

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

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

### Introduction

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

There are many levels at which the performance of a bacterial test method can be evaluated with respect to the performance of

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

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

### Materials and methods

#### *The Ames II test*

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

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

**Liquid exposure.** The chemicals were tested in TAMix and TA98 using a modified liquid fluctuation test. In the absence of S9 fraction, 0.190 ml of Ames II Exposure medium (Xenometrix) per well of a 24-well plate and 0.050 ml of each overnight culture per well were mixed gently. Each test chemical was added in 0.010 ml aliquots. In experiments with Aroclor 1254-induced rat liver S9 fraction (Moltox, Boone, NC), the aliquot of Exposure medium was decreased to 0.152 ml to accommodate 0.038 ml of the S9 reagent. This provided a final concentration of 4.5% S9 fraction. The S9 mix contained 33 mM KCl, 8 mM MgCl<sub>2</sub>, 5 mM glucose-6-phosphate, 4 mM nicotinamide adenine dinucleotide phosphate, 102 mM NaH<sub>2</sub>PO<sub>4</sub> buffer (all Sigma) and 30% S9 (Moltox). The 24-well plates were incubated at 37°C for 90 min, with shaking at 250 r.p.m.

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

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

**Data acquisition.** A pH indicator dye in the Indicator Media turns yellow as the pH drops ( $pK_a \cong 5.2$ ) as catabolites accumulate from the metabolically active revertant cells which grow in the absence of histidine. The number of yellow positive wells out of a total of 48 wells is an indication of the frequency of reversion per replicate per dose and was compared to the number of spontaneous revertant wells obtained in the solvent control sections. Each 48-well section of the 384-well plates was scored for the number of revertant wells (yellow) by an SLT Spectra Image plate reader (Tecan U.S., Research Triangle Park, NC) at optical density (OD)<sub>492 nm</sub> 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. The data were organized further into summary tables per compound code for its classification.

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

Ten compounds were chosen to be retested in triplicates to allow for statistical analysis and to evaluate the robustness of the original screen. The data from the initial tests were taken into account when test concentrations were chosen for the repeated experiments. The average number of wells containing revertants per culture per dose was calculated from the triplicates, and the increases above the zero dose baseline (mean of zero dose plus 1 SD) were

**Table I.** Salmonella tester strains used

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

•, Strain used.

**Table II.** Evaluation criteria of compounds with single data points

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

EQ, equivocal.

<sup>a</sup>Possibly positive if >4× baseline at highest concentration tested.

determined at each dose of test chemical. If the zero dose baseline was <1, it was set to 1. Results from this triplicate experiment were used for evaluation rather than the less reliable single-point measurements.

Detailed evaluation data are available at the Xenometrix homepage ([www.xenometrix.ch](http://www.xenometrix.ch))

#### Traditional Ames test

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

#### Selection of chemicals

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

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

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

## Results and discussion

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

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

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

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

Table III. Chemicals tested

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

Table III. Continued

Code No.	CAS number	Chemical name	Solvent	Concentrations ( $\mu\text{g/ml}$ ) <sup>a</sup>	
				-S9	+S9
30	115-96-8	Tris(2-chloroethyl) phosphate	DMSO	5.00-5000	5.00-5000
95	51-79-6	Urethane	H <sub>2</sub> O	4.44-4400	4.44-4400

CAS, Chemical Abstracts Service; DMSO, dimethyl sulfoxide; THF, tetrahydrofuran.

<sup>a</sup>Concentration range tested in Ames II.

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

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

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

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

three required it in the Ames II procedure and one in the NTP procedure.

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

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

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

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

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

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

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

**Table IV.** *Continued*

Chemical name	Cancer	Ames II	S9	TAMix	TA98	NTP Ames	S9	TA100, 1535	TA98
Tris(2-chloroethyl) phosphate	+	– <sup>a</sup>				–			
Urethane	+	– <sup>a</sup>				E	y	E <sup>d</sup>	

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

<sup>a</sup>Based on triplicate data.

<sup>b</sup>Positive in TA97/TA1537, negative in TA98.

<sup>c</sup>Positive only with reductive metabolism.

<sup>d</sup>Only with hamster S9.

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

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

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

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

Acetamide has shown liver tumours in rats which were nearly completely suppressed by co-feeding of arginine glutamate (15). Aniline has shown mostly negative responses in other *in vitro* and *in vivo* assays (16); tumorigenic responses at high doses in the spleen of rats have been regarded as a sequel of methaemoglobin formation with iron overload of splenic tissues oxidative stress (17,18). Methyl carbamate has shown liver tumours in rats but not in mice which appear to hydrolyse the material at a faster rate than rats (19) and no mutagenic effects were observed in a number of *in vitro* experiments (20). This is a profile very different from ethylcarbamate (urethane) which is metabolized to vinylcarbamate and other DNA-reactive chemicals (21). Thiourea inhibits iodine uptake and showed equivocal evidence of

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

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

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

Note: Agreement summations are in bold.

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

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

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

carcinogenicity in a number of earlier studies for thyroidal tumours (22) and hepatoma (23,24). Several Ames tests have been mostly negative (25–28) and neither initiating nor promoting activities were detected in the rat liver foci bioassay (29).

#### *Use of the tests and regulatory implications*

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

**Table VII.** Summary performance of the Ames and Ames II assays for predicting rodent carcinogenicity<sup>a</sup>

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

<sup>a</sup>The various measures of performance are sensitivity, proportion of carcinogens correctly identified; specificity, proportion of non-carcinogens correctly identified; positive/negative predictivity; the proportion of positives/negatives that correctly predict cancer/non-cancer; false positive/negative, the proportion of non-carcinogens/carcinogens that are incorrectly predicted; concordance, overall agreement between *Salmonella* and carcinogenicity test results and prevalence, the proportion of carcinogens in the tested population.

<sup>b</sup>The same chemicals were tested in the Ames, Ames II and cancer tests.

<sup>c</sup>Data from Zeiger, 1998 (based on 363 chemicals). The 56 chemicals reported in this paper are included in the Zeiger, 1998, chemicals.

<sup>d</sup>These values were 67, 89, 20, 33 and 64%, respectively, before the results were adjusted to ignore the two positive responses dependent on hamster liver S9 and use the negative, rat liver S9 responses instead.

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

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

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

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