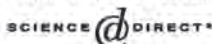




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## Assessment of the performance of the Ames II<sup>TM</sup> assay: a collaborative study with 19 coded compounds

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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: cyclophosphamide, 2-naphthylamine, benzo(a)pyrene, pyrene, 2-acetylaminofluorene, 4,4'-methylene-bis(2-chloroaniline), 9,10-dimethylanthracene, anthracene, 4-nitroquinoline-*N*-oxide, diphenylnitrosamine, urethane, isopropyl-*N*-(3-chlorophenyl)carbamate, benzidine, 3,3'-5,5'-tetramethylbenzidine, azoxybenzene, 3-aminotriazole, diethylstilbestrol, sucrose and methionine. The results of both assay systems were compared, and the inter-laboratory consistency of the Ames II test was assessed. Of the eight mutagens selected, six were correctly identified with the Ames II assay by all laboratories, one compound was judged positive by five of six investigators and one by four of six laboratories. All seven non-mutagenic samples were consistently negative in the Ames II assay. Of the four chemicals that gave inconsistent results in the traditional Ames test, three were uniformly classified as either positive or negative in the present study, whereas one compound gave equivocal results. A comparison of the test outcome of the different investigators resulted in an inter-laboratory consistency of 89.5%.

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

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**Keywords:** Ames II test; *Salmonella* mutagenicity test; Validation study

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## 1. Introduction

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

The Ames II assay is a liquid microtiter modification of the Ames test and consists of the 'strains' TAMix and TA98. TAMix is a mixture of the *Salmonella typhimurium* strains TA7001, TA7002, TA7003, TA7004, TA7005 and TA7006 [3]. The genetic complementation among the six 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  $\beta$ -lactamase gene that confers ampicillin resistance. These gene products increase the cell's ability to perform mutagenic lesion bypass repair during DNA replication.

This study had two goals: (1) to corroborate the use of the Ames II test as a suitable alternative screening assay [4,5] to the traditional Ames plate-incorporation method, and (2) to test the Ames II assay system for its reproducibility among different laboratories. The 19 compounds included in this study were selected on the basis of traditional Ames data published as a

report of the International Collaborative Program for the Evaluation of Short-Term Tests for Carcinogens (ICPESTTC study) [6]. The chemicals selected were either Ames-positive, -negative or equivocal: among the compounds that were positive in the traditional Ames assay, weak and strong mutagens were chosen, and the necessity of metabolic activation (S9 mix) for a positive response as well as the target site (frameshift mutation versus base-pair substitution) were considered. The equivocal chemicals that were chosen gave either inconsistent results in the ICPESTTC study or are known to be difficult to detect in bacterial mutagenesis assays. Although the discrimination between carcinogens and non-carcinogens played a secondary role in the present study, some chemical 'pairs' (carcinogens and their non-carcinogenic analogs) were included.

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

## 2. Materials and methods

### 2.1. Bacterial strains

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

Freshly thawed frozen strains of 10  $\mu$ 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	<i>hisD3052 Δara9 Δchl008 (bio chl uvrB gal)rfa1004/pKM101</i>	Frameshifts
TAMix	TA7001, TA7002, TA7003, TA7004, TA7005, TA7006	Base-pair
TA7001	<i>hisG1775 Δara9 Δchl004 (bio chlD uvrB chlA)galE503 rfa1041/pKM101</i>	A:T → G:C
TA7002	<i>hisC9138 Δara9 Δchl004 (bio chlD uvrB chlA)galE503 rfa1041/pKM101</i>	T:A → A:T
TA7003	<i>hisG9074 Δara9 Δchl004 (bio chlD uvrB chlA)galE503 rfa1041/pKM101</i>	T:A → G:C
TA7004	<i>hisG9133 Δara9 Δchl004 (bio chlD uvrB chlA)galE503 rfa1041/pKM101</i>	G:C → A:T
TA7005	<i>hisG9130 Δara9 Δchl004 (bio chlD uvrB chlA)galE503 rfa1041/pKM101</i>	C:G → A:T
TA7006	<i>hisC9070 Δara9 Δchl004 (bio chlD uvrB chlA)galE503 rfa1041/pKM101</i>	C:G → 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

non-mutagenic and 4 with conflicting responses in the different laboratories of the ICPESTTC study using the traditional Ames assay.

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

Table 2  
Chemicals tested

Chemical	CAS no.	MW <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-N(3-chlorophenyl) carbamate	101-21-3	213.7	–	–	Sigma	95
L-Methionine	63-68-3	149.2	–	–	Sigma	98
4,4-Methylene-bis(2-chloroaniline)	101-14-4	267.2	+	+	Fluka	99+
2-Naphthylamine	91-59-8	143.2	+	+	Sigma	Unknown
4-Nitroquinoline-N-oxide	56-57-5	190.2	+	+	Aldrich	98
Pyrene	129-00-0	202.3	–	?	Fluka	99
D-Sucrose	57-50-1	342.3	–	–	Sigma	99+
Tetramethylbenzidine	54827-17-7	240.5	–	–	Fluka	98
Urethane	51-79-6	89.1	+	?	Aldrich	99

(+) Positive; (–) negative; (?) equivocal.

<sup>a</sup> Molecular weight.

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

Table 3  
Positive control chemicals used in the Ames II assay

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

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

### 2.3. Positive controls

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

### 2.4. Metabolic activation

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

### 2.5. Study design

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

and chemicals. Unless stated otherwise, all procedures were performed manually.

#### 2.5.1. Repeat testing

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

#### 2.5.2. Test concentrations

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

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

#### 2.5.3. Liquid exposure

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

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

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

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

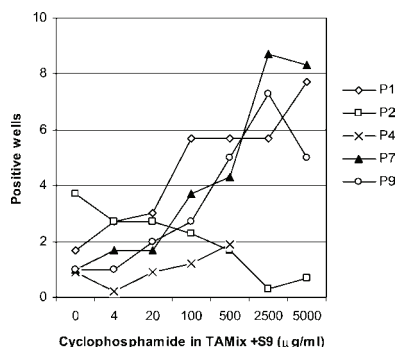


Fig. 1. Cyclophosphamide-induced reversion events in TAMix in the presence of S9 mix. P: participating laboratory; each number represents a specific company. Positive wells: the number of wells out of 48, where mutation occurred (see Section 2). Factors 3F greater than 2.0 were observed by P1, P7 and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

48 positive wells) differ from those of the weak mutagenic and equivocal compounds. Clearly negative results are not shown graphically. The robotic results of laboratory P1 are not shown in the following figures since another dose range was used. If there were discrepancies between the manual and robotic system, they will be commented on under the chemicals concerned.

3.1. Code 1: cyclophosphamide

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

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

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

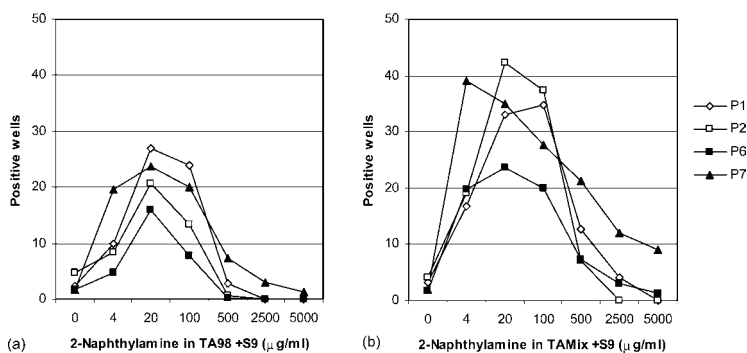


Fig. 2. 2-Naphthylamine-induced mutagenicity in the presence of metabolic activation: (a) strain TA98 and (b) strain TAMix.

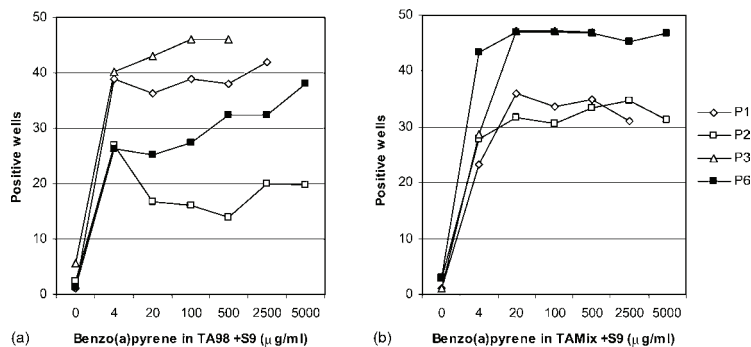


Fig. 3. Benzo(a)pyrene-induced reversion events in the presence of S9: (a) strain TA98 and (b) strain TAMix.

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

3.2. Code 2: 2-naphthylamine

All four laboratories that tested the compound found 2-naphthylamine to be positive in TA98 and TAMix. The responses were more pronounced in TAMix than

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

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

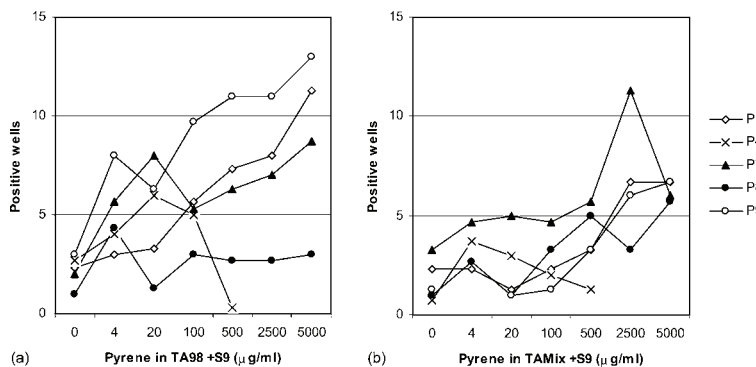


Fig. 4. Dose response curve of pyrene with TA98 and TAMix in the presence of S9 mix: (a) strain TA98 and (b) strain TAMix.

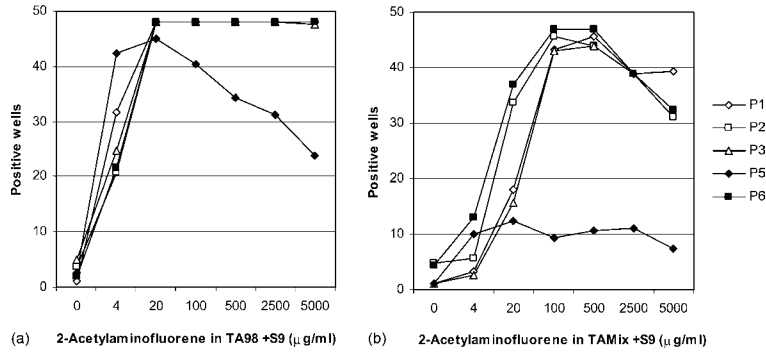


Fig. 5. 2-Acetylaminofluorene-induced mutagenicity in the presence of S9: (a) strain TA98 and (b) strain TAMix.

3.3. Code 3: benzo(a)pyrene

Benzo(a)pyrene gave consistently positive results in the Ames II test and S9 mix was likewise typically required for this activity. The maximum response was observed between 4 and 100  $\mu\text{g/ml}$  (Fig. 3). One laboratory (P2) repeated the test with a lower dose range and observed a mutagenic effect beginning at 0.5  $\mu\text{g/ml}$  (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  $\mu\text{g/ml}$ ) than in TA98 (20–100  $\mu\text{g/ml}$ ). When using the robotic system with a top dose of 1000  $\mu\text{g/ml}$ , group P1 obtained a clear positive result only in TA98 (not

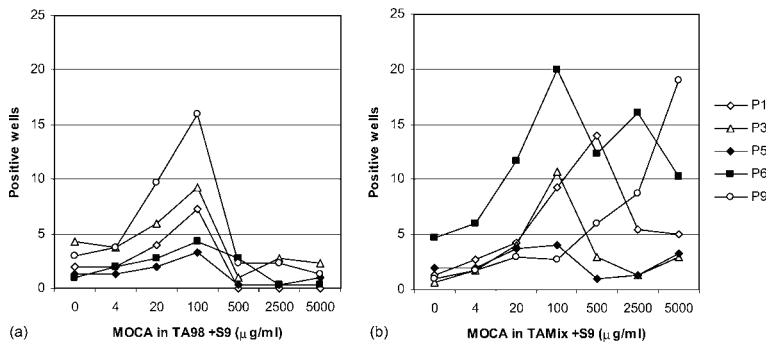


Fig. 6. 4,4'-methylene-bis(2-chloroaniline)-induced reversion events in the presence of S9 mix: (a) strain TA98 and (b) strain TAMix.



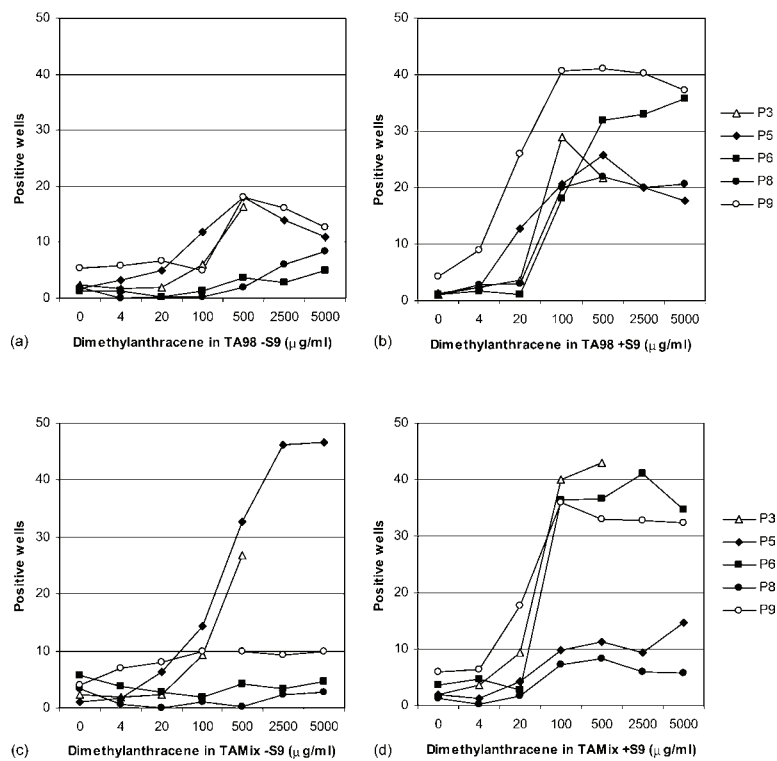


Fig. 7. 9,10-Dimethylanthracene-induced mutagenicity in the Ames II assay: (a) TA98 without S9; (b) TA98 with S9; (c) TAMix without S9 and (d) TAMix with S9.

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 ICPSTTC study. The majority of laboratories did not detect mutagenicity in the traditional Ames test, and where positive effects were seen they were variable. However, it had been considered a mutagen that was difficult to detect because of differences in protocol or evaluation criteria [6].

### 3.5. Code 4: 2-acetylaminofluorene

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

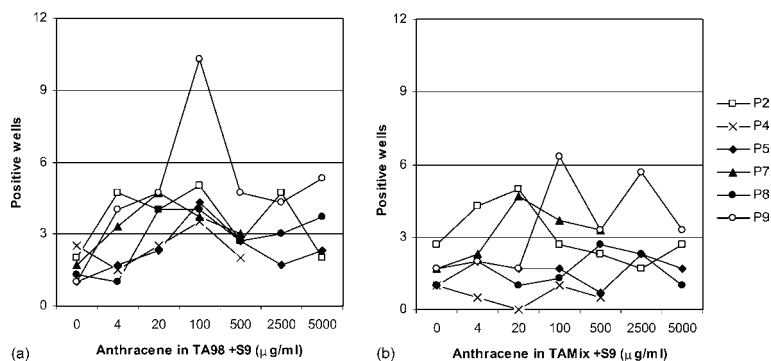


Fig. 8. Anthracene-induced reversion events in the Ames II assay in the presence of S9 mix: (a) strain TA98 and (b) strain TAMix.

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

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

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

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

### 3.7. Code 6: 9,10-dimethylanthracene

9,10-Dimethylanthracene gave positive results in all laboratories. S9 mix was not required for TA98, whereas for TAMix it was essential in three of five

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

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

### 3.8. Code 18: anthracene

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

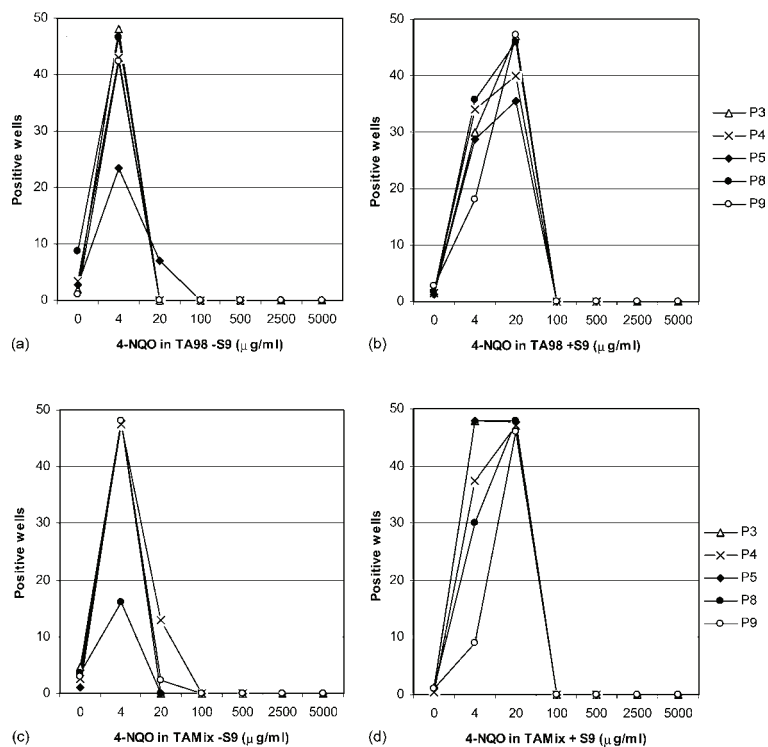


Fig. 9. 4-NQO-induced mutagenicity in the presence and absence of S9 mix: (a) TA98 without S9; (b) TA98 with S9; (c) TAMix without S9 and (d) TAMix with S9.

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

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

4-NQO was highly mutagenic in the Ames II test for both TA98 and TAMix in all laboratories that tested the chemical, and there was no requirement for metabolic activation (Fig. 9). The doses chosen

in this study were extremely toxic; without S9 toxicity started at 20 μg/ml and with S9 at 100 μg/ml. Higher concentrations caused cell death. These results were confirmed by laboratory P1 after the study (not shown). One laboratory (P9) repeated the test with a lower dose range in which mutagenicity started at 0.16 and 0.8 μg/ml in the absence and presence of S9, respectively (not shown). In the traditional Ames assay [6], TA98 and TA100 were the most useful strains for detecting 4-NQO activity, and S9 mix was, in general, not necessary for a mutagenic effect.

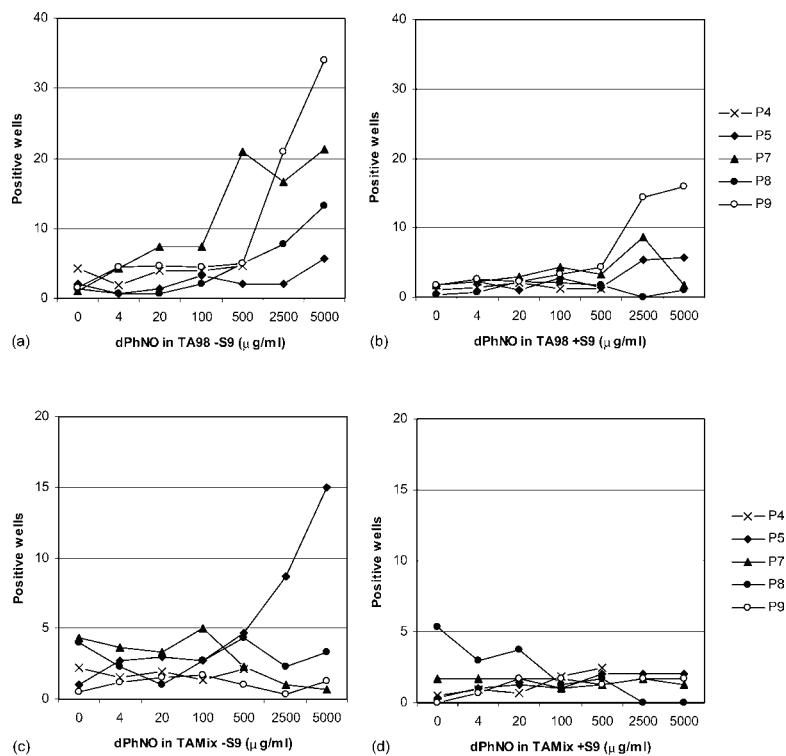


Fig. 10. Diphenylnitrosamine-induced mutagenicity in TA98 in the absence (9a) and presence (9b) of S9 mix.

### 3.10. Code 8: diphenylnitrosamine (dPhNO)

Diphenylnitrosamine was mutagenic in all laboratories that tested the chemical at concentrations higher than 500 µg/ml. But as in the ICPESTTC study [6], there was little consistency in the pattern of results and the scale of positive responses. Three laboratories (P7, P8 and P9) found diphenylnitrosamine mutagenic in TA98 without S9 mix (Fig. 10a), and three (P5, P7 and P9) found it positive in TA98 with S9 (Fig. 10b), one of which (P5) obtained also significant responses

in TAMix in the absence of S9 (Fig. 10c). Investigator P4 that tested with a top dose of 500 µg/ml obtained an equivocal result in TAMix with S9 mix (Fig. 10d). The positive responses in TA98 without S9 were dose-dependent with an onset of around 500 µg/ml. Of the two laboratories that re-tested diphenylnitrosamine (P7 and P9), only one (P9) could reproduce the positive result with TA98 in the presence of S9. Laboratory P1 obtained a positive, dose-related effect in TA98 without S9 mix in the manual as well as in the robotic system in its supplementary test (not shown).

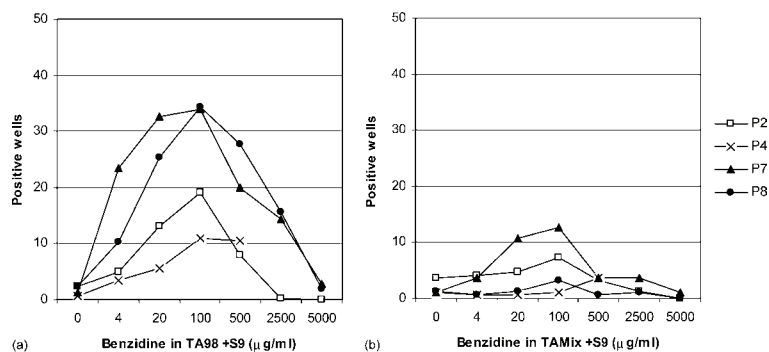


Fig. 11. Benzidine-induced mutagenicity in the presence of S9 mix: (a) strain TA98 and (b) strain TAMix.

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

### 3.11. Code 9: urethane

In the present study, no mutagenic response was obtained in all four laboratories that tested urethane (P4, P7, P8 and P9). Urethane was also negative in the robotic system in the supplementary test of laboratory P1, but it was clearly positive at 500 and 2500  $\mu\text{g/ml}$  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 ac-

tivity 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  $\mu\text{g/ml}$  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 µg/ml in the manual but not in the robotic system, and the other (P7) at 20 and 100 µg/ml. The latter positive result was confirmed upon repeating the test after the study with a dose response from 20 to 500 µg/ml. Laboratory P4 judged azoxybenzene negative according to its proper evaluation criteria.

Azoxybenzene gave equivocal results in the ICPESTTC study [6]. In those laboratories where a mutagenic effect was observed, S9 mix was essential. It has therefore been suggested that the capacity of S9 mix may be critical for demonstration of azoxybenzene mutagenicity. In the present study, it was mainly negative. The concentration of S9 mix in the Ames II test is 4.5% and therefore considerably lower than the 10 and 30% used in the traditional Ames assay. The lower S9 concentration may have been the reason that the majority of the laboratories in

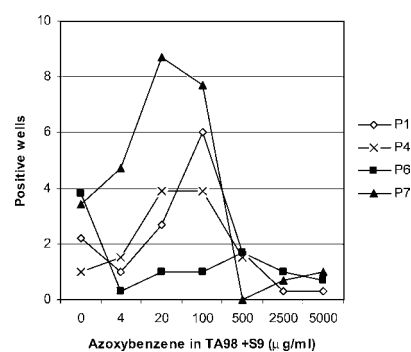


Fig. 12. Azoxybenzene-induced reversion events in TA98 with S9 mix.

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

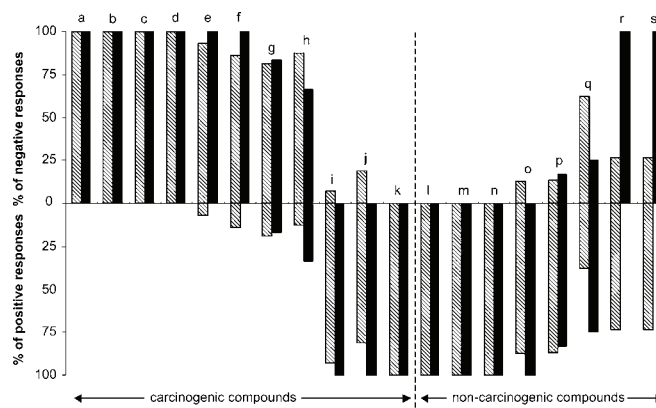


Fig. 13. Relative performances of the traditional Ames (light bars) and the Ames II (black bars) assays. Responses have been normalized (%) because of different group sizes. Questionable responses have been ignored. (a) benzo(a)pyrene; (b) 2-acetylaminofluorene; (c) 4-nitroquinoline-*N*-oxide; (d) benzidine; (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; (l–s) non-carcinogenic compounds.

the standard Ames results of the ICPESTTC study [6]. All eight mutagenic chemicals that were selected from the ICPESTTC report (Fig. 12a–h) were also positive in the Ames II test, except cyclophosphamide (h)

which was found to be positive in 4 of 6 laboratories in the present study. All seven Ames-negative compounds were also clearly negative in the Ames II test (Fig. 13i and k–p).

Table 4  
Inter-laboratory consistency

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

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

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

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

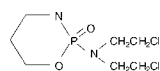
The present international collaborative study, in 9 laboratories with 19 chemicals, shows that: (1) the Ames II test results are well reproducible among the different laboratories and (2) that the sensitivity of both Ames assays, the Ames II and the traditional Ames, are comparable. The Ames II assay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

A screening assay should be performed with a relatively high throughput as there is an increased need to screen many compounds efficiently and in a cost-effective manner in the early phase of development. The Ames II assay meets these criteria. It offers a higher speed format than the traditional Ames assay even when performed manually. The simplicity of the protocol allows employing auto-

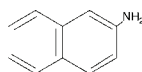
mated pipetting stations to perform the bulk of labor. The mix of the six new strains (TAMix) allows to record all possible base-pair substitutions in one culture. As the Ames II is a colorimetric assay, it is easy to score. The assay consumes a substantially lower amount of test chemical for yielding information useful in making decisions about a given compound.

#### Appendix A. Structural formulae of the test chemicals

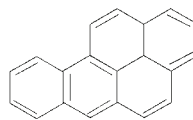
Carcinogenic/non-carcinogenic pairs are placed next to each other. Code numbers are in brackets.



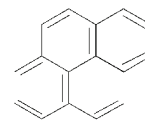
Cyclophosphamide (1)



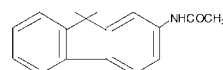
2-Naphthylamine



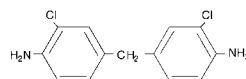
Benzo(a)pyrene (3)



Pyrene (11)

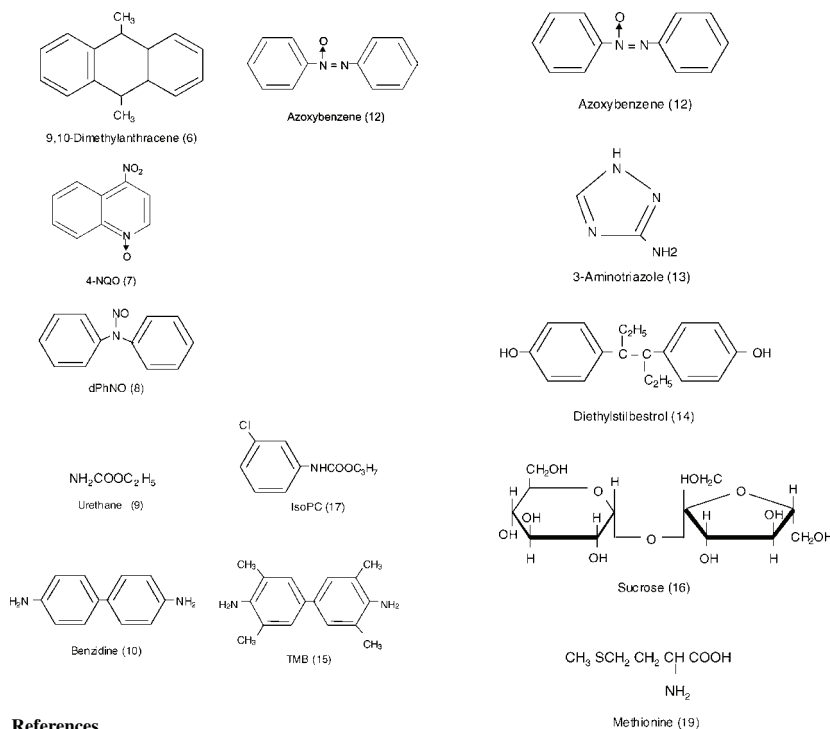


2-Acetylamino fluorene (4)



MOCA (5)





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