test results. Some of these chemicals had additional, unpublished, tests performed,

<sup>c</sup> Contains 0.25% propylene oxide.

(4NQO) and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical (St. Louis, MO), while 2-nitrofluorene (2NF) and 2-aminoanthracene (2AA) were purchased from Aldrich Chemical (Milwaukee, WI). Ethanol was obtained from Aaper Alcohol and Chemical (Shelbyville, KY). N4AC was prepared in sterile deionized water ( $\Omega \ge 10^{15}$  ohms) for a final concentration of 50 µg/ml and used as a positive control chemical for TA7001. STN was dissolved in DMSO and used at a final concentration of 5 ng/ml as a positive control for TA7003. MMS was used as a positive control chemical for TA7002, TA7004, TA7005, TA7006, and the Mix at a final concentration of 500 µg/ml. In experiments where MMS was not consistent in reverting the cultures, 4NQO was used as a positive control chemical for strains, TA7004, TA7005, TA7006, and the Mix at a final concentration of 1 µg/ml dissolved in DMSO. The

positive control chemicals used were those shown to be mutagenic by Gee et al. [9]. 2NF was prepared in DMSO at a final concentration of 1  $\mu$ g/ml as a positive control for both TA98 and TA1537. 2AA was prepared in DMSO at a final concentration of 5.0  $\mu$ g/ml and used as the positive control in the presence of S9 fraction. The degree of reversion in response to these control chemicals was within the limits typical of results observed over a minimum of 10 experiments performed over the previous year. In cases where the results were ambiguous or negative with respect to these control chemicals, the entire set of experiments were repeated before the test chemicals were decoded.

The final concentration of solvent in each experiment was 2% in a final exposure volume of 0.5 ml. The range of doses used was determined by a combination of viability measured in the number of positive wells in non-selective media and the optical density of exposed cultures after 90 min of incubation at 37°C in an environmental shaker at 250 rpm. Where there was no detectable toxicity and no limitations in the solubility of the chemical, a maximal concentration of 5 mg/ml was tested. The density of unknown liquids was assumed to be 1.0 at the time of testing and concentrations were adjusted after decoding for Table 2.

#### 2.4. Study design

Data were collected for a total of 4 doses of each test chemical plus a solvent (zero dose) and positive control. Each culture was treated and dispensed into microtiter plates in triplicate. Three independent cultures were dosed either on the same day and/or up to 3 weeks later. Each culture came from a separate frozen vial, although all the vials were from the same production lot. Therefore, 9 measurements were obtained at each dose for each chemical. Doses were selected using a preliminary range-finding experiment with the Mix. All strains and the Mix were used at the same time. Approximately 2–8 chemicals were tested in 1 day.

#### 2.5. Liquid exposure

The chemicals were tested in the Ames II strains using a liquid fluctuation test (the AMAX test procedure: Ames II<sup>™</sup> Mutagenicity Assays by Xenometrix). Unless stated otherwise, all procedures were performed manually (i.e. without using the robotics station). In the absence of S9 fraction, 9 ml of Ames II Exposure medium (Xenometrix), and 1.0 ml of each overnight culture were mixed gently. In experiments where the S9 fraction was used, the Exposure medium was decreased to 7.5 ml to accommodate 1.5 ml of the S9 reagent to provide a final concentration of 4.5% S9 fraction. The S9 mix, in a total volume of 20 ml, contained: 0.66 ml of 1 M KCl (J.T. Baker, Phillipsburg, NJ), 0.64 ml of 0.25 M MgCl<sub>2</sub> (J.T. Baker), 0.50 ml of 0.2 M glucose-6phosphate (Sigma), 2 ml of 0.04 M NADP (Sigma), 10 ml of 0.2 M NaHPO<sub>4</sub> buffer (J.T. Baker) and 6 ml of rat liver S9 fraction induced by Aroclor 1254 (Molecular Toxicology, Annapolis, MD, or Microbiological Associates, Rockville, MD).

Aliquots of the appropriate stock concentrations of each test chemical, control chemicals, and solvents were dispensed into individual wells of 24-well, microtiter plates (not treated for tissue culture; Falcon, Becton Dickinson Labware, Costar, Cambridge, MA: Nunc, Roskilde, Denmark) to a final volume of 500  $\mu$ l per well. The 24-well plates were incubated at 37°C for 90 min, with shaking at 250 rpm. The S9 reagent was stored on ice until it was added to the culture mixture.

#### 2.6. Prototrophic selection

After the 90-min incubation, the 24-well plates were removed from the incubator to room temperature. An aliquot of 2.5 ml of Ames II Reversion Indicator medium (Xenometrix) was pipeted manually or by a programmable automated pipeting station (Hamilton, Reno, NV) into each well of the 24-well plates containing chemically treated cultures. The histidine-deficient Indicator medium which selects for prototrophic reversion was mixed gently by pipeting and dispensing the volume in place several times, either manually or in the pipeting station. When adequately mixed, the contents of each well of a 24-well microtiter plate were distributed in 50-µl aliquots over 48 wells of a 384-well microtiter plate (Nunc) by Eppendorf Repeater Plus/8<sup>™</sup> pipets (Brinkmann, Westbury, NY) manually or by the robotics pipeting station. Each column (4 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. The 384-well microtiter plates were sealed in Ziplock " plastic bags to prevent evaporation, and incubated at 37°C for 48 h.

#### 2.7. Robotics

Several robots were evaluated for their ability to distribute the exposed liquid cultures into 384-well microtiter plates for prototrophic selection. Many criteria, including speed of pipeting, adequate mixing, minimal cross-contamination of cultures and chemicals without disposable pipet tips, flexibility and most importantly, the cost of the instrument, operating supplies and long-term maintenance, were considered. We selected the ML 2200 pipeting station manufactured by Hamilton (Reno, NV) to reduce the time needed for the performance of the study.

The ML 2200 pipeting station used a liquid level detector and wash station to minimize carry-over from well to well of the 24-well and 384-well plates. The system was controlled by ECLIPSE software (Hamilton) from a Hewlett-Packard Vectra 486/25N computer (Grenoble, France). The pipeting head consists of 8 Teflon-lined probes for aspiration and dispensation of liquids. Liquid was transferred by powered precision syringe pumps which controlled by DOS-based software. Each probe was programmed to flush with 5 ml of 70% ethanol, followed by 5 ml of sterile deionized water ( $\Omega \ge 10^{15}$ ohms) 8 to 10 times between pipeting steps. The Indicator medium was dispensed into each well of the 24-well microtiter plate. Probes were rinsed twice with sterile deionized water before each transfer of Indicator medium from a sterile stock. The probes mixed Indicator media with the exposed culture three times before distributing 50 µl into each well of a 384-well microtiter plate. The 384-well plates were covered, removed from the platform of the pipeting station, and incubated at 37°C as described above. The entire robotics workstation was enclosed in an HEPA-filtered positive pressure bubble (Biobubble, Ft. Collins, CO) to minimize contamination.

#### 2.8. Data acquisition

Bromocresol purple, an essential constituent of the Indicator media, turns yellow as the pH drops  $(pK_a \cong 5.2)$  as catabolites accumulate from the metabolic activity of revertant cells which grow in the absence of histidine. The number of 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 a SLT Spectra Image plate reader (Tecan U.S., Research Triangle Park, NC) at OD492 nm normalized at OD<sub>623 nm</sub> as a reference wavelength. The optical density was digitized by the SLT data Capture software and exported to Microsoft Excel, Version 7.0 (Microsoft, Bothell, WA) for data analysis. The digitized optical densities were converted to positive (1)

or negative (0) wells and summated by macros written in Excel to determine the number of positive wells for each sample. The data were organized further into summary tables of triplicate data per culture and pooled data per three independent cultures for each dose.

The average number of wells containing revertants per culture per dose were calculated from the triplicates, and the mutational ratios and increases above the zero dose were determined at each dose of test chemical. If the mean spontaneous reversion was I well or greater out of 48 wells, the mutational ratio was calculated as a ratio of the mean number of wells of revertants in the test dose divided by the mean number of wells in the zero/solvent dose control. If the mean spontaneous reversion was zero then the absolute difference between the numbers of revertants in the test dose and the zero dose control was used to look for a dose effect. These increases and ratios were tested for preliminary statistical significance by the Student's *t*-test at p < 0.05. Those chemicals that were negative based on these preliminary results were tested again in the presence of the S9 fraction.

## 2.9. Complementary-log generalized linear model (GLiM)

To ask whether there was a dose effect significant from the control cultures, the data were analyzed according to a model that assumed mutagenic events followed a Poisson process ([29–31]; W. Piegorsch and B. Margolin, personal communication). In preparation for these analyses, the variability among sets of triplicate plates and from culture to culture were determined.

The number of positive wells (containing revertants) from each of the triplicate plates within each given culture per dose were tested for excessive plate-to-plate variability [32,33]. Since there were no consistent patterns of significant extra binomial variation at the plate-to-plate level, there was sufficient homogeneity to pool these data sets. The pooled triplicates were analyzed for excessive variability amongst three independent cultures of each tester strain for each chemical tested with and without S9 fraction. There were consistent patterns of significant extra-binomial variation at the culture-to-culture level due to jackpot mutations that were observed at every dose level for some cultures.

In these analyses, it was assumed that there were no culture-by-dose interactions present in these experiments. One data set for a chemical chosen at random, was tested for such possible interactions. Significant interactions at  $\alpha = 0.05$  were found in two of the 18 possible strain and S9 conditions. This was thought to be an insubstantial interactive effect; therefore the results were not modified for a possible culture-by-dose interaction for this chemical, nor for any others. The small-sample operating characteristics of this form of likelihood ratio test under a complementary-log GLiM are not known, and further applied statistical research is required before this methodology can be recommended unequivocally. Because of this concern, only p-values lower than the extreme value of  $\alpha = 0.001$  were chosen as a reasonable limit for significance.

The statistical treatment of variability among replicates within a culture, and among cultures will be addressed elsewhere (Piegorsch et al., in preparation).

#### 2.10. NTP test results

Detailed descriptions of the test protocols and criteria for evaluation of the data can be found in Zeiger et al. [2,20,34,35]. Briefly, chemicals were tested under code using a preincubation procedure, and the data evaluated prior to deciphering the code. All chemicals were tested up to a toxic dose or to a dose limited by solubility. In the absence of toxicity, the highest dose tested was 10 mg/plate. The strains used were TA97. TA98, TA100, TA102, TA104, TA1535, and TA1537. Not all strains were used for all chemicals. However, all chemicals were tested in TA98 and TA100; only 1 chemical was tested in TA102 and TA104. All tests contained 5 chemical concentrations at half-log intervals, in triplicate, and concurrent solvent and positive controls. Chemicals were tested without metabolic activation, and with 10% and/or 30% S9 from Aroclor-induced rat and hamster livers. All positive results were repeated. In general, a chemical was judged mutagenic if a reproducible, dose-related response was obtained, and statistical procedures were not used. A non-mutagenic chemical was one that was judged negative in at

least 4 tester strains (TA98, TA100, TA1535, and TA97 or TA1537).

For the purposes of this evaluation chemicals were selected which had been judged mutagenic (+), weakly mutagenic (+w), and non-mutagenic (-). A mutagenic response was defined as a reproducible, dose-related response. A weakly mutagenic response was a low-level, reproducible, dose-related response. The difference between a response judged mutagenic or weakly mutagenic was highly subjective. It was not necessary to achieve a 2-fold increase over background for a chemical to be considered (+) or (+w) [2,20,34,35].

#### 3. Results

A summary of the qualitative results from the Ames II test and the NTP database are presented in Table 3. The tables containing the raw data from this study are available on the Xenometrix web site at http://www.xeno.com or can be obtained from P.G.

If a chemical was found to revert any of the strains in the Ames II test, the chemical was classified as mutagenic. A study design such as the one used here allows several different analyses of the data. A number of useful combinations of the Ames II and NTP test results have been evaluated for concordance, and are presented in Table 4.

#### 3.1. Concordances

When the results from the individual TA7000 strains, the Mix, TA98 and TA1537, were compared to the summary NTP results for each of the 25 chemicals, the overall concordance was 88% in agreement (Table 4<sup>1</sup>). This was calculated from a total of 22 chemicals (16 NTP mutagens and 6 NTP non-mutagens). There were two mutagens in the NTP (1-chloro-2-propanol and isobutyl nitrite) preincubation test that were not classified as mutagens, and one NTP non-mutagen (benzaldehyde) in the NTP procedure that was mutagenic in this study.

1-Chloro-2-propanol and isobutyl nitrite were not identified as mutagens by any of the strains in the Ames II test (Table 3). The overall NTP results classified equivocal (?) for TA98 in both the presence and absence of S9 fraction. 1-Chloro-2-proP. Gee et al. / Mutation Research 412 (1998) 115-130

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Reversion results

Chemical	$\pm S9$	AMA	Х								NTP F	oreincul	batio	n test		
		7001	7002	7003	7004	7005	7006	Mix	1537	98	1535	1537	97	98	100	102/4
9 Aminoacridine HCl · H <sub>2</sub> O	NA	-	2	-	-	-	-	-	+	-	-	+		-	-	
	59										-	+		-	+ w	
2-Amino-5-nitrophenol	NA		-	-	-	-	-	-	-	+	-	+		+	+ w	
	S9											+		+	+w	
2-Amino-5-nitrophenol	NA	-	-	-	-	-	-	-	-	+	-	+		+	+ w	
	<b>S</b> 9										-	+		+	+w	
5-Azacytidine	NA	-	-	8	+	-	+	+	-	+	+ w		-	-	?	+
	S9										+		-	-	?	+
Benzaldehyde	NA	-	-	-	-	-	-	-	-	-	-	-		-	-	
	S9	-	9.1	-	-	+	-	-	-	-	S. 1	~		-	-	
Benzo[a]pyrene	NA	-	-	-	-	-	-	-	-	-	-	-		-	-	
	S9	+	+	-	+	+	+	+	+	$\rightarrow$	2	+		+	+	
Benzyl chloride	NA	-	-	-	+	+	-	-	-	-	-	-		-	-	
Course Courses	59										-	-	-	-	+ w	
Benzyl chloride	NA	2	<u></u>	-	+	+	-	+		-	-	-	-	-	-	
	59	2	2	-	-	-	-	4	-	_	2	-	-	_	+ w	
1-Chloro-2-propanol	NA	1.2.11	-	-	-	_	-	-	-	-	+	-	-	-	-7	
r entero e propanor	59	-	_	-	-	-		2	-	-	+	-	-	-	- 2	
Coumarin	NA	1		100	-	1	12	-		12	1	1		1		
countarin	50	12.1	+	20	÷	0	0.1			1.	2	2.1		1	4	
Coursein	NIA	-E. 1	Ŧ	2.1	E		2.1	S.,	2		1.1			121	T	
Countain	cn cn	31	-	100	200		51		1.1	1	100			8	5	
Centonaldabuda	S9		T	0.0	6.	-	4	-		-		-		-	Ť	
Crotonaldenyue	E0	-	a.	-	+	+	Ŧ	Ŧ		4	-				+	
Contraction in the	59					5	120	1	7	1		-			+	
Cumene hydroperoxide	NA		+	~	- <del>1</del> -	+	+	+	t	t		-		-	÷	
	59	+	-	-	+	+	+	+	+	_	-	-		+	+w	
Dicumyl peroxide	NA	-	-	_	-	-	_	-	-	-	2		-	-	-	
	59	-	-	-	-	-	-	-	-	1	5		-	-	-	
Di(2-ethylhexyl)phthalate	NA	-	-	-	-	-	-	-	1.1	-	÷	-	-	-	-	
22- 31 C 190 A 2	\$9	3	-	-	-	-	-	-	-	-	-	-	-	-	-	
Dimethyl sulfoxide	NA	_	-	-	- C	-	-	-	-	-	~	-	-	-	-	
	S9	-	-	-	-	-	-	1.00	-	8	-	-	-	$\sim$	~	
1,2-Epoxybutane	NA	-	-	-	+	-	-	-	-		+	-		-	+	
	S9	-	-	-	-	-	-	-	-	-	+	-		-	+	
Ethylenediamine	NA	-	-	$\sim$	+	+	+	+	-	$\sim$	+	-	2	-	+ w	
	S9										+	-	-	-	+w	
Ethylenediamine	NA	~	-	-	+	+	-	+	-	-	+	-	्रस्	-	+w	
	S9										+	-	-	-	+w	
8-Hydroxyquinoline	NA	-	-	-	8.	-	-	-	-	$\sim$	-		-	$\sim$	-	
	59	-	<del>.  </del>	-	-	+	-	+	+	+	-		+	-	+	
Isobutyl nitrite	NA	5	-	-	-	-	-	-	~	-	7	-		-	+	
	S9	-	2	2	-	-	~	-	-	-	$+\mathbf{w}$	-		-	+	
Isobutyl nitrite	NA	8	-	÷	÷	-	-	-	÷	(-	?	-		-	+	
	S9	-	-	-	÷1	-	-	-	-	-	+w	1		-	+	
Nitrofurantoin	NA	-	+	-	+	+	+	+	-	+	-	-		+	+	
	59										-	7		+	+	
4,4'-Oxydianiline	NA	-	-	-	-	÷	-	+	-	-	-		-	-	+ w	
	59							( )			-	+ w.	÷	+	+	
Phenol	NA	-	-	-	~	-	2	8	÷.	2	2	-		-	2	
A																

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Table	3	(continued)
2	100	and the second second

Chemical	± \$9	AMA	Х								NTP p	oreincul	oation	test		
		7001	7002	7003	7004	7005	7006	Mix	1537	98	1535	1537	97	98	100	102/4
Proflavin HCl · 1/2 H <sub>2</sub> O	NA	3	-	-	$\tau$	+	~	+	÷	+	-	+		?	-	
Quercetin	NA	-	+	-	+	÷.	-	+	+	+				+	+	
Tetracycline HCI	S9 NA	-	-	-	4	-	-	-	-	-	2.7	-		+	+	
	\$9	-	2	2	-	-	8	-	-	-	-	-		-	-	
Trichloroacetonitrile	NA	÷	8	÷.,	-	-	20	5	-		5	20		~	3	
	S9	-	-	-	-	-	-	+	-	-	-	-		-	+w	
Tricresyl phosphate	NA	-	-	-	-	-		-	-	-	-	-		-	-	
	S9	-	-	-	-	-	-	-	-	-	~	-		-	-	

NA, not tested with S9; -, negative; +, positive; +w, weakly positive; ?, equivocal,

panol was judged to be equivocal (?) in 3 tests and weakly positive (+w) in 2 tests out of the 5 experiments performed in the presence of 10% rat S9 fraction. Similarly, it was negative (-) in 2, equivocal (?) in 2 and weakly positive (+w) in 1 out 5 experiments performed in the presence of 10% hamster S9 in TA100 [19]. However, it was clearly positive with and without 10% hamster and rat S9 in TA1535. There were no strains with comparable genetic backgrounds to TA1535 in this Ames II test.

Isobutyl nitrite, diluted with 95% ethanol, was tested as two independent unknown samples and the results were negative in both replicates. Positive results were observed in the NTP preincubation assay using TA100 in the presence of 10% hamster and 10% rat S9 when isobutyl nitrite was dissolved in 95% ethanol. Isobutyl nitrite, diluted in DMSO was also positive in the presence of 30% hamster and 30% rat S9 [17]. If the S9 fraction in the Ames II test was increased to 10%, it is possible that positive results might have been obtained.

Benzaldehyde was positive in this study, in disagreement with the negative results in TA1535, TA1537, TA98 and TA100 with and without 10% hamster and rat S9 [20]. Results of testing benzaldehyde in strains TA102, TA104 or TA97 were not available. This chemical reverted only TA7005, and was not detectable in the Mix culture, TA1537 or TA98. Strain TA7005 has a C:G base-pair sequence at the target site and reverts only to a A:T base pair to regain functional histidine biosynthesis. Crotonaldehyde was also successful in reverting TA7005 in this study and we have obtained positive results with formaldehyde (unpublished data). There is a G:C base pair both 5' and 3' of the target mutation which is similar to the context of the 1 base pair deletion in TA1537 (*hisC3076*) which has the sequence, 'CGCGCG'. However TA1537 was not reverted by crotonaldehyde or benzaldehyde in this study, or by formaldehyde in a NTP study [20]. TA97 also has C:G base pair at its target site, but it has not been reported as being particularly responsive to these aldehydes.

#### 3.2. Ames II test vs. NTP TA100 and TA98

The overall concordance is 88% (Table 4<sup>2</sup>) when the results of all of the strains (TA700x, Mix, TA1537 and TA98) in the Ames II test were compared to the NTP results obtained from just TA100 and TA98 in the preincubation assay. The discordances for isobutyl nitrite and benzaldehyde remain, however, there is a disagreement for 5-azacytidine, which is balanced by an agreement for 1-chloro-2-propanol. In this case, TA100 and TA98 classify 5-azacytidine as a non-mutagen in the NTP database (although it is mutagenic in TA102 and TA104). 5-Azacytidine was mutagenic in TA7004, TA7006, TA98 and the Mix in the Ames II test.

#### 3.3. Ames II test vs. NTP (all strains)

The results from strains TA7001 to TA7006 in the Ames II test agreed with the NTP preincubation test

	TA700x <sup>4</sup>	Mix	TA700x + Mix	TA98	TA1537	Mix+TA98 +TA1537	Mix+TA98	TA700x + Mix + TA98	Ames II all strains
NTP. all strains TA98	19/25 (76%)	18/24 (75%)*	20/25 (80%)*	21/25(84%) <sup>8</sup>	01	20/25 (80%)	19/24 (79%)7	21/25 (84%)	22/25 (88%)'
TA100+TA98 TA100		17/24 (71%) <sup>12 h</sup>			19/70/07/21		19/24 (79%) <sup>10</sup>		22/25 (88%) <sup>2</sup>
TA100+TA98 +TA1537						20/24 (83%) <sup>11</sup>			

Table 4

N.B.: In comparisons where there were only 24 chemicals in total, benzyl chloride was not included because of conflicting results in the duplicate samples.

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for 13 of the 18 NTP mutagens (72%) and 6 out of 7 NTP non-mutagens, which becomes a total of 19 out of the 25 chemicals (76%) tested (Table 4<sup>3</sup>). When the Mix was included, there was one additional chemical detected, to increase the agreement to 14 (78%) mutagens, which became a total of 20/25 (80%) (Table 4<sup>4</sup>). When the results from the Mix, TA98 and TA1537 were compared with all the strains used in the NTP database, the agreement between the AMAX test and the NTP test was 13 (72%) out of 18 (72%), NTP mutagens, and 7 (100%) out of 7 (100%) non-mutagens. This resulted in an overall agreement of 80% (20/25, Table 4<sup>5</sup>).

The results from the Mix alone, when compared to that of the NTP database, gave an overall agreement of 75% (18/24, Table 4<sup>6</sup>). Benzyl chloride was tested twice in this study as two independent chemicals, however the results for the Mix did not agree. Therefore, benzyl chloride results were not included in these calculations. The agreement increases by I chemical (2-amino-5-nitrophenol, Table 3) to 79% when the Ames II TA98 is added to the comparison (Table 4<sup>7</sup>).

#### 3.4. Strains TA98 and TA1537

The tester strains, TA98 and TA1537, which detect small deletions, additions and suppressions of the frameshift mutations, have been included for routine use in the Ames II test. This allowed for a somewhat limited comparison of the NTP's preincubation method to the liquid format used in this study. Because the results for these two strains were reproducible among the 5 duplicated chemicals, these chemicals are considered here as single tests.

There was overall agreement in the TA98 results for 21 (84%) out of 25 (84%) chemicals (Table 4<sup>8</sup>). In this comparison, the Ames II test appeared to be more sensitive because there was only one chemical positive in the NTP test and negative in the Ames II test (4,4'-oxydianiline). By comparison, there were three positive (5-azacytidine, crotonaldehyde, 8-hydroxyquinoline, Table 3) in the Ames II test and negative in the NTP test. 4,4'-Oxydianiline (Table 3) was positive in the preincubation test only with S9, but negative in the Ames II test which was run only without S9. Because 4,4'-oxydianiline was positive in other strains, it was not tested with S9 in this study. Similarly, proflavin was positive without S9 so it was not tested with S9 in the Ames II test. Proflavin was equivocal and therefore classified as negative, but was positive when tested with S9 in the NTP preincubation test. In contrast, cumene hydroperoxide was positive in the Ames II test without S9, and negative with S9, while S9 activation was required for mutagenicity in the NTP preincubation test. Another disagreement was with 8-hydroxyquinoline, which was negative in TA98 in the NTP study with and without S9, and positive in the Ames II test with S9. However, the sulfate salt of 8-hydroxyquinoline was mutagenic with S9 in the NTP protocol [22].

Of the 20 chemicals tested in TA1537 by the NTP, the test results agreed for 18 (94%, Table 4<sup>9</sup>); 3 were positive in both test systems, and 15 were negative. 4,4'-Oxydianiline was positive in the NTP tests only with S9. Its responses were considered to be concordant because it was non-mutagenic in the absence of S9 in both procedures. 2-Amino-5-nitrophenol was negative in TA1537 without S9 in the Ames II test, and positive in the NTP preincubation test. Cumene hydroperoxide was positive in the Ames II test with and without S9, and negative under these same conditions in the preincubation test. Benzaldehyde, dicumyl peroxide, 8-hydroxyquinoline and quercetin were not tested in TA1537 in the NTP preincubation protocol.

#### 3.5. Ames II Mix + TA98 vs. NTP TA100 + TA98

The NTP test protocol uses TA98 and TA100, with and without S9 as an initial screen. If the chemical is mutagenic in one of these two strains, it is usually not tested in other strains. Chemicals not mutagenic in either of these two strains are tested in at least two additional strains [2,20,34]. This procedure was adopted because an analysis of the NTP database showed that 89% of the mutagenic chemicals would have been correctly classified if only TA98 and TA100 had been used [36]. This parallels the role proposed for the Mix + TA98 screen. Benzyl chloride was not included in this compilation because one of its duplicates yielded a positive response with the Mix, and the other sample was negative. When the Mix and TA98 were used, 11 (46%) of the chemicals were judged mutagenic, as

compared to the 15 (63%) chemicals which were classified as mutagens by TA100 and TA98 in the NTP preincubation test. There were 4 chemicals that were mutagenic in TA98 and/or TA100 in the preincubation test that were not detected by the Mix + TA98 strategy, and one chemical (5-azacytidine) that was mutagenic in the Mix and TA98, but not in TA98 or TA100 in the preincubation test. Overall, there was agreement in the test results for 19 (79%) out of the 24 chemicals considered (Table  $4^{10}$ ).

These results demonstrate that the use of TA98 and TA100 detected a higher proportion of mutagens in the preincubation test than did TA98 and the Mix in the Ames II test. Of the 4 chemicals that were negative in the Ames II protocol, 9-aminoacridine was only weakly positive with S9 in the NTP preincubation assay, while 9-aminoacridine was not tested with S9 in the Ames II protocol because it was positive in TA1537 in the absence of S9. The addition of TA1537 to the Mix and TA98 in the Ames II test results would have allowed detection of 9aminoacridine (Table 4<sup>11</sup>).

#### 3.6. Mix vs. individual strains

The Mix is designed to respond to treatments that mutate any one of the 6 strains, from TA7001 to TA7006. The Mix and one or more of the individual strains were positive for 20 (83%) out of 24 chemicals. Because the results with the Mix were discordant for the duplicate benzyl chloride samples, this chemical is not included in this compilation (Table 3). Both samples of benzyl chloride were mutagenic in TA7004 and TA7005, but only one was mutagenic in the Mix. Three chemicals (benzaldehyde, mutagenic in TA7005 with S9; coumarin, mutagenic in TA7002 with S9; 1,2-epoxybutane, mutagenic in TA7004 without S9) were detected by the individual strains, but not by the Mix, and one (trichloroacetonitrile) was detected by the Mix, but not by the individual strains.

#### 3.7, Strain responsiveness

TA7004 and TA7005 were considered to be the most sensitive strains because they detected 12 and 14 out of the 18 mutagens, respectively (Table 3),

This was followed closely by TA98 which was positive for 10 mutagens. TA7002, TA7006 and TA1537 were reverted by 7 to 8 mutagens, and TA7001 was reverted by only two mutagens. TA7003 did not respond to any of the test chemicals. Streptonigrin, 5 ng/ml, was used as positive control chemical for strain, TA7003, in the liquid protocol. However, in the protocol used here, it never induced more than  $5 \pm 2$  positive wells out of 48 possible wells in a total of 165 experiments that used streptonigrin in this study (data not shown). In these same experiments, the untreated controls were between 0 and 1 positive wells. In contrast, streptonigrin (0.1  $\mu$ g/plate) resulted in 63 ± 7 revertants per plate for TA7003 from a spontaneous response of 0.1 to 0.2 revertants per plate in the standard plate incorporation assay [9].

#### 3.8. Reproducibility

There were 5 mutagens that were tested as duplicate samples unknown to the personnel performing the test. The overall conclusions from each of the duplicate tests were the same, although there were some differences with respect to the responding Salmonella strains. Four of the chemicals were reproducibly mutagenic and one, isobutyl nitrite, was non-mutagenic (Table 3). With respect to comparisons by strain and activation, two of the positive chemicals, 2-amino-5-nitrophenol and coumarin responded similarly in both tests. Benzyl chloride was mutagenic in TA7004 and TA7005 without S9 in both tests, but only one of the duplicate samples was mutagenic in the Mix. Ethylenediamine was mutagenic in TA7004, TA7005 and the Mix without S9 in both tests, but only one of the duplicate samples was positive in TA7006.

#### 4. Discussion

#### 4.1. Comparisons

This study evaluated the ability of the basespecific set of Salmonella tester strains (designated Ames II)<sup>TM</sup>, when tested in a high-throughput, fluctuation protocol, to duplicate the mutagenic responses of the standard Salmonella strains when tested in a preincubation protocol. Thus, the two data sets generated differ in the Salmonella strains used, in the cell and chemical exposure regimen, and in the mutant expression conditions.

Of the 30 coded substances tested, 5 of the mutagens were duplicates, a factor that was not divulged to the laboratory personnel until after the testing had been completed. This allowed an evaluation of the intra-laboratory reproducibility. Identical patterns of positive and negative responses were obtained for 3 of the 5 chemicals. Of the remaining two chemicals, one (ethylenediamine) differed in its response to strain TA7006; both samples showed increased responses, however there was no consistent dose response for all three cultures for one sample. The other chemical (benzyl chloride) differed in its response to the Mix. Both samples of benzyl chloride gave dose related increases but one sample was not significant ( $\alpha = 0.008$ ) at  $\alpha = 0.001$  to score positive in the Mix. Neither of these differences affected the overall evaluation of each of the test samples.

The concordances between the TA7000 series strains, and the NTP results must be measured within the limitations of the reproducibility of the NTP results. The intra-assay agreement of the Salmonella test, as performed by the NTP, is 84.5%, when measured as a strict, positive-versus-negative concordance, and 86.9% when measured as a pair-wise concordance [35]. These values reflect the reproducibility of the Salmonella test when tested in the same or different laboratories at different times, but with the same protocol. As such, they can be considered to provide an upper bound for the agreement to be expected from testing or test validation exercises.

#### 4.2. Protocol effects

There are a number of differences between the Ames II test, as performed in this study, and the NTP preincubation test protocol, that could have contributed to the differences between the two sets of results. These differences are best seen in the TA98 and TA1537 results. These two strains were used in both procedures, and it may be assumed that the differences in the responses of these strains between the NTP and the Ames II tests resulted from the different testing procedures. The Ames II test identified mutagens in each strain that were not detected by the preincubation test; and visa versa.

However, the overall concordances were 84% and 94% for TA98 and TA1537, respectively, so the results from the two different protocols can be considered comparable.

The final concentration of Aroclor-induced rat liver S9 was 4.5% in the Ames II test, This was considerably lower than the 10% and 30% S9 used in the NTP protocol. For many of the NTP chemicals, the initial test used was 30% S9, based on observations that the higher S9 concentrations allowed more mutagens to be identified; therefore, it is not known if a positive chemical under these conditions would also have been positive if the S9 concentrations were decreased. Although, the NTP protocol used both rat and hamster liver-derived S9, all of the S9-dependent mutagenic chemicals chosen for this study were positive with rat S9.

Because the Ames II test is a colorimetric assay that is dependent on pH changes, raising the percentage of S9 fraction overcomes the buffering capacity of the colored Indicator media. Therefore it is technically difficult and costly to match the S9 concentrations used in the NTP preincubation tests. To further minimize the cost of testing, we tested only those chemicals that were negative in all strains in the presence of S9. For example, 4,4'-oxydianiline was not tested with S9 because it was positive in TA7005 and the Mix; however, it was positive in TA1537 and TA98 the NTP studies only in the presence of S9 [2].

The histidine concentration in the fluctuation test wells was optimized to 130 µM in the Ames II protocol such that there was a doubling in the optical density of the culture as measured at 600 nm in the Exposure medium during the 90-min exposure period for the solvent control. Since the cells were not washed after the exposure period, 130 µM histidine was estimated to be sufficient to support 1-2 cell divisions over the course of the assay. This in contrast to the preincubation test where the added histidine is sufficient for 6-8 cell divisions over the course of the assay (unpublished). This difference in the numbers of cell divisions in the presence of the mutagen is expected to have a significant effect on the sensitivity of the test. Therefore the sensitivity of the Ames II test might be improved by increasing both the histidine concentration and exposure time to allow fixing of more pre-mutagenic lesions.

The low spontaneous mutant response seen with some of the TA7000 series strains may increase the sensitivity of the strain to mutagenic damage, but could actually reduce the sensitivity of the test (particularly TA7003). It has been shown in the preincubation test using multiple well plates that maximum statistical sensitivity is obtained when the spontaneous response is 10% of the maximum number of wells [37]. When the solvent control response is 0, 1, or 2 positive wells, the increase in mutant wells cannot be accurately determined in practice. For example, a 3-fold increase of the spontaneous response could still leave the numbers of induced mutants too low to be adequately evaluated, or to provide confidence in a positive response.

Although TA7003 was shown to be responsive to a number of substances in the standard plate incorporation protocol ([9]; unpublished data), it did not respond to any of the chemicals used here. Whether because of its low spontaneous response, or as a function of the selection of test chemicals, it should be noted that none of the test chemicals produced an increase in this strain, whereas all other strains responded to at least one chemical. Because this strain was more responsive under plate test conditions [9], it is possible that a number of the chemicals tested here might revert TA7003 had the exposure been carried out under those conditions.

#### 4.3. Genetic targets

Many factors affect the sensitivity or the ease with which a strain is reverted by potential mutagens. Some parameters such as consistent pipeting were addressed somewhat by robotics liquid transfers, and consistent dosing procedures; however, others depend on the reactivity of the target base and the context of the adjacent DNA sequence. The unresponsiveness of the TA7003 target site may be related to its context in the gene, and putting this base change in a different context may change its reactivity to mutagens.

Because TA100 has been shown to revert by 4 out of the 6 possible base substitutions, and by missense suppression [4], and it had been tested with all 25 chemicals, we compared it with the results from the Mix. We found that the Mix was only 71% in agreement with TA100; however, if the S9 specificity was ignored, that agreement was increased to 79% (Table 4<sup>12</sup>). Thus in spite of the two extra pathways of reversion offered by the Mix (TA7001 and TA7002) not found in TA100, it did not identify 25% of the mutagens tested in this study. T:A to A:T transversions and A:T to G:C transitions, which are detectable by the strains in the Mix, have not been detected in reversions of *hisG46* strains, including TA100.

While it was expected that the response from any culture of an individual strain would be more predominant than that of the Mix, trichloroacetonitrile was positive only when tested in the Mix. The significance levels of  $\alpha = 0.007, 0.016, \text{ and } 0.003$ for TA7004, TA7006 and TA98, respectively, did not reach the level ( $\alpha = 0.001$ ) required to classify the responses as positive. Previous reports of the mutagenicity of trichloroacetonitrile were inconsistent. It was weakly positive in the NTP preincubation test [17], but was judged non-mutagenic in another laboratory using the same protocol [38]. A study using the plate incorporation assay found it to be non-mutagenic [39]. However, another study using only TA100 in the fluctuation test, which is most comparable to the Ames II test, found trichloroacetonitrile to be mutagenic [40].

The Ames II test failed to classify 1-chloro-2-propanol as a mutagen, although one experiment out of the triplicate gave a dose response up to 6-fold in TA7004, but this was not reproducible. Although responses were weak in TA100, 1-chloro-2-propanol was clearly mutagenic in TA1535. The strains TA1535 and TA100 both carry the *hisG46* mutation in very similar genetic backgrounds, except that TA1535 does not carry the R factor [41]. The genetic background of the TA7000 strains (Table 1) are thought to be comparable to that of TA100, although not isogenic with TA100. Others have reported positive results with this chemical using both the liquid preincubation and the plate incorporation protocols [42,43].

Isobutyl nitrite reverted TA1535 in a dose-related manner in the presence of 10% hamster S9, and there was an approximate 2-fold dose response in TA100 with 10% rat and hamster S9 up to 6.6 mg per plate, and slightly less than the response without S9 [17]. These results were consistent with others reported in the literature in which the standard plate incorporation, liquid preincubation and vapor exposure protocols were used [44,45].

Benzaldehyde has been reported to be non-mutagenic using both preincubation and plate incorporation protocols in the traditional strains, TA98, TA100, TA1535 and TA1537 [20,46,47]. We found that it caused C:G to A:T transversions as identified by a reproducible response in TA7005. Although the target codon in mutant allele (*his46*) in both TA100 and TA1535 has been known to revert by C:G to A:T transversions, the sequence context and/or the difference in genetic background probably accounts for the difference in the response to benzaldehyde.

#### 4.4. Summary

The AMAX test, partial automation of a modified fluctuation assay using base-specific strains developed to determine mutagenicity in a Salmonella/microsomal reversion system, gave results comparable to those found in the NTP database for 25 chemicals. Some of the differences seen between the two procedures may result from differences in test protocol. The high concordance with the traditional Salmonella test, and the reproducibility among cultures and replicates, demonstrate that the Ames II test procedure using the Ames II tester strains with TA98 and TA1537, is an effective screen for identifying Salmonella mutagens.

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# Statistical modeling and analyses of a base-specific Salmonella mutagenicity assay

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#### Abstract

Statistical features of a base-specific *Salmonella* mutagenicity assay are considered in detail, following up on a previous report comparing responses of base-specific *Salmonella* (Ames  $II^{TM}$ ) strains with those of traditional tester strains. In addition to using different *Salmonella* strains, the new procedure also differs in that it is performed as a microwell fluctuation test, as opposed to the standard plate or preincubation test. This report describes the statistical modeling of data obtained from the use of these new strains in the microwell test procedure. We emphasize how to assess any significant interactions between replicate cultures and exposure doses, and how to identify a significant increase in the mutagenic response to a series of concentrations of a test substance. © 2000 Elsevier Science B.V. All rights reserved.

*Keywords:* Ames II strains; *Salmonella typhimurium*; Complementary log–log link function; Culture-by-dose interaction; Fluctuation test; Generalized linear model; *His*<sup>-</sup> mutant tester strains; Many-to-one testing; Multiple comparisons with a control; Statistical methods

#### 1. Introduction

The *Salmonella*/microsome reversion assay has been used extensively in genetic toxicology testing [1-4]. The procedure employs bacterial tester strains that identify the reversions of missense and small frameshift mutations in the *his* operon. Despite the widespread acceptance of this test, new *Salmonella* 

tester strains are constantly being developed and studied. In a previous report [5], we discussed the use and validity of a series of six new  $his^-$  mutant strains (TA7001, TA7002, TA7003, TA7004, TA7005 and TA7006), each of which was designed to revert to *his* independence by unique base-pair substitutions [6]. The TA700X series of tester strains has been designated "Ames II<sup>TM</sup>" (Xenometrix, Boulder, CO, USA).

In order to help automate the data collection process, and to allow the assay to be adapted to high-throughput, robot-controlled procedures, a modified fluctuation protocol [7,8] has been developed

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for use with the TA700X tester strains (AMAX<sup>™</sup>: Ames II Mutagenicity Assays by Xenometrix). In our previous report on the AMAX<sup>TM</sup> procedure, the performance of these strains was compared with the results obtained using the traditional Salmonella tester strains in a preincubation procedure [4,5]. Thirty coded chemicals (five of which were duplicates with different code numbers) were tested in the individual strains TA7001-TA7006 to identify mutagens that produced base-pair substitutions, in a mixture of these six strains, and in the traditional strains TA98 and TA1537 to detect frameshift mutagens. All testings were done using a modified liquid fluctuation test procedure as designated in the AMAX<sup>TM</sup> protocol. The results were compared to results obtained previously using a preincubation protocol with strains TA98, TA100, TA1537 (or TA97) and TA1535 [5].

One important component of the validation effort in this previous study was proper recognition and adjustment for the various sources of statistical variability in the AMAX<sup>™</sup> data. However, the statistical analysis of those data was conducted at an introductory level only. The current manuscript presents more detailed statistical aspects of the AMAX<sup>TM</sup> assay, based on the data from the previous validation study. The issues discussed include the aspects of the sampling protocol, the possible interactions between cultures and exposures to mutagens, and the identification of exposure-related effects using a form of generalized linear statistical model. Recommendations are made for statistical analysis of microwell fluctuation test (e.g., AMAX<sup>™</sup>) data from these Salmonella strains.

#### 2. Methods

#### 2.1. AMAX<sup>TM</sup> protocol and experiments

Eight individual *Salmonella typhimurium* strains and a mixture of the base-specific strains were employed in our previous report, where the strains and their genotypes are described [5]. Each of the six base-specific strains (TA7001–TA7006) carries a target missense mutation in the histidine operon, which reverts to prototrophy by base-substitution events unique to each strain. Strains TA7001, TA7002 and TA7003 detect base substitutions at A:T base pairs, while TA7004, TA7005 and TA7006 detect base changes at G:C base pairs.

The TA700X strains are not designed to identify mutagens that induce only frameshift mutations. Thus, routine tests of unknown chemicals must also use one or two of the traditional strains that are designed to detect frameshift mutations, viz. TA98 and TA1537. For the purposes of the previous study [5], these two strains were used in the AMAX<sup>TM</sup> procedure along with the TA700X strains.

All 30 coded chemicals were studied without exogenous metabolic activation. Those that appeared to be negative in the initial test (18 out of 30) using a subjective, non-statistical assessment were also tested in the presence of S9 (4.5% rat liver S9 fraction), in order to allow for possible metabolic activation. Protocols used to generate the data analyzed herein were detailed in our previous report [5]. Data were collected for a positive control, five doses of each test chemical (including a zero-dose control), and for a solvent control. Each culture was treated independently with the test chemical in a total exposure volume (Exposure Medium) of 0.5 ml per dose, in triplicate. Following exposure, 2.5 ml of selective medium (Indicator Media) was added and 50 µl aliquots were dispensed into 48 wells of 384-well microtiter plates, either manually or using a ML 2200 pipeting station (Hamilton, Reno, NV). Each experiment was performed using three independent cultures by one of two teams of experimenters on the same day and/or up to 3 weeks later. Each culture was inoculated from separate frozen vials, although each vial of cells used for the study was from the same production lot. Each chemical was tested up to 5 mg, the maximal tolerated dose, or the limits of the solubility, whichever was reached first.

A concern noted previously [5] with this assay system is that under otherwise-homogeneous preparation, a few cultures may exhibit an extreme response, called *jackpot mutations*. Jackpots result from reversion events that occur early in the growth of the overnight culture, such that the wild-type revertant population expands during the overnight growth and pre-exists in the culture before exposure to the test agent. High spontaneous counts are usually attributed to jackpot mutations, and may obscure any increase in reversion events caused by the test agent. The frequencies of jackpots for the different strains correspond to each strain's individual inherent genetic instability. For example, 6% of cultures of TA1537 exhibited high spontaneous positive wells, while TA7001 and TA7006 cultures did not exhibit any jackpot mutations.

Where jackpot mutations were observed to obscure the results, the experiments were repeated. The final data set may have included experiments where jackpot mutations did not affect the overall results and were therefore included in the analysis. Statistically, the presence, or even the possibility of jackpots associated with individual cultures must be viewed as a source of additional variability in this assay, and some adjustment or correction must be made to account for it in the analysis (see below).

#### 2.2. Statistical models

The basic experimental design of the AMAX<sup>TM</sup> assay for any given chemical (in the presence or absence of S9) consists of an exposure regimen involving an untreated control, a solvent control, a positive control, and four increasing doses of the chemical. The doses are indexed via k = 0, 1, ..., 4, where k = 0 indicates the untreated (zero-dose) control. Each dose was tested in triplicate in aliquots of each independent culture, and each experiment was performed three times, thus using three different overnight cultures of the *Salmonella* strains. This design yielded three experiments, each consisting of a series of solvent controls and chemicals tested in triplicate.

Statistically, the experimental response is a set of dichotomous outcomes in 48 wells of a 384-well plate. Each well is scored for growth of  $his^+$  reverent bacteria (growth is indicated by a positive yellow well, vs. an otherwise purple well), and we record 0 for purple/negative growth, or 1 for yellow/positive growth.

For the *i*th plate (i = 1,2,3) in the *j*th culture (j = 1,2,3) at the *k*th dose level, we denote by  $X_{ijk}/48$  the proportion of mutagenic wells observed for a given combination of chemical/strain/S9. In this design, cultures are *crossed* with dose levels, representing a form of *two-way design* [9]. Because

each culture appears at each dose level an equal number of times, the two-way design is *balanced*.

The standard statistical model for  $X_{ijk}$  is the binomial distribution [10]. Data across plates within any culture/dose combination are assumed homogeneous; we pool these values into a single summary proportion, say  $Y_{jk}/144 = \{X_{1jk} + X_{2jk} + X_{3jk}\}/144$ , and write  $Y_{jk} \sim \text{Binomial}(144, \pi_{jk})$ , where  $\pi_{jk}$  is the (unknown) probability of mutation in the *j*th culture at the *k*th dose level.

To adjust for the effect of jackpots and to analyze more generally the mutant proportions for any chemical/strain/S9 combination, we chose to take advantage of the balanced feature of the treatment design through a form of analysis of variance (ANOVA) appropriate for binomial proportions. Specifically, we applied a generalization of the common ANOVA model, known as the Generalized Linear Model, or GLiM [11]. A GLiM can involve two specialized components: (i) a statistical model for the data other than the normal (in our case, based on the observed binomial proportions), and (ii) a function that links the unknown mutation probability  $\pi_{ik}$  to the features of the treatment design. For component (i), we incorporated the binomial assumption on  $Y_{ik}$ ; for component (ii), we recognized an experimental feature that induces a specialized form of link function. Specifically, we assumed that the random number of mutations per well, say  $U_{iik}$ , is described by a Poisson distribution with unknown, positive mutation rate  $\lambda_{ijk} > 0$ . Of course,  $U_{ijk}$  is unobservable; all that is recorded is whether any mutations occurred in a given well. Thus, the per-well Poisson variate  $U_{ijk}$  is truncated to the dichotomous observation  $X_{iik}$ , which equals 1 if any mutations occurred in that well, and 0 otherwise. Denote the probability that  $X_{ijk}$  equals 1 by  $\phi_{iik}$ . Then under this truncated Poisson model,  $\phi_{iik}$  is given by

$$\phi_{ijk} = P[X_{ijk} = 1] = P[U_{ijk} \ge 1]$$
  
= 1 - P[U\_{ijk} = 0] = 1 - exp{-\lambda\_{ijk}},

the latter equality following from the basic form of the Poisson probability mass function [10]. Assuming that triplicate wells are homogeneous, and thus, that no per-plate effects are present, we may drop the *i* subscript in  $\lambda$ . The usual ANOVA formulation for the mean response under a two-way design is a linear combination of the unknown effect parameters:

$$\mu + \gamma_j + \delta_k + \psi_{jk}, \tag{1}$$

where for our setting,  $\mu$  is an overall effect parameter,  $\gamma_j$  is a term for the culture effect,  $\delta_k$  is a term for the dose effect, and  $\psi_{jk}$  represents a possible *interaction* between culture and dose. In Eq. (1),  $\gamma_j$ represents a "blocking" term that accounts for any jackpot-related culture-to-culture variability.

In most ANOVA settings, the linear expression in Eq. (1) is set equal to the mean response and analyzed accordingly. Here, however, equating (1) to the mean mutation rate  $\lambda_{jk}$  fails to account for the constraint that  $\lambda$  must be positive. To overcome this, we can model  $\lambda_{ik}$  itself as an exponential form:

$$\lambda_{jk} = \exp\{\mu + \gamma_j + \delta_k + \psi_{jk}\}.$$
 (2)

Under Eq. (2),  $\lambda_{jk}$  is guaranteed to be positive for any realization of the linear effect parameters in Eq. (1).

Collecting all of these model components together, the resulting GLiM may be written as  $Y_{jk} \sim$ Binomial(144, $\pi_{jk}$ ), where  $\pi_{jk} = 1 - \exp\{-\exp[\mu + \gamma_j + \delta_k + \psi_{jk}]\}$  and  $Y_{jk}$  is the sum over the three replicate plates of the mutant wells at the *j*th culture (*j* = 1,2,3) and the *k*th dose (*k* = 0,1,2,3,4) for any chemical compound being tested. Inverting this model for  $\pi$  gives:

$$\log\left\{-\log(1-\pi_{jk})\right\} = \mu + \gamma_j + \delta_k + \psi_{jk}, \qquad (3)$$

which is known as a complementary log-log GLiM [10,11].

#### 2.3. Statistical analyses

Under the complementary log-log GLiM in Eq. (3), we can assess whether there is an effect due to the dose after correcting for possible culture-to-culture variability. As is well known, however, it is inappropriate to test for any main effects due to individual factors, such as dose, in the presence of a significant interaction. Indeed, *P*-values for testing the main dose effect possess no sensible interpretation if given in the presence of a significant interaction (see Ref. [9]). Thus, before assessing the dose-related effects for any chemical/strain/S9

com-bination under study, we first must test the null hypothesis of no interaction. This translates to H<sub>o</sub>:  $\psi_{jk} = 0$  for each *j*,*k*, vs. an alternative hypothesis that  $\psi_{jk} \neq 0$  for some combination of *j* and *k*. H<sub>o</sub> may be assessed via a likelihood ratio test, which is similar in form to the usual *F*-test for the interaction in a block design/ANOVA. Under our design, the likelihood ratio statistic,  $G_{\psi}^2$ , for the culture × dose interaction is distributed as  $\chi^2$  with (5 - 1)(3 - 1)= 8 degrees of freedom (*df*). Departure from H<sub>o</sub> is indicated if the *P*-value  $P = P[\chi^2(8) \ge G_{\psi}^2]$  drops below a pre-assigned  $\alpha$ -level.

The likelihood ratio computations must be performed on a computer. We employ the SAS<sup>®</sup> computer package via its GLiM procedure Proc Genmod [12]. [Sample SAS code for fitting the complementary log-log model under Eq. (3) is given in Fig. 1. To identify the likelihood ratio statistic in Proc Genmod, invoke the Type 1 option in the Model statement, being sure to order the model components with culture first, dose second, and culture \* dose last.]

If a particular chemical/strain/S9 combination of interest tests negative for culture × dose interaction, we can move directly to testing the main effect due to dose. This translates to the null hypothesis  $H_o: \delta_0 = \delta_1 = \cdots = \delta_4$ . The alternative hypothesis,  $H_a$ , is that some departure from pure equality exists among the  $\delta_k$ s. The SAS output again provides a likelihood ratio statistic,  $G_{\delta}^2$ , that is referenced to a  $\chi^2$  distribution with  $5 - 1 = 4 \ df$ ; the corresponding *P*-value is  $P = P[\chi^2(4) \ge G_{\delta}^2]$ . When *P* drops below a pre-assigned  $\alpha$ -level, there is a departure from  $H_o$ , and hence, some dose effect is indicated.

If significant, the dose effect can take on many forms. Of interest in a mutagenicity testing setting is the set of one-sided departures from the control,  $H_{ak}$ :  $\delta_k > \delta_0$  (k = 1, ..., 4). If any such alternative hypothesis is significant at the *k*th dose, it indicates a significant, dose-related mutagenic effect. Notice that we can also write  $H_{ak}$  as  $H_{ak}$ :  $\delta_k - \delta_0 > 0$ .

To test against these one-sided alternatives, SAS' Proc Genmod is particularly useful. First, refit the model after removing the culture × dose interaction. Then, under the Proc Genmod output for Analysis of Parameter Estimates, find the point estimates of the dose effect parameters. Information in these point estimates is employed in testing against the alternatives  $H_{ak}$ :  $\delta_k - \delta_0 > 0$ .

```
*Sample SAS code to fit complementary log-log GLiM;
*Includes test for culture*dose interaction;
data work1;
infile 'data_file_name_here' ;
    *Ensure that the control level is coded as last;
input dose culture y n;
proc genmod;
class culture dose;
model y/n = culture dose culture*dose / dist=b link=cll type1;
* if interaction is insignificant, re-fit with interaction term removed;
proc genmod;
class culture dose;
model y/n = culture dose / dist=b link=cll ;
run;
```

Fig. 1. Sample SAS Proc Genmod complementary log-log GLiM code for fitting a two-way model with interaction terms.

Users are cautioned to proceed carefully, however, since the outputs from the Proc Genmod analysis do not produce point estimates of each  $\delta_k$ . As is common with two-factor models such as Eq. (1), there are certain identifiability constraints required to complete the fit [9]; SAS defaults to a reference-cell constraint, where the last  $\delta$ -parameter is set equal to zero. As a result, the reported SAS "parameter estimates" turn out to be estimates of the *differences*  $\delta_k - \delta_4$ ,  $k = 0,1,\ldots,4$ . Admittedly, this SAS artifact can add confusion to the analysis, but it is a necessary consequence of the two-factor model being employed in Eq. (1).

For testing against  $H_{ak}$ , this differencing can nonetheless be manipulated to our advantage. The quantities we wish to study are differences from the control, so if in the SAS input code, we enter and code the dose levels such that the control level is *last* — say, discard the k = 0 subscript and *relabel the control as* k = 5 — the corresponding "parameter estimates" will relate to the differences  $\delta_1 - \delta_5$ ,  $\delta_2 - \delta_5, \dots, \delta_4 - \delta_5$ . These are the precise differences we wish to estimate.

Under this relabeled scheme, denote the SAS estimates as  $d_k - d_5$ . The SAS output also supplies standard errors, se $[d_k - d_5]$ , from which a Wald statistic for testing against  $H_{ak}$  is calculated as  $W_k = (d_k - d_5)/\text{se}[d_k - d_5]$ . This is referenced in large

samples to a standard normal distribution, with corresponding one-sided *P*-value  $P_k = 1 - \Phi(W_k)$ . [The function  $\Phi(z)$  is the cumulative distribution function of the standard normal.] However, further caution is advised here: the SAS output also reports *P*-values under the heading Pr > Chi, but these are actually *two*-sided *P*-values. To convert them to the one-sided values we desire, use the following rule: (i) if the output "parameter estimate"  $d_k - d_5$  is zero or positive, divide SAS' output *P*-value by two to find  $P_k$ , or (ii) if the "parameter estimate"  $d_k - d_5$  is negative, divide SAS' output *P*-value by two *and subtract this from* 1.0 to find  $P_k$ .

It is important to recognize that in most cases, this analysis of the dose-effect will be performed at all non-zero dose levels. Thus, e.g., under our k = 5construction, there are four separate significance tests being performed for the dose effect. Each is a comparison of a specific dose level against the control level; hence this is often called a *multiple comparison with the control*, or a "MCC." (Some authors also call this a *many-to-one* analysis.) Due to the multiple comparisons being performed, however, there will be an inflation in the false positive error rate for testing the dose effect. One possible MCC adjustment to account for error inflation that operates well with binomial GLiMs is a simple Bonferroni correction [13]: this amounts to multiplying the raw

```
*Sample SAS code to fit complementary log-log GLiM;
*Assumes culture*dose interaction is significant;
data work1;
infile 'data_file_name_here' ;
 *Ensure that the control level is coded as last;
input dose culture y n;
proc sort; by culture;
proc genmod;
by culture;
class dose;
model y/n = dose / dist=b link=cll type1;
run;
```

Fig. 2. Sample SAS Proc Genmod complementary  $\log -\log$  GLiM code for fitting a one-way model of only dose. Model assumes a significant culture  $\times$  dose interaction and consequently stratifies the analysis over levels of culture.

*P*-value by the number of individual comparisons being made. In our case, the MCC-adjusted *P*-value at each of the four dose levels is  $P_k^* = 4\{1 - \Phi(W_k)\}$ . Reject in favor of the one-sided alternative  $H_{ak}$  if  $P_k^*$  drops below  $\alpha$ .

## 2.4. Statistical analysis under significant culture $\times$ dose interaction

When a particular chemical/strain/S9 combination tests positive for culture × dose interaction, the MCC analysis described above must be modified, since we cannot make interpretable inferences about the main dose effect in the presence of a significant interaction. In this case, we are forced to assess the possible dose effects at a simpler level. Specifically, we test for a dose-related increase by assessing the dose effect at each level of culture — under our design this is at each j = 1,2,3. In effect, we *stratify* the dose analysis over the levels of culture.

The computations for this stratified analysis are no more complex than those for testing the main effects; sample SAS code for this is given in Fig. 2. The resulting output contains a dose analysis at every level of the culture indicator. In each case, conduct the analysis in the same manner as above, i.e., read the parameter differences  $d_k - d_5$  from the Analysis of Parameter Estimates output, calculate the correct one-sided  $P_k$ -values, adjust the  $P_k s$  for multiplicity via a Bonferroni correction, etc. If any of the three per-culture tests of dose effect indicates a significant increase over the corresponding control response, we judge the dose effect to be significant.

## **3.** Results: example with cumene hydroperoxide and nitrofurantoin

To illustrate the complementary log-log analysis, we selected two different chemicals from our previous study [5]: cumene hydroperoxide (CASRN 80-15-9) and nitrofurantoin (CASRN 67-20-9). The first represents a case of no culture  $\times$  dose interaction so that main-effect testing is warranted; the second illustrates a case where a significant culture  $\times$  dose interaction requires a stratified analysis.

#### 3.1. Example 1: cumene hydroperoxide

Cumene hydroperoxide is a chemical intermediately used to synthesize organic peroxides for the

Table 1

Proportions of positive wells in Ames  $II^{\rm \tiny TM}$  Strain TA7006 after exposure to cumene hydroperoxide. Case: no S9 activation

Replicate	Dose inde	ex (doses (i	n µg/ml)	)	
culture	k = 5(0)	k = 1 (1)	k = 2 (5)	k = 3 (10)	k = 4 (25)
j=1	3/144	1/144	6/144	21/144	4/144
j = 2	4/144	3/144	15/144	17/144	3/144
j = 3	1/144	3/144	11/144	13/144	2/144

Dose level, <i>k</i>	MCC comparison	Estimated difference	SAS two-sided <i>P</i> -value <sup>a</sup>	Upper one-sided <i>P</i> -value	Bonferroni adjusted $P_k^*$ -value
1	$\delta_1 - \delta_5$	-0.1353	0.7938	0.6031	N.S. <sup>b</sup>
2	$\delta_2 - \delta_5$	1.4156	0.0003	0.0002	0.0007
3	$\delta_3 - \delta_5$	1.9054	$5.43 \times 10^{-7}$	$2.72 \times 10^{-7}$	$1.09 \times 10^{-6}$
4	$\delta_4 - \delta_5$	0.1185	0.8073	0.4037	N.S.

Results from complementary log–log analysis of data from Ames II<sup>™</sup> Strain TA7006 after exposure to cumene hydroperoxide. Case: no S9 activation (data from Table 1)

<sup>a</sup>SAS typically limits its output *P*-values to only four digits of accuracy. For values of P < 0.0001, we have supplied more accurate values based on direct computations.

<sup>b</sup>N.S. = Not significant.

Table 2

manufacture of plastic resins and polymerization catalysts. Table 1 contains proportion response data from the original study in Ames II<sup>TM</sup> Strain TA7006 with no S9 activation. Notice the coding of the control dose as k = 5.

Applying the SAS code in Fig. 1 to these data results in the following likelihood ratio test for the culture × dose interaction:  $G_{\psi}^2 = 8.3326$ , with *P*-value P = 0.4017. At  $\alpha = 0.05$ , this is insignificant, so we continue with this analysis and move to the tests of the dose main-effect. The overall likelihood ratio statistic for the dose effect is  $G_{\delta}^2 = 71.163$ , with a *P*-value of P < 0.0001. Although significant, this omnibus *P*-value provides no guidance regarding which dose levels deviate significantly from the control, and also judges departures below the control level equal in importance to those that exceed the control level. For a more pertinent analysis, the one-sided MCC analysis described above is required.

The SAS output for the main-effect MCC analysis gives the results in Table 2. From the Bonferroni-adjusted  $P^*$ -values, we see that a significant increase in mutagenic response over the control is observed at the middle two dose levels (P < 0.001 in both cases). This represents definitive evidence of mutagenicity for this chemical in Ames II<sup>TM</sup> Strain TA7006.

#### 3.2. Example 2: nitrofurantoin

The pharmaceutical product nitrofurantoin is a potent germicide employed to treat urinary tract infections. Proportion response data from the original study in Ames II<sup>TM</sup> Strain TA7004 with no S9 activation are given in Table 3. Again, notice the coding of the control dose as k = 5.

Applying the SAS code in Fig. 1 to these data results in the following likelihood ratio test for culture  $\times$  dose interaction:  $G_{\psi}^2 = 47.3442$ , with a Pvalue of P < 0.0001. At  $\alpha = 0.05$ , this is significant, so to analyze the dose effect, we must turn to a culture-stratified analysis. Applying the SAS code in Fig. 2 yields the results given in Table 4. (Notice that the Bonferroni-adjusted P\*-values are the raw one-sided values multiplied now by 12. This is because there are  $4 \times 3 = 12$  different MCC comparisons being performed for this data set.) From the Bonferroni-adjusted P\*-values, we observe significant increases in mutagenic response at the two middle doses for all the three cultures, along with significant increases at high dose in the first culture and at the low dose in the last culture. We view this pattern of consistent increases across cultures as indicative of a significant mutagenic effect, rather

Table 3

Proportions of positive wells in Ames II<sup>™</sup> Strain TA7004 after exposure to nitrofurantoin. Case: no S9 activation

Replicate	Dose index (do	ses (in μg/ml))				
culture	k = 5 (0)	$k = 1 \ (0.1)$	k = 2 (0.5)	k = 3 (1.0)	k = 4 (5.0)	
j = 1	5/144	6/144	44/144	76/144	36/144	
j = 2	6/144	18/144	50/144	70/144	5/144	
j = 3	2/144	18/144	53/144	92/144	13/144	

Table 4

Results from a complementary log-log analysis, stratified by level of culture, of data from Ames II<sup>TM</sup> Strain TA7004 after exposure to nitrofurantoin. Case: no S9 activation (data from Table 3)

Dose level, k	MCC comparison	Estimated difference	SAS two-sided <i>P</i> -value <sup>a</sup>	Upper one-sided <i>P</i> -value	Bonferroni adjusted $P_k^*$ -value
Culture: $j = 1$					
1	$\delta_1 - \delta_5$	0.1859	0.7588	0.3794	N.S. <sup>b</sup>
2	$\delta_2 - \delta_5$	2.3339	$7.71 \times 10^{-7}$	$3.85 \times 10^{-7}$	$4.62 \times 10^{-6}$
3	$\delta_3 - \delta_5$	3.0555	$3.90 \times 10^{-11}$	$1.95 \times 10^{-11}$	$2.23 \times 10^{-10}$
4	$\delta_4 - \delta_5$	2.0969	$5.63 \times 10^{-6}$	$1.13 \times 10^{-5}$	0.0002
Culture: $j = 2$					
1	$\delta_1 - \delta_5$	1.1434	0.0153	0.0076	0.0918
2	$\delta_2 - \delta_5$	2.3047	$9.82 \times 10^{-8}$	$4.91 \times 10^{-8}$	$5.89 \times 10^{-7}$
3	$\delta_3 - \delta_5$	2.7500	$1.08 \times 10^{-10}$	$5.40 \times 10^{-11}$	$6.48 \times 10^{-10}$
4	$\delta_4 - \delta_5$	-0.1859	0.7588	0.6206	N.S.
Culture: $j = 3$					
1	$\delta_1 - \delta_5$	2.2563	0.0025	0.0013	0.0150
2	$\delta_2 - \delta_5$	3.4909	$1.27 \times 10^{-6}$	$6.35 \times 10^{-7}$	$7.62 \times 10^{-6}$
3	$\delta_3 - \delta_5$	4.2881	$2.05 \times 10^{-9}$	$1.02 \times 10^{-9}$	$1.23 \times 10^{-8}$
4	$\delta_4 - \delta_5$	1.9118	0.0118	0.0059	0.0708

<sup>a</sup>SAS typically limits its output *P*-values to only four digits of accuracy. For values of P < 0.0001, we have supplied more accurate values based on direct computations.

<sup>b</sup>N.S. = Not significant.

than as a series of random jackpot increases. This represents definitive evidence of mutagenicity for this chemical.

#### 4. Discussion

We find the complementary log-log GLiM to be a useful model under which to test interaction and MCC dose effects when presented with data in the form of proportions (i.e., number of positive wells divided by total number of wells) from the AMAX<sup>™</sup> protocol. Facilitated by the use of simple SAS programming code, the statistical methods can be easily implemented. Using the SAS output, multiplicity-adjusted  $P^*$ -values are straightforward to calculate and can yield proper inferences on the ability of a chemical or environmental agent to induce mutagenesis in these Ames II<sup>™</sup> strains. The examples displayed above were chosen to be representative of the larger body of experimental results reported from our previous, larger study of the AMAX<sup>™</sup> protocol [5]. Both illustrate the methodology and show qualitative agreement with our corresponding previous results.

It is interesting to note that in both examples, the dose response is non-monotone, i.e., in both Tables 1 and 3, there is a consistent increasing-then-decreasing trend in the response as dose increases. This is not uncommon with Ames test data [14], and we were not surprised by the phenomenon here. The MCC methods we apply to detect the increases above the background response are designed to be unaffected by such downturns. (Although this is at the cost of some sensitivity to detect a monotone-increasing trend over dose, if one did exist. If desired, a trend test that accounts for the downturns may be useful, such as that suggested in Ref. [15] or Ref. [16]. The issue of testing non-monotone trend specifically with the proportion data is problematic, however [17,18], and is open for further statistical research.)

One additional problem for further statistical study concerns the small-sample properties of the Bonferroni-adjusted MCC inferences that form the core of our dose analysis. Previous research [13] has suggested that the Bonferroni correction exhibits acceptable false positive error properties for binomial-based GLiMs under a simple one-way model (say, with only a single factor such as Dose). The correction is generally conservative in that it protects against false-positive errors too strenuously, but as the sample size increases, this conservative nature tends to lessen somewhat. Whether this performance carries over to the two-way setting with interaction, as studied herein, is unclear. Clearly, more research is required in this area.

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## **AMES II ASSAY: RESULTS OF A VALIDATION STUDY**

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## A) AMES II ASSAY / METHOD

#### L TEST SYSTEM

#### The Ames II assay of Xenometrix is a liquid microtiter modification of the traditional Ames test for the detection of potential mutagens in Salmonella typhimurium.

- Media and tester strains, except S9-mix, are available as a kit
- The test is performed in microwell plates
- Mutagenicity (growth of bacteria) is measured colorimetrically from purple to yellow (pH change)
- The Ames II assay uses the so-called "mixed strains" (TAMix) → a mixture of 6 newly developed base-pair strains of the TA7000 series for the detection of base-pair mutations. Each strain will be reverted by only one specific base-pair substitution
- The Ames II assay is available in two versions: → "N → "H

lanual kit" (benchtop ve	rsion for routine analysis)	
igh throuput screening	(HTS)" (automatable version)	

e Ames I Mix (mix	assay is perfi ed strains TA7	ormed with th '001 - 7006, b	e tester strains ase-pair subsi	TA98 (framshift itutions).	mutations) ar	nd
STRAIN	MUTATION	TYPE	TARGET	CELL WALL	REPAIR	pKM10
TA98	hisD3052	frameshift	GC	rfa	uvrB	+



II. TESTER STRAINS

Th



## 384-Well Plate Medium 37°C, 48 h

#### IV: ADVANTAGES OF THE AMES II ASSAY

- ♦ Routine analysis → compound throughput is ~ 5 times higher with the "Ames II Manual System" than with the traditional Ames test
- Screening (HTS) → ~ 1'000 compounds / year / robot / technician with a partly automated version

#### V: LIMITATIONS

 At present not applicable for registrations/authorizations of new chemicals/pesticides/drugs

→ until now no existing guideline → until now no acceptance by the authorities

### **B) AMES II ASSAY / VALIDATION STUDY**

SENSIT

SPECIE

ACCUR

SPECIFIC

+

+

+

#### I. AIM

Validation of a high throughput screening version (HTS) of the Ames II assay (= automated version > single experiment without replicates) using selected genotoxic/non-genotoxic compounds. Comparison with the classical Ames assay (Ames I assay) with regard to:

Concordance of the results between the two test systems

 Sensitivity (percentage of correctly identified genotoxic/carcinogenic compounds) and specificity (percentage of correctly identified non-genotoxic/non-carcinogenic compounds) of the two test systems

#### II. TEST COMPOUNDS

- 127 compounds (1st comparison) including different chemical classes were selected according to the criteria listed below
- → negative in the traditional Ames assay, possibly positive in other,
- non-bacterial genotoxicity tests → positive in the Ames plate incorporation assay, partly in different tester strains → positive only when using a modification of the Ames assay. (e.g. pre-incubation test, prival modification, liquid suspension assay, addition of norharman etc.)
- For 95 compounds with different genotoxic profiles there are sufficient additional in vitro- and/or in vivo data to allow an assessment for genotoxicity
- (2<sup>nd</sup> comparison)
- For 70 compounds there are sufficient data to allow an assessment for carcinogenicity (3rd comparison)

#### III RESULTS

#### 1. COMPARISON OF THE TWO AMES TEST SYSTEMS **RESULTS OBTAINED WITH 127 COMPOUNDS**

AGREEME	NT ca. 75%	AMES	IIASSAY	
		negative	positive	
AMES I	negative	41 (32.3%)	16 (12.6%)	
ASSAY	positive	16 (12.6%)	54 (42.5%)	

#### IV. CONCLUSIONS

#### The percentage of correctly identified >genotoxic/carcinogenic compounds (= sensitivity)

> non-genotoxic/non-carcinogenic compounds (= specificity) of the two Ames test versions is comparable About ¾ of all compounds are correctly identified by both assay systems

In addition, each assay system correctly detects different compounds (possible reasons: different methodology, different strains, different concentrations of S9-mix)

The Ames II assay is therefore suitable for the screening of mutagens/genotoxic carcinogens



#### 2. COMPARISON OF THE RESULTS OF THE TWO AMES TEST SYSTEMS: GENOTOXICITY DATA (95 COMPOUNDS)

	AMESIASSAY	AMES II ASSAY
VITY <sup>1)</sup>	55/75 73.3%	50/75 66.7%
CITY <sup>2)</sup>	18/20 90.0%	16/20 80.0%
ACY <sup>3)</sup>	55+18 = 73/95 76.8%	50+16 = 66/95 69.5%

#### 3. COMPARISON OF THE RESULTS OF THE TWO AMES TEST SYSTEMS: CARCINOGENICITY DATA (70 COMPOUNDS)

	AMES I ASSAY	AMES II ASSAY
SENSITIVITY <sup>1)</sup>	36/52 69.2%	35/52 67.3%
SPECIFICITY <sup>2)</sup>	11/18 61.1%	11/18 61.1%
ACCURACY <sup>3)</sup>	36+11 = 47/70 67.1%	35+11 = 46/70 65.7%





Correctly identified + = carcinogenic - = non-carcinogenxic



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## SENSITIN

# From Hazard to Risk

European Environmental Mutagen Society 33<sup>rd</sup> Annual Meeting

> organised by the UK Environmental Mutagen Society

> > August 24-28, 2003

Aberdeen Exhibition and Conference Centre, Aberdeen, Scotland, UK

> Form AA08 01-2012

The test was performed with rapid end-point determination, with good concordance of results, confirming the lack of inter-laboratory and inter-personnel variation, therefore supporting the use of this screen as a useful and rapid genotoxicity assessment tool.

Considering the original plating procedure was the first trial outside of the developer's laboratory, the results gained were pleasing.

The assay can be taught to skilled technicians within a single day, increasing its ease and frequency of use as a screening tool. Twelve test compounds can be set-up using the manual protocol by a single operator in 2/3 hours, which is a marked improvement over the standard regulatory Ames test or screen.

P120 Assessment of a screening experience with the Ames II™ test and future prospects Véronique Gervais ①, Didier Bijot ① and Nancy Claude ②

Drug Safety Assessment, Servier, Orléans-Gidy, France, DIRIS. Servier, Courbevoie, France

Most pharmaceutical companies look for miniaturized genotoxicity tests which require a minimum amount of drug candidates for an early selection in the discovery process.

The prerequisites for the choice of a miniaturized genotoxicity test are its consumption of small amounts of compound, its possibility to automate, its rapid achievement of results and its good concordance with other genotoxicity tests.

For these reasons, the Servier Drug Safety Department has selected the Ames II™ test, a liquid fluctuation version of the Salmonella mutagenicity assay, provided by Xenometrix GmbH.

This test is composed of the *TA7000* series of tester strains (*TA7001*, *TA7002*, *TA7003*, *TA7004*, *TA7005*, and *TA7006*), which revert by a specific base substitution in the histidine operon. This mixture of six base-specific Salmonella typhimurium strains (also called "mix") is used as if it was one single strain. In addition to the "mix", the frameshift tester strain *TA98* is also used. The treatment performed in microtiter plates allows partial automation, and consequently it requires less test substance than the standard Ames test (about 60-fold less).

Three hundred and fifty compounds were tested, including molecules issued from our own chemistry department, known non- or genotoxicants, or molecules producing equivocal results. The concordance, between the results achieved in this Ames II<sup>™</sup> test and those reported in the literature or in the standard Ames test performed in our company, ranged from 79 to 85 %. No false positive results were obtained with known non-mutagenic substances. But false negative results with the "mix" may arise when chemicals revert only specific strains like TA1535 or E. coli WP2 uvrA (pKM101), which have no equivalent in the "mix".

All these results supported the Ames II<sup>TM</sup> test as a reliable screening tool. However, we are still exploring ways to reconcile the Ames II<sup>TM</sup> test product consumption required (typically 50 mg) with much lower substance amounts supplied by chemistry without lowering the prediction of the test.

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## Assessment of the performance of the Ames II<sup>TM</sup> 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. © 2003 Elsevier B.V. All rights reserved.

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 TA700x 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. ty-phimurium* 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  $10 \,\mu$ l 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  $\mu$ g/ml ampicillin (Xenometrix by Endotell GmbH).

Table 1					
Bacterial	strains	used,	and	the	mixture

Strain	Genotypes	Mutation <sup>a</sup>
TA98	hisD3052 <i>Aara9 Achl008 (bio chl uvrb gal)rfa1004/pKM101</i>	Frameshifts
TAMix	TA7001, TA7002, TA7003, TA7004, TA7005, TA7006	Base-pair
TA7001	hisG1775 $\Delta$ ara9 $\Delta$ chl004 (bio chlD uvrb chlA)galE503 rfa1041/pKM101	$A:T \rightarrow G:C$
TA7002	hisC9138 $\Delta$ ara9 $\Delta$ chl004 (bio chlD uvrb chlA)galE503 rfa1041/pKM101	$T:A \rightarrow A:T$
TA7003	hisG9074 Dara9 Dchl004 (bio chlD uvrb chlA)galE503 rfa1041/pKM101	$T:A \rightarrow G:C$
TA7004	hisG9133 Δara9 Δchl004 (bio chlD uvrb chlA)galE503 rfa1041/pKM101	$G:C \rightarrow A:T$
TA7005	hisG9130 $\Delta$ ara9 $\Delta$ chl004 (bio chlD uvrb chlA)galE503 rfa1041/pKM101	$C:G \rightarrow A:T$
TA7006	hisC9070 $\Delta$ ara9 $\Delta$ chl004 (bio chlD uvrb chlA)galE503 rfa1041/pKM101	$C:G \rightarrow G:C$

<sup>a</sup> 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

Table 2

Chemicals tested

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,

Chemical	CAS no.	MW <sup>a</sup>	Carcinogenicity <sup>b</sup>	Mutagenicity <sup>b</sup>	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- <i>N</i> (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.

<sup>a</sup> Molecular weight.

<sup>b</sup> Assessment according to the ICPESTTC study.

Table 3Positive 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,  $25 \times$  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  $25 \times$  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  $4 \mu g/ml$ , 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  $(500 \,\mu\text{g/ml})$  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  $37 \,^{\circ}$ 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 ( $\times$ ) 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 3F 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  $500 \mu g/ml$  and higher (Fig. 1). Using the robotic system with  $1000 \mu g/ml$  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  $500 \mu g/ml$  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  $5000 \mu g/ml$ .

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 basepair strains of *S. typhimurium* in the presence of metabolic activation [6]. The degree of positive responses varied and concentrations  $500 \mu g/plate$  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  $\mu$ g/ml) and reached a maximum at 20–100  $\mu$ g/ml. 2-Naphthylamine was toxic for both strains at concentrations of 500  $\mu$ g/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  $\mu$ g/ml (Fig. 3). One laboratory (P2) repeated the test with a lower dose range and observed a mutagenic effect beginning at 0.5  $\mu$ g/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  $\mu$ g/ml) than in TA98 (20–100  $\mu$ g/ml). When using the robotic system with a top dose of 1000  $\mu$ g/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  $\mu$ g/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  $\mu$ g/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  $100 \mu g/ml$ . 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  $\mu$ g/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 noncarcinogenic 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  $\mu$ g/ml and with S9 at 100  $\mu$ g/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  $\mu$ g/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  $\mu$ g/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  $\mu$ g/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  $\mu$ g/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  $\mu$ g/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  $500 \,\mu$ g/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  $\mu$ g/ml in the manual but not in the robotic system, and the other (P7) at 20 and 100  $\mu$ g/ml. The latter positive result was confirmed upon repeating the test after the study with a dose response from 20 to 500  $\mu$ g/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



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

the present study did not identify azoxybenzene as a mutagen.

#### 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 3F) 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; (1–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 auto-

mated 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.





MOCA (5)

Form AA08 01-2012



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Azoxybenzene (12)



3-Aminotriazole (13)



Diethylstilbestrol (14)





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#### The AMES II<sup>™</sup> Mutagenicity Assay:

#### An International Validation Study Performed With Nineteen Coded Compounds

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#### Introduction

The Ames II™ assay, a liquid microtiter modification of the standard Ames plate incorporation test («Ames I»), was used for an international col-laborative study with nineteen coded chemicals. The goal of this study was to (1) validate the Ames II as a suitable alternative screening assay to the Ames I test, and (2) to test the Ames II™ assay system for its reproducibility amos 9 different laboratories.

#### Test Method

The Ames II<sup>TM</sup> assay is performed with the histidine auxotroph Salmonella typhimurium tester strains TA98 (frameshift mutations) and TAMix (base-pair substitutions). TAMix is a mixture of six base-pair stains, TA700-11-A7006 in equal proportions, each of which reverts by only one specific base substitution (Ref. 1). The test is performed in microtitre plates. Tester strains and media are available at Xenometrix by Endowli GmbH. Chemical treatment is performed

med in 24-well plates (6 concentrations in triplicate, concurrently with solvent and positive controls) in the absence and presence of 59 mix. After treatment, a medium containing a pH indicator and lacking histidi-ne is added. Each well of the 24-well plate is then aliquoted into 48 wells of a 384 well-plate and incubated for two days to allow reverant bacte-ria to form colonies. Mutagenicity (bacterial growth) is measured colori-metrically by a color change (pH drop) from purple to yellow (Fig. 1).



Table 1 Test Chemical

Code No. Chemical

- The 19 chemicals selected from the literature (Ref. 2) included know mutagens, non-mutagens and compounds producing conflicti results in the standard Ames plate incorporation test. If possible, ch mical pairs were chosen, i.e. carcinogens and non-carcinogens wi closely related chemical structure (Table 1). inogens with
- The chemicals were coded at random and distributed among nine independent laboratories to allow for an inter-laboratory comparison of the Ames II test system.
- · Each chemical was tested by 4-6 investigators.

2-Actstyaminofluorene 3-Anricio 12-Artisaole Anthracene Berucidine Berucid

#### Fig. 1: Ames II Assay Procedure Culture Overnight Stored at -80° C Culture Assay Preparation Exposure Culture 24-Well Plate TAMix Ø TAMX ODADD 100 <sup>1°</sup> C, 12–17 h 250 rpm 37° C, 90 Min, 250 rpm 384-Well Plate 37° C, 48 h D1

#### Results

- The present Ames II study revealed an overall agreement of 84% with the standard Ames plate incorporation test (Ames I, Fig. 2).
- No false positive results were obtained
- All mutagenic chemicals selected were correctly identified with the Ames II™ assay (e.g. Fig. 3), except cyclophosphamide (Cphos) which was Ames II positive in only 4 of 6 laboratories.
- Two of the compounds with equivocal results in the Ames I test (pyre ne, Pyr and diphenylnitrosamine, dPhNO) were weakly but consistently positive in the Ames II test, whereas one (azoxybenzene AzoxB) produced also conflicting results in the Ames II test.
- Table 2 summarizes the Ames II results obtained by the different par-ticipating laboratories.
- All laboratories agreed to 100% in 15 of 19 chemicals (individual questionable results are ignored). Furthermore, all except one laboratory came to the same conclusion for 17 out of 19 test compounds which results in an inter-laboratory consistency of 89.5%.



#### Conclusion

This study shows that the Ames II<sup>TM</sup> Assay is a well reproducible test alternative to the traditional Ames test (Ames I) and that the sensitivity of both test systems (Ames I and AmesII) is comparable, **making the Ames II** Mutagonicity<sup>TM</sup> Assay a cost-effective per-egulatory screening test.

#### Advantages of Ames II over Ames I:

- Higher speed format Microplate format Automatable
- TABIX allows to record all possible base-pair substitutions in one culture Ready to use reagents Less hands on time Colorimetric assay Substantially lower consumption of test chemical and plasticware

#### pos neg pos pos pos pos pos pos pos pos pos pos

pos pos pos pos pos



#### References

Р3

P5

P6

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veight according to the ICPESTTC study (Ref. 2)



Abbreviation

2AAF

AzoxB Bzd B(a)P

CAS No.

53-96-3 61825-120-12-7 495-48-7 92-87-5 50-32-8 6055-19-2 56-53-1 781-43-1 86-30-6 101-21-3 63-68-3 101-14-4 91-59-8 56-57-5 129-00-0 57-50-1 54827-17-7 51-79-6

MW-

223.3 84.1 176.2 198.2 252.3 279.1 268.3 206.3 198.2 213.7 149.2 267.2 143.2 190.2 202.3 342.3

neg neg neg neg

neg neg neg neg neg

neg neg neg neg neg

Mutagenicity\*

Table 2: Inter-laboratory Consi Participant CODE # 
 LODE#
 1
 2
 3
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 8
 9
 10
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 12
 13
 14
 15
 16
 17
 18
 19
 P1 pos pos pos pos pos pos equ neg neg neg neg pos pos pos neg neg neg neg neg P2





## Comparison of DEREK, Ames and Ames II

Sanofi aventis Das Wichtigste ist die Gesundheit

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#### Introduction

The Ames II<sup>™</sup> assay is performed with the histidine auxotroph *Salmonella typhimurium* tester strains TA98 (frameshift mutations) and TAMix (base-pair substitutions). TAMix is a mixture of six base-pair strains, TA7001-TA7006 in equal proportions, each of which reverts by only one specific base substitution

The Ames II test is an valuable screening tool in early drug development due to the low amount of compound needed (5 mg), the higher throughput and its supposed predictivity for the Standard Ames Test. The aim of this work was to compare the results of the AmesII assay with results predicted in DEREK in comparison to data from literature for the standard Ames protocol. 16 different chemicals revealing 9 different DEREK alerts and known results in the Standard Ames were selected for testing in the Ames II assay with and without S9 mix.

#### Purpose

The Ames II<sup>™</sup> assay, a liquid microtiter modification of the standard Ames plate incorporation test ("Ames I"), was used for an internal study with sixteen coded chemicals. The goal of this study was to 1) compare the Ames II with data from literature with reference chemicals and 2) to identify DEREK alerts which were not recognized by the Ames II assay. For this purpose, 16 compounds revealing different DEREK alerts were investigated

#### Results

It could be demonstrated that 12 out of 16 compounds of different structural classes showed congruent results between the Ames II and standard Ames. 3 compounds were found to be negative in the Ames II, but positive in the standard Ames (5-Amino-4-imidazolecarboxamide, N,N-Dimethylinitros -amine and Glutaraldehyde). All these compounds were detected in the standard protocol in the strains detecting base pair substitutions. One of the compounds (N-Hydroxymethylacrylamide) was found to be positive in the Ames II but not in the standard Ames.

Compared with the the mutagenic alerts predicted by DEREK 5 compounds out of 16 were found to be negative in the Ames II. For DEREK alert M311 two Acrylamides were selected which were known to be negative in procaryotic mutagenicity assays.

#### Material and Methods

The Ames II<sup>™</sup> assay is performed with the histidine auxotroph *Salmonella typhimurium* tester strains TA98 (frameshift mutations) and TAMix (base-pair substitutions). TAMix is a mixture of six base-pair strains, TA7001-TA7006 in equal proportions, each of which reverts by only one specific base substitution (Ref. 1).

The test is performed in microtiter plates. Tester strains and media are available at Aniara. Chemical treatment is

performed in 24-well plates (6 concentrations in triplicate, concurrently with solvent and positive controls) in the absence and presence of S9 mix. After treatment, a medium containing a pH indicator and lacking histidine is added. Each well of the 24-well plate is then 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 (Fig. 1).



Since a 100 % correlation between standard Ames and Ames II is not expected, the purpose of this investigations was to identify classes of compounds that gave controversial results. Although a selection of two compounds per alert is not sufficient to completely estimate the two screening methods, this approach was used as a first step for further investigations.

We identified three classes of compounds for which further investigations should be conducted to improve the predictivity of the Ames II. In conjunction with DEREK analysis it seems to be possible to provide differentiated alerts for a mutagenic potential of a drug candidate.

#### Conclusions

### **Results - con't**

DEREK Alert	Compound	Results Ames II	Results AMES
1) Monosubstituted aromatic amine/amide (M351)	2-aminophenol	Mixed strains positive at 3000 µg/ml and 5000 µg/ml TA98 negative	positive: TA100 +/-S9
1) Monosubstituted aromatic amine/amide (M351)	m-Aminoacetanilide	TA 98 + S9 positive at 100 - 300 µg/ml, Mixed strains negative	positive: TA100 +S9, TA97 +S9, TA98 +S9
2) Polysubstituted aromatic amine/amide (M352)	2,4,5-Trimethyl- aniline	TA 98 + S9 positive at 100 µg/ml Mixed strains +S9 positive at 3 µg/ml	positive: TA100 +S9, TA98 +S9
2) Polysubstituted aromatic amine/amide (M352)	2-Acetamidofluorene	TA98 +S9 positive at 3 µg/ml Mixed strains + S9 positive at 10 - 30 µg/ml	positive: TA98 +S9, TA100 +S9
3) Heterocyclic aromatic amine/amide (M353)	2-Amino-5- chlorothiazole	Mixed strains +/S9 positive at 10-30 µg/ml +S9 and 1000 µg/ml -S9 TA98 +S9 positive at 3000 µg/ml	positive: TA100 +S9
3) Heterocyclic	5-Amino-4-imidazole- carboxamide	TA98+/- S9 negative, Mixed strains+/- S9 negative	positive: TA100 +S9
aromatic amine/amide (M353)			
4) Deluguelle	2-Acetamido-	Mixed +S9 positive	positive:
aromatic amine/amide (M354)	napritnalene	TA98 negative	TA100 +S9
5) Aromatic nitro compound (M329)	o-Nitroanisol	Mixed strains+ positive at 1000 - 3000 µg/ml TA98 negative	positive: TA100-/+ S9
5) Aromatic nitro compound (M329)	1-Methyl-5- nitroimidazole	Mixed strains +/- S9 positive at 30-100 µg/ml +S9 1000 µg/ml -S9, ТА98 +/- S9 positive at 30 - 100 µg/ml	positive: TA100 -S9
6) N-Nitro-/Nitroso compound (M007)	N,N-Dimethyl- nitrosamine	TA98 and mixed strains negative	positive: TA100 +S9, TA104 +S9, TA1535 +S9
6) N-Nitro-/Nitroso compound (M007)	N-Methyl-N- nitrosourea	Mixed strains +S9 positive at 30 - 100 µg/ml TA98 negative	positive: TA100 -/+S9, TA104 -/+S9, TA1535 -/+S9
7) Quinoline (MD16)	4-Methylquinoline	TA98 +S9 poitive, at 300 µg/ml Mixed strains +S9 positive at 1000 µg/ml	positive: TA100 +S9
7) Quinoline (MD16)	6-Methoxyquinoline	Mixed strains and TA98 weakly positive with S9	positive: TA100 +S9
8) Alkylaldehyde or precursor (M306)	Glutaraldehyde	TA98 and mixed strains negative	positive: TA100 -/+S9, TA102 -/+S9, TA104 -/+ S9
10) ∝, β-unsaturated amide or thioamide (M311)	Acrylamide	TA98 and mixed strains negative	negative
10) ∝, β-unsaturated amide or thioamide (M311)	N-Hydroxymethyl- acrylamide	Mixed strains+/- S9 positive at 1000 µg/ml TA98 negative	negative

It could be shown that the major number of compounds with DEREK alerts was identified with the Ames II assay. For some classes of compounds further investigations seem to be necessary to improve the meaningfulness of this assay.

However, the Ames II is a valuable screening tool for the prediction of the outcome of the standard Ames assay.



#### ASSESSMENT OF A SCREENING EXPERIENCE WITH THE AMES II<sup>TM</sup> TEST AND FUTURE PROSPECTS

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Tab.1: Results of a collaborative study by Xenometrix GmbH (March 2003)

#### INTRODUCTION

The Ames II<sup>TM</sup> test, a liquid fluctuation version of the Salmonella mutagenicity assay, provided by Xenometrix GmbH, was used for an early compound selection in the discovery process. The aim of this work was to validate the Ames II compared to the standard Ames test and to explore a way to reduce the required compound quantity without lowering the predictability of the test.

#### MATERIALS and METHODS

This test is composed of a mixture of 6 Salmonella typhimurium strains: TA7001, TA7002, TA7003, TA7004, TA7005 and TA7006, which revert to histidine autotrophy by a specific base substitution in the histidine operon. This "mix" is used as a single strain. In addition, the frameshift tester strain TA98 is used. The mix and TA98 strains are inoculated in the medium for growth overnight at 37℃. The treatment, performed in 24-wells microtiter plates, allows partial automation and consequently requires about 60-fold less compound (50 mg) than the standard Ames. After a 90 minincubation treatment with or without Aroclor-induced S9 mix, concurrently with solvent and positive controls, an indicator medium lacking histidine is added to each well. Each well is then aliguoted into 48 wells of a 384-well plate. Within two days, revertants to His grow into colonies. The metabolism of the bacterial colony reduces the pH of the medium, changing the colour of the wells. The number of discoloured wells are counted for each concentration and compared to the solvent control (Fig.1). Each concentration is evaluated in triplicate to allow statistical analysis. 350 compounds were tested and three modified conditions were also evaluated to reduce the compound use, namely: test with one strain only, with S9 mix only or with lower concentrations.



Tab.2: Comparative results between Ames II and Ames I tests (42 proprietary compounds)





Sensibility : 50 % Specificity : 100 % Concordance : 83 %

#### Fig.3: Distribution of 56 positive responses vs concentration





#### RESULTS

350 compounds were tested, including molecules issued from our own research, known non- or genotoxicants, or molecules producing equivocal results. The concordance between the results achieved in this Ames II<sup>™</sup> test and those reported in the literature or in the standard Ames test ranged from 79 (Ref.1) to 83% (Tab.2). The concordance reached 89% in a collaborative study (Tab.1). No false positive results were obtained with known non-mutagenic substances. False negative results may arise when chemicals revert only specific strains like TA1535 or E. coli WP2 uvrA (pKM101) which meet no equivalent in the "mix".

The positive responses were randomly distributed among the strains or the concentration range (Fig.2 and 3). In contrast, only 11% of positive results emerged specifically in the absence of S9 (Fig.4), while 89% of genotoxicants should be detected using S9 mix as the only treatment condition.

#### **DISCUSSION - CONCLUSION**

Based on the acquired experience on a large number of compounds, the Ames II<sup>TM</sup> test is a reliable screening tool. When used with the recommended conditions by the supplier, it allows an early identification of genotoxicants, otherwise likely discarded at a later stage of development. The two proposed strains (mix and TA98) as well as a high level of tested concentrations are essential to keep an acceptable level of predictability. However, as the compound availability is always of high concern at a screening stage, it is possible to reduce by half the required quantity to be tested (i.e. 25 mg) when performed with the metabolic activation as a unique treatment condition. In that case, the number of false negative would be increased by only 2% (decreased specificity).

#### REFERENCES

1. Gee P. et al. (1998). Mutat. Res., 412: 115-130

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responses among strains:



Fig.4: Distribution of 56 positive

## responses vs metabolic activation