

Chapter 2

The Ames II and Ames MPF Penta I Assay: A Liquid Microplate Format Modification of the Classic Ames Test

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Abstract

This chapter describes a protocol modification of the bacterial reverse mutation test (Ames test). It is based on the same principle as the Ames test but uses a liquid, low-volume microplate version of the fluctuation method. Clear strengths are the low compound requirement and the increased throughput as compared to the standard format. The liquid system allows for processing several replicates at once with the possibility of using pipetting robots and has an easy colorimetric readout. The Ames II/Ames MPF also uses less S9 and produces less hazardous waste due to the low-volume multiwell format.

Key words Ames II, Ames MPF, Liquid microfluctuation method, Test kits, High throughput

1 Introduction

The bacterial reverse mutation test using *Salmonella typhimurium* [1, 2] or *Escherichia coli* [3] is the most widely used in vitro test to evaluate mutagenicity of chemical substances and environmental samples. The principle of this test is described in detail in Chap. 1 of this book.

With at least two strains detecting base-pair and frameshift mutations, the bacterial reverse mutation test is generally used for genotoxicity screening of drugs, chemicals, environmental samples, and food additives. The bacterial reverse mutation test is also required for regulatory genotoxicity testing and performed according to the guidelines of various regulatory agencies such as OECD (Organisation for Economic Co-operation and Development) and ICH (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use). The traditional format of a bacterial reverse mutation test described in OECD guideline 471 [4] relies on scoring bacterial growth (colonies) on selective agar plates after exposure of the bacterial cells to a test chemical, either by incorporation of the test chemical into the agar plates or by preincubation prior to plating.

This format uses a lot of agar plates and consumes large amounts of test sample and S9. The need for relatively large amounts of test chemicals and the low-throughput format limit the use of the standard Ames plate incorporation or preincubation assay during early phase of drug development. Similar constraints apply for the testing of surface waters or drinking water which requires large amounts of raw material for extraction and concentration steps. Scientists’ efforts have therefore led to the development of scaled-down and high-throughput genotoxicity assays which allow screening of a greater number of samples. One of such tests is the Ames II/Ames MPF assay, a liquid microplate version of the bacterial reverse mutation test which has a very good conformity to the standard testing procedure [5], decreases the amount of test sample required for a study and allows for automation.

2 Ames II Assay

2.1 Principle

The Ames II assay is a 384-well microplate format modification of the classic bacterial reverse mutation test. It uses a small volume, liquid format for exposure of the bacterial tester strains to test samples in the absence and presence of S9, and a colorimetric read-out for ease of scoring the revertant bacteria. In this assay, the frameshift mutations are detected by the traditional strain TA98, and base-pair substitutions by a mixture of six strains named TAMix (Table 1). The TAMix strains and the microfluctuation test procedure that is used in the Ames II assay were developed by Gee et al. [5, 6]

Table 1
Genotypes of TA98 and TAMix *Salmonella typhimurium* strains

Strain	Mutation	Type	Target	Cell wall ^a	Repair ^b	pKM101 ^c
TA98	<i>hisD3052</i>	Frameshift	GC	<i>rfa</i>	<i>uvrB</i>	Yes
<i>TAMix contains</i>						
TA7001	<i>hisG1775</i>	b.p. subst.	T:A>C:G	<i>rfa</i>	<i>uvrB</i>	Yes
TA7002	<i>hisC9138</i>	b.p. subst.	T:A>A:T	<i>rfa</i>	<i>uvrB</i>	Yes
TA7003	<i>hisG9074</i>	b.p. subst.	T:A>G:C	<i>rfa</i>	<i>uvrB</i>	Yes
TA7004	<i>hisG9133</i>	b.p. subst.	C:G>T:A	<i>rfa</i>	<i>uvrB</i>	Yes
TA7005	<i>hisG9130</i>	b.p. subst.	C:G>A:T	<i>rfa</i>	<i>uvrB</i>	Yes
TA7006	<i>hisC9070</i>	b.p. subst.	C:G>G:C	<i>rfa</i>	<i>uvrB</i>	Yes

^a(*rfa*): This mutation leads to a defective lipopolysaccharide (LPS) layer that coats the cell surface, making the bacteria more permeable to bulky chemicals
^b(*uvrB*): The *uvrB* deletion mutation eliminates the accurate excision repair mechanism, thereby allowing more DNA lesions to be repaired by error-prone DNA repair mechanisms. The deletion through the biotin gene makes the bacteria biotin dependent
^c(pKM101): This R factor plasmid enhances chemical and UV-induced mutagenesis via an error-prone recombinational DNA repair pathway. The plasmid also confers ampicillin resistance

and originally designed to serve both as a screen for mutagenic substances and, by using the TAMix strains individually, to allow the identification of the specific base-pair substitution mutations produced. The TAMix comprises six *his*⁻ *Salmonella* tester strains, TA7001–TA7006, each with a different base-pair substitution. Each of these mutants can be reverted only by one specific transition or transversion such that all possible base-pair changes can be detected and identified. Like the traditional strains, the genetic background of the TA700x series of strains has been modified to improve the sensitivity of their reversion (*uvrB*, *rfa*, plasmid pKM101).

2.2 General Assay Description

Freshly prepared overnight cultures of TA98 and TAMix are exposed to six concentrations of a test agent, as well as to a positive and negative control for 90 min in medium containing sufficient histidine to support a few cell divisions. After 90 min the exposure cultures are diluted in pH indicator medium lacking histidine and aliquoted into 48 wells of a 384-well plate. Within 2 days, cells that have undergone the reversion to histidine prototrophy—either spontaneously, or as a result of the exposure to a mutagen—will grow into colonies. Bacterial metabolism reduces the pH of the medium, changing the color of that well from purple to yellow. The number of wells containing revertant colonies is counted for each dose and compared to a solvent (negative) control. Each dose is tested in triplicate to allow for statistical analysis of the data. An increase in the number of revertant wells relative to the solvent controls indicates that the chemical is mutagenic in the Ames II assay. The mutagenic potential of substances is assessed directly and in the presence of liver S9 (Fig. 1).

2.3 Advantages

Important advantages of this test system are that it consumes much less test sample than the standard plate or preincubation tests. It requires less hands-on time since the low-volume liquid format allows working with multichannel pipettes and processing several replicates at once. It needs considerably less S9 and plasticware, and it can be partly processed by pipetting stations. The assay has an easy colorimetric readout in 48-well sections of a 384-well plate. Typically, triplicate plates with six sample dilutions, negative and positive controls are scored by eye in 1–2 min. The Ames II assay is available as a kit including all necessary ingredients ready to use and with detailed instruction for use (*see* Sect. 2.5.1). The Ames II procedure has found its use as an early mutagenicity screening procedure for pharmaceutical and chemical companies, as well as for environmental samples.

2.4 Validation Studies

There are several validation studies comparing the Ames II with the traditional agar plate assay using several strains. In all these studies, many chemical classes were evaluated. The first Ames II

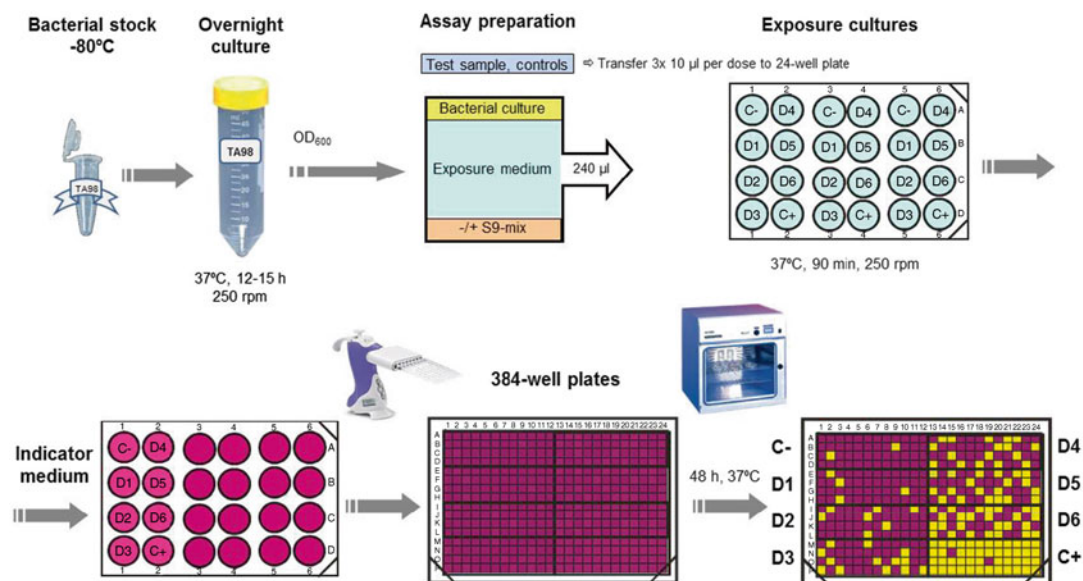


Fig. 1 Ames II/Ames MPF test procedure

validation study with 25 compounds was published in 1998 and gave a concordance of 79 % for TA98 and TAMix with traditional data obtained with up to five strains [5]. The authors concluded that the high concordance with the traditional *Salmonella* test and the reproducibility among cultures and replicates demonstrate that the Ames II test procedure is an effective screen for identifying *Salmonella* mutagens. An international Ames II round robin study performed with 19 coded compounds [7] gave an 84 % concordance with published traditional results and an almost 90 % inter-laboratory consistency. Another validation study was performed by Gervais et al. [8]. They tested 42 proprietary compounds and obtained a concordance of 83 %. The disagreement in the test results was obtained mostly with compounds that specifically revert *E. coli* or TA1535. In a study released in 2009 [9], 71 compounds tested with the Ames II assay were compared with published data for the traditional test, resulting in an agreement of 84 %, which is comparable to the intra- and interlaboratory reproducibility of 87 % for the traditional test itself.

The Ames II assay is routinely used for the screening of early drug candidates by several pharmaceutical companies such as BASF, Janssen Pharmaceutica NV, Sanofi-Aventis GmbH, and Servier, or for the investigation of genotoxic impurities. Sanofi-Aventis reports a predictability of ~92 % when comparing the results of the Ames II with the full-scale traditional test, and a throughput of 40 compounds weekly [10]. The Servier Group uses the Ames II test for early evaluation of in-house compounds [11].

All compounds that resulted positive in the Servier Ames II screening assay were confirmed positive in the regulatory bacterial reverse mutation test. The Ames II test is also regularly used for investigating drinking water quality [12].

2.5 Detailed Assay Description

2.5.1 Materials

The TA98 strain which is also used in the traditional bacterial reverse mutation assay can be re-isolated and stored as described earlier [1, 2]. The individual TA700x strains of the TAMix are patent protected. The TAMix is an equimolar mixture of the six individually grown strains. Strains have to be stored at ≤ -70 °C until use. TAMix, as well as growth, exposure, and indicator media, which are stored at room temperature, can be purchased ready to use [13, 14]. S9 can be purchased from different vendors, either in liquid (storage -80 °C) or lyophilized form (storage -20 °C), and has a shelf life of ≥ 2 years when stored correctly. Positive control chemicals and ampicillin are available from different suppliers. Reagents for preparing the S9 mix (buffer-salts, G-6-P, and NADP) can be prepared individually or are available as ready-to-use solutions [13]. The necessary plasticware for performing the assay (culture tubes, multiwell plates, reagent reservoirs) can be bought individually. Complete test kits for performing the Ames II assay are also commercially available [13, 14], and the procedure is described in the Ames II Instructions for Use [15].

2.5.2 Equipment

Apart from the abovementioned items, a laboratory should feature the following equipment to perform the Ames II assay: an environmental shaker with approximately 2.5–3 cm amplitude capable of 37 °C, 250 rpm incubations, a 37 °C dry incubator, an autoclave, a spectrophotometer for measuring optical density at 600 nm, 1.5 mL spectrophotometer cuvettes, single and multichannel adjustable pipettes and sterile tips, a programmable stepper for dispensing 2.6 mL (recommended), an 8-channel repeating pipette for the transfer of the exposed cultures to the 384-well plates (highly recommended), 5 and 10 mL serological pipettes and solvents for sample dilution and solvent control. A light box facilitates the scoring of the 384-well plates.

***Note:** Shakers with different characteristics may be used but might require different incubation flasks for optimal bacterial growth.*

2.5.3 Overnight Culture Preparation

Using sterile technique, overnight cultures of TA98 and TAMix are prepared by performing the following steps: 10 μ L of freshly thawed and carefully mixed bacterial stocks are added to 50 mL culture tubes containing 10 mL growth medium and 10 μ L of 50 mg/mL ampicillin. The culture tubes are capped loosely (or filter caps are used) to allow for sufficient aeration. The cultures are grown for 12–15 h at 37 °C, 250 rpm in an environmental shaker to the late exponential phase. Incubation time varies depending on the shaker type and amplitude.

2.5.4 Determination of Optical Density (OD_{600})

One hundred microliter aliquots of the overnight cultures are diluted 1:10 with growth medium and the OD_{600} is measured in semi-micro-cuvettes (light path: 1 cm). The OD_{600} reading is multiplied by 10 to obtain the actual optical density and should be at least 2.0.

Note: The OD measurement of bacterial cultures is actually based on the amount of light scattered by the culture rather than the amount of light absorbed. In their standard configuration, spectrophotometers are not optimized for light scattering measurements, thus resulting in differences in measured absorbance between instruments.

2.5.5 Preparation of Test Sample Dilutions, Positive Controls

Test samples and positive controls are prepared as 25-fold concentrated stock solutions because they will be diluted 1:25 in the assay (10 μ L in a final volume of 250 μ L). Sample dilutions are performed in a 96-well plate. The dilution scheme is configured for six concentrations, plus a negative (solvent) and positive control; every other well is used to allow for the transfer to the 24-well exposure plate with a multichannel pipette (Fig. 2). The dilution scheme is performed by first adding the appropriate amount of solvent to wells #0–5 of the plate. The 25X stock is transferred to well #6 of the dilution plate. The serial dilutions are performed by transferring a defined volume of the test sample from well #6 to #1. The “0” well is the solvent control and the “+” well is the positive control for the assay.

For the testing of solid chemicals, a top dose of 5 mg/mL (corresponding to a 25-fold stock solution of 125 mg/mL) is recommended. If there are solubility limitations, the lowest workable suspension may be used as the highest concentration. For liquid test samples, a top dose of 5 μ L/mL is recommended. Environmental samples (e.g., surface waters, wastewater, sediment) can be tested in the Ames II assay after appropriate extraction/concentration.

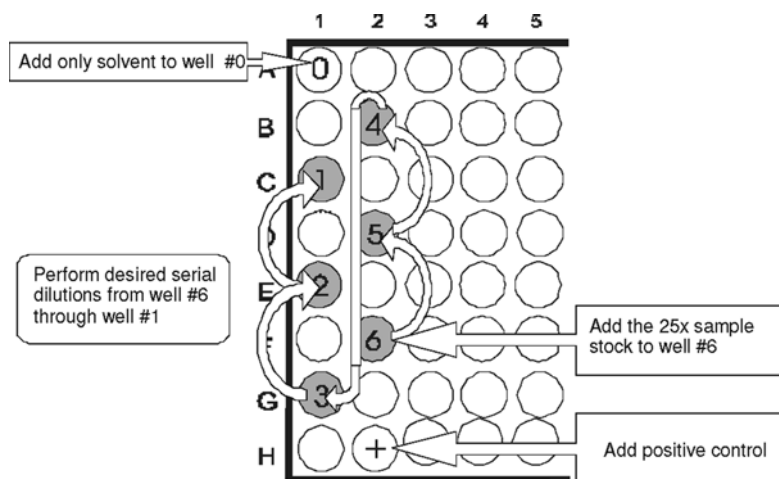


Fig. 2 Preparation of the 96-well dilution plate

Table 2
Preparation of 30 % S9 mix using self-made reagents (one test compound in TA98 and TAMix)

Stock (M)	Reagent	Volumes (mL)
1.00	KCl	0.076
0.25	MgCl ₂ × 6H ₂ O	0.074
0.20	Glucose-6-phosphate	0.058
0.04	NADP	0.230
0.20	NaH ₂ PO ₄ buffer, pH 7.4	1.173
	S9 fraction	0.690
	Final volume	2.301

2.5.6 Amount of Test Sample Needed in the Ames II Assay

The required volumes are dependent on the number of replicates (usually 3) and the dilution factor used. 60 µL of each stock concentration will be required for dosing each strain when testing triplicates with and without S9 (6 × 10 µL). Therefore, enough of the 25X stock should be added to well #6 such that there will be sufficient volume for the serial dilutions. It is recommended to calculate also a dead volume (pipetting reserve) of approximately 20 µL. The required test sample amount and volume can be automatically calculated by using the “Ames MPF dilutions calculator” which is included in the “Ames calculation sheet” and can be downloaded from the Internet [13]. For example, an Ames II assay (two strains) performed in triplicates in the presence and absence of S9, and using a 5 mg/mL top dose and ½ log dilution steps ($10^{0.5} = 3.16$ -fold dilutions), requires 205 µL of a 125 mg/mL stock solution, resulting in a total test sample amount of 25.6 mg. The dilution scheme for the abovementioned example corresponds to the following volumes for Fig. 2: 205 µL of a 125 mg/mL stock is added to well #6 and 140 µL solvent is added to wells #0–5. 65 µL is transferred from well #6 to #5 and mixed. The serial dilutions are completed from well #5 to #4, #4 to #3, #3 to #2, and #2 to #1; the 0 well is the solvent control for the assay.

2.5.7 Preparation of 30 % S9-Mix

The Ames II assay is performed in the absence and presence of exogenous metabolic activation. Aroclor-1254- or phenobarbital/β-naphthoflavone-induced rat liver S9 is usually used. The instructions for preparing the S9 mix are given in Table 2.

Immediately before use, a 30 % S9 mix is prepared by combining the volumes of reagents listed in Table 2 in a sterile tube. All (thawed) reagents are kept on ice.

2.5.8 Preparation of the Exposure Cultures

Note: This dosing protocol applies to one 24-well plate corresponding to an assay with one test sample at six concentrations in triplicates in one strain without or with S9.

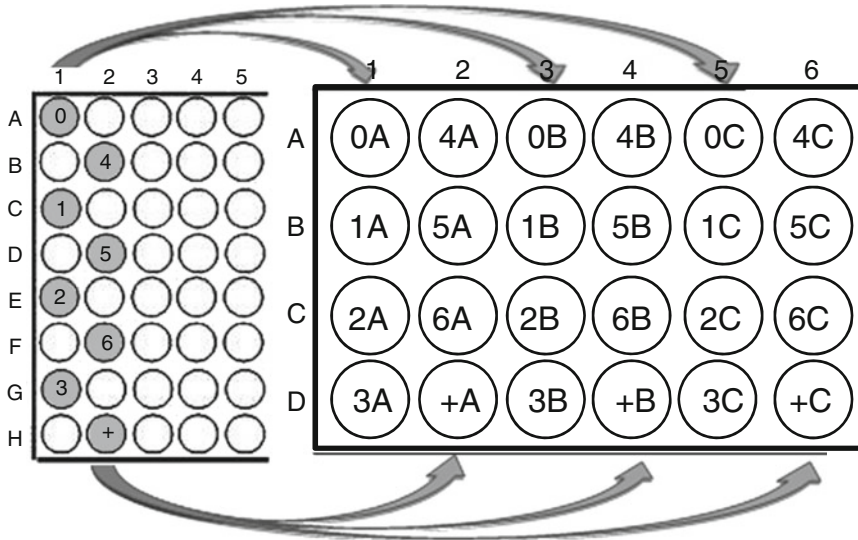


Fig. 3 Transfer of test sample from 96-well dilution plate to 24-well exposure plate

Using an 8-channel pipette with four evenly spaced tips (every other channel), 10 μL from the first column of the dilution plate (wells #0–3) is transferred to columns 1, 3, and 5 of the 24-well plate. Then, 10 μL from the second column of the dilution plate (wells #4–6 and +) is transferred to columns 2, 4, and 6 of the 24-well plate (Fig. 3).

The exposure culture is prepared by adding 6.3 mL exposure medium and 0.7 mL bacterial culture to a sterile reagent reservoir (assay without S9), or by adding 5.25 mL exposure medium, 0.7 mL bacterial culture, and 1.05 mL of freshly prepared 30 % S9 mix to a sterile reagent reservoir (assay with S9). Using an 8-channel pipette, 240 μL is transferred to all wells of the 24-well exposure plate. (The pipette is set to 120 μL since two tips dispense into each well.) Each well of a 24-well plate now contains 10 μL of test sample diluted to the appropriate concentration, 25 μL of bacterial culture, and 215 μL of exposure medium (or 177.5 μL of exposure medium and 37.5 μL of 30 % S9 mix), giving a total volume of 250 μL . This mixture is incubated for 90 min at 37 °C with agitation at 250 rpm.

Precipitation of the sample in the exposure mixture should be recorded.

2.5.9 Transfer of the Exposed Cultures to 384-Well Plates

After the 90 min incubation, 2.6 mL indicator medium is added to each well of the exposure plates. Each replicate (8 wells) is transferred to a separate 384-well plate. Using an 8-channel repeating pipette, 50 μL aliquots of column 1 are dispensed into columns 1–12 of a first 384-well plate. Column 2 of the 24-well exposure plate is dispensed accordingly into columns 13–24 of the first 384-well plate. This procedure is repeated for the remaining columns of the

24-well plates. Columns 3 and 4 of the 24-well plate are dispensed into a second 384-well plate, and columns 5 and 6 are dispensed into a third 384-well plate. This procedure is repeated for each 24-well plate. The transfer can also be processed by a pipetting workstation. After completion of the transfer, the 384-well plates are placed into a sealable plastic bag to prevent evaporation during incubation. The plastic bag is placed into a 37 °C dry incubator for 2 days.

2.5.10 Scoring, Calculation, and Evaluation Criteria

After 2 days, the 384-well plates are scored ideally by placing them on top of a light box. The use of a transparent template that divides the 384-well plate in eight 48-well sections can be helpful. The number of positive wells in each 48-well section is scored. Positive wells are those that have turned yellow or have a bacterial colony visible on the bottom of the well (Fig. 4). Any indication of a color change from purple to yellow should be included in the positive count.

Note: *The plates are easily scored by eye, but they can also be measured with a microplate reader.*

An Ames II assay is considered valid if the solvent control and the positive control values (mean of positive wells of all replicates) are within the acceptable range (Table 3). Toxicity can be assessed

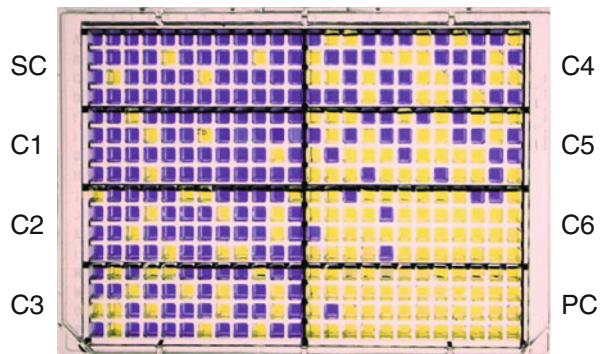


Fig. 4 Plate scoring. *SC* solvent control, *C1–C6* sample concentrations, *PC* positive control

Table 3
Positive controls and acceptable range for solvent and positive controls in the Ames II assay

Strain	S9	Chemical	Acceptable range (positive wells/48)
TA98 and	–	None	≤8
TAMix	–	2-NF/4-NQO (2 µg/mL/0.5 µg/mL)	>25
	+	None	≤8
	+	2-AA (5 µg/mL)	>25

2-NF 2-nitrofluorene, 4-NQO 4-nitroquinoline-*N*-oxide, 2-AA 2-aminoanthracene

by a significant decrease of positive wells relative to the solvent control (generally at high test sample concentrations) and by an increase of the brilliance of the purple medium as compared to the solvent control (cell lysis, absence of bacterial cells).

The fold increase of revertants relative to the solvent control is determined by dividing the mean number of positive wells at each dose by that of the solvent control baseline. The solvent control baseline is derived from the mean number of positive wells in the solvent control plus 1 standard deviation. If the baseline is less than 1, the value is set to 1 for calculation. A fold increase greater than two times the baseline level is generally considered as an alert. Multiple alerts with a dose-response will lead to the test sample being classified as a clear positive. A test sample is classified negative when no response greater than two times the baseline is recorded.

Fold inductions are calculated from the baseline of the actual overnight culture and not from historical data. Although the acceptable range for most cultures is between 0 and 8, the overnight cultures of different experiments have a distinct narrower range (e.g., 2-2-1 or 4-5-3). The use of the baseline will accommodate for larger standard deviations. If more than one compound is tested with the same overnight culture, the negative (solvent) control wells can be pooled. For example, when three samples were tested with the same culture on the same day (e.g., TA98-S9), the three corresponding triplicate negative control scores are pooled to a mean of nine replicates. A large number of negative control counts give a more reliable range of the spontaneous revertants of a given culture.

3 The Ames MPF Penta I Assay

The Ames II test has found its use as an early mutagenicity screening procedure with pharmaceutical and chemical companies [8, 10], as well as in the field of water testing [12]. Recently, the liquid microplate procedure used in the Ames II screen has been adapted to the strains listed in the OECD guideline 471. The regulatory full-scale bacterial reverse mutation assay uses several strains of *S. typhimurium* and *E. coli*, and has been used extensively in genetic toxicology testing. The OECD guideline 471 recommends using at least five tester strains that carry different target sites in order to be sensitive to a broad range of chemicals. They include four strains of *S. typhimurium*, TA98, TA100, TA1535, and TA1537 or TA97a or TA97. These strains all have GC base pairs at the primary reversion site and may therefore not detect certain classes of chemicals. Such chemicals can be detected by either *E. coli* WP2 strains or *S. typhimurium* TA102 which have an AT base pair at the primary reversion site. The Ames microplate fluctuation format (Ames MPF) assay can be performed with *S. typhimurium* TA98, TA100, TA1535,

Table 4
Genotypes of *S. typhimurium* and *E. coli* strains

Strain	Mutation	Type	Cell wall	Repair	pKM101
<i>S. typhimurium</i> (histidine auxotrophic)					
TA98	<i>hisD3052</i>	Frameshift	<i>rfa</i>	<i>uvrB</i>	Yes
TA100	<i>hisG46</i>	Base-pair subst.	<i>rfa</i>	<i>uvrB</i>	Yes
TA1535	<i>hisG46</i>	Base-pair subst.	<i>rfa</i>	<i>uvrB</i>	No
TA1537	<i>hisC3076</i>	Frameshift	<i>rfa</i>	<i>uvrB</i>	No
<i>E. coli</i> wp2 (tryptophan auxotrophic)					
uvrA	<i>trpE65</i>	Base-pair subst.	–	<i>uvrA</i>	No
[pKM101]	<i>trpE65</i>	Base-pair subst.	–	–	Yes

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

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

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

TA1537, and *E. coli* WP2 *uvrA* and *E. coli* WP2 [pKM101] (Table 5). All reagents and the strains can be purchased separately or as a kit (Ames MPF Penta I, [13]). The *Salmonella* strains are histidine auxotrophs and are therefore tested in media with limiting histidine (and biotin) supplementation (exposure medium) or lacking histidine (indicator medium). The *E. coli* WP2 strains are tested in the same way as the *Salmonella* strains with the exception that the exposure medium is supplemented with limiting tryptophan and the indicator medium lacks tryptophan. The genotypes of the strains used in the Ames MPF Penta I assay, including additional mutations which enhance sensitivity to certain mutagens, are shown in Table 4.

3.1 Assay Description

The procedure for the Ames MPF Penta I assay is basically the same as described for the Ames II assay (Fig. 1, paragraphs 2.5.3–2.5.10), except that different strains are used (some of them being differently diluted in the exposure medium), and that the *E. coli* strains with their tryptophan requirement need a different exposure and indicator medium than the histidine-requiring *Salmonella* strains.

The Ames MPF Penta I assay, in brief, is as follows:

1. Growth of tester strains overnight.
2. Exposure to test sample and S9 if employed.
3. Distribution and plating of cells in a medium which selects for revertants. This medium contains a pH indicator dye that turns from purple to yellow upon bacterial growth.

4. Incubation in 384-well plates for 48 h to allow growth of revertant bacteria.
5. Scoring of plates for positive (yellow) wells, data entry, and evaluation of mutational potential.

3.2 Advantages

A main advantage of the Ames MPF over the Ames II assay is that strains recommended in the OECD guideline 471 [4] are used, thus making the microfluctuation procedure a valid alternative to the regulatory plate incorporation/preincubation assay. The technical advantages of the Ames MPF test system are the same as those for the Ames II test:

- Almost four times less test chemical is needed than in the standard plate or preincubation test.
- It requires less hands-on time since the low-volume liquid format allows working with multichannel pipettes and robotic systems.
- It needs less plasticware and 10 times less S9 mix.
- The assay has an easy colorimetric readout in 48-well sections of a 384-well plate.
- The Ames MPF assay is available as a kit including all necessary ingredients ready to use and constantly quality controlled, and with detailed instruction for use.
- Easy and quick colorimetric readout.

3.3 Validation Studies

In the first validation study of 1998 with base-specific tester strains, Gee et al. [5] included also the traditional frameshift strains TA98 and TA1537 for comparing the liquid microplate format with published data of the preincubation method. There was an overall agreement of 84 % (21/25) and of 94 % (18/20) in the TA98 and TA1537 results, respectively. The liquid format seemed to be more sensitive in this comparison. Several posters [16–18] presented at different congresses showed the excellent concordance of the Ames MPF test results obtained with a combination of the standard set of tester strains with those of published data for the traditional test. The Ames MPF assay using the four *Salmonella* strains TA98, TA100, TA1535, and TA1537 showed the best correlation with a miniaturized plate incorporation assay (“Mini Ames”) when compared to the Vitotox and the Ames II at UCB Pharma [19]. In 2010, Umbuzeiro et al. [20] compared the Ames MPF method and the microsuspension assay developed by Kado et al. [21] by concurrently testing environmental samples (air, surface water, effluent water) with strain TA98. The results of both assays correlated very well, and the authors considered the Ames MPF method a valid alternative to the microsuspension assay due to its easy handling. In their 2012 publication [22], Flückiger-Isler and Kamber tested 15 equivocal to weakly positive chemicals selected

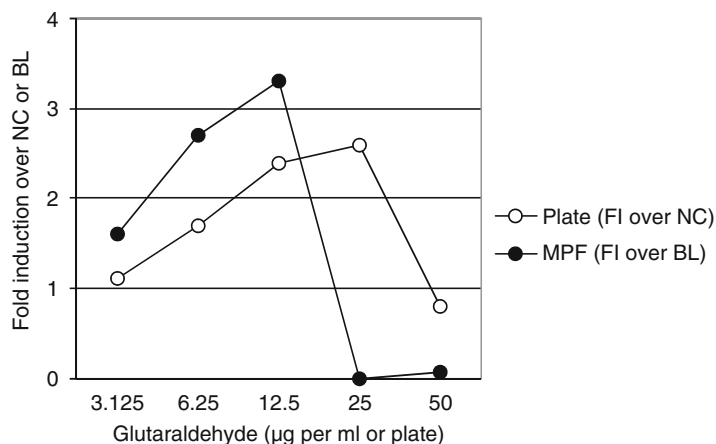


Fig. 5 Dose–response of glutaraldehyde in TA100 without S9. *NC* negative control, *BL* baseline (mean negative control + 1 SD), *FI* fold induction, *Plate* preincubation method, *MPF* microplate fluctuation method

from the National Toxicology Program (NTP) database concurrently in the Ames MPF and the standard Ames preincubation method. Thirteen of the 15 chemicals showed concordant results in both tests despite the challenging choice of chemicals. The Ames MPF method appeared to be more sensitive, since in half of the positive results, the mutagenic effect or cytotoxicity, if present, was seen at lower doses in the Ames MPF than in the Ames plate incorporation method (Fig. 5).

3.4 Deviations from the Ames II Test Protocol

The assay procedure is described in detail in the “Ames MPF Penta I Instructions for Use” [23]. Furthermore, a “visual guide for Ames MPF” illustrates all steps of the test procedure and the evaluation of the results [13].

3.4.1 Preparation of Strains and Media

The *Salmonella* strains were developed in the laboratory of Dr. Bruce Ames, University of California, Berkeley, and have been established in many laboratories. The *E. coli* strains are available at the National Collections of Industrial and Marine Bacteria Limited, Aberdeen, Scotland, UK.

Ready-to-use kits for performing the Ames microfluctuation assay with the standard tester strains are commercially available [13, 14]. The tester strains are cryopreserved and delivered in a semisolid form which allows for shipment at ambient temperatures for up to 10 days [13]. The kits also include ampicillin, positive controls, and S9. Reagents for preparing the S9 mix (buffer–salts, G-6-P, and NADP) can be prepared individually or are available as ready-to-use solutions [13]. The necessary plasticware (culture tubes, multiwall plates, reagent reservoirs) can be bought individually.

3.4.2 *Equipment* *see* Ames II (Sect. 2.5.2).

3.4.3 *Overnight Culture Preparation* The strains of the Ames MPF Penta I assay [23] are cryopreserved in a different medium (semisolid) than those of the Ames II assay (liquid). This necessitates a small additional step in the preparation of the overnight cultures.

After thawing and the addition of 200 μL growth medium, the semisolid pellet is disrupted mechanically by pipetting up and down until a uniform suspension is obtained which can be pipetted repeatedly without clogging the tip and which shows visually a homogeneous distribution of the dark fragments. 25 μL of the dispersion is added to 50 mL culture tubes containing 10 mL growth medium with ampicillin (strains TA98, TA100, *E. coli* [pKM101]) or without ampicillin (strains TA1535, TA1537, and *E. coli* WP2 *uvrA*). The culture tubes are capped loosely (or filter caps are used) in order to allow for sufficient aeration. The cultures are grown for 12–15 h at 37 °C, 250 rpm in an environmental shaker to the late exponential phase.

3.4.4 *Determination of Optical Density (OD_{600})* *see* Ames II (Sect. 2.5.4).

3.4.5 *Preparation of Test Sample Dilutions, Positive Controls* The procedure is the same as described for the Ames II assay (Sect. 2.5.5), except that the different positive controls should be added directly to the corresponding wells of the 24-well exposure plates.

The recommended sample top dose for the Ames MPF assay is the same as described for the Ames II assay.

3.4.6 *Amount of Test Sample Needed in the Ames MPF Assay (See also Sect. 2.5.6)* As in the Ames II protocol, 60 μL of each stock concentration will be required for dosing each strain when testing in triplicates with and without S9 ($6 \times 10 \mu\text{L}$), and there should be sufficient volume (including a dead volume) of the 25X stock added to well #6 to perform the serial dilutions. When the calculated volume of the sample in well #6 is $>360 \mu\text{L}$, it is recommended to use a 24-well plate as the dilution plate and to increase the dead volume to 40–50 μL . The required test sample amount and volume can be automatically calculated by using the “Ames MPF dilutions calculator” which is included in the Ames calculation sheet and can be downloaded [13]. For example, a full-scale Ames MPF Penta I assay with five tester cultures performed in the presence and absence of S9, and using triplicates, a 5 mg/mL top dose and $\frac{1}{2}$ log dilution steps, requires 497 μL of a 125 mg/mL stock solution, resulting in a total test sample amount of 62.2 mg. The dilution scheme for the abovementioned example corresponds to the following volumes for Fig. 2: 497 μL of a 125 mg/mL stock is added to well #6 and 340 μL solvent is added to wells #0–5. 157 μL is transferred from well #6 to #5 and mixed. The serial dilutions are completed from well #5 to #4, #4 to #3, #3 to #2, and #2 to #1; the 0 well is the solvent control for the assay.

3.4.7 Preparation of 30 % S9-Mix

The S9 mix is prepared as described for the Ames II assay (Sect. 2.5.7). Instructions for preparing appropriate volumes of the S9 mix for one to five strains are given in Table 5.

3.4.8 S9 Booster Solution (Optional)

Some batches of S9 can lead to signs of toxicity when tested with the positive control chemical 2-AA, especially in strains TA100 and TA1537. Xenometrix AG therefore provides the S9 together with a “S9 booster solution” to protect strains TA100 and TA1537 from possible toxic S9 effects. This solution will be mixed with the exposure medium when using S9 in the Ames MPF assay.

Procedure for assays with S9: The S9 booster solution is mixed with the *Salmonella* exposure medium at a ratio 1:667 (e.g., 10 mL exposure medium + 15 µL booster solution). The required volume of exposure medium/S9 booster solution mixture is prepared at the day of the assay.

3.4.9 Preparation of Exposure Cultures

The transfer from the dilution plate to the 24-well exposure plates is performed as described for the Ames II (Sect. 2.5.8).

The preparation of the TA98, TA1535, and TA1537 exposure cultures is the same as described for the Ames II, whereas TA100 and the *E. coli* are more diluted in the exposure medium. The two *E. coli* strains are grown individually overnight and then exposed together to a test sample. This “EC Combo” has the following advantages over an individual exposure: The two strains are not equally sensitive to different chemicals and it is always the more sensitive strain that dominates in the EC Combo assay which allows to detect more mutagens than with the single strains alone [17]. Table 6 lists the volumes of the exposure culture components.

The 24-well plates are incubated at 37 °C for 90 min (*Salmonella*, *E. coli* without S9) or 20 min (*E. coli* with S9) at 37 °C, 250 rpm. It is strongly recommended to use a shortened

Table 5
Preparation of 30 % S9 mix using self-made reagents (for one test compound)

Stock (M)	Reagent	Volume for one strain (mL)	Volume for two strains (mL)	Volume for three strains (mL)	Volume for four strains (mL)	Volume for five strains (mL)
1.00	KCl	0.043	0.076	0.110	0.146	0.179
0.25	MgCl ₂ × 6H ₂ O	0.042	0.074	0.106	0.141	0.172
0.20	G-6-P	0.033	0.058	0.083	0.111	0.136
0.04	NADP	0.130	0.230	0.330	0.440	0.540
0.20	NaH ₂ PO ₄ buffer	0.663	1.173	1.683	2.244	2.754
	S9 fraction	0.390	0.690	0.990	1.320	1.620
	Final volume	1.301	2.301	3.302	4.402	5.401

Table 6
Preparation of exposure cultures

Strain		Strain dilution	Exposure medium (mL)	Bacterial culture (mL)	S9
TA98		1:10	6.3	0.7	–
		1:10	5.25	0.7	1.05 mL
TA100		1:20	6.65	0.35	–
		1:20	5.6	0.35	1.05 mL
TA1535		1:10	6.3	0.7	–
		1:10	5.25	0.7	1.05 mL
TA1537		1:10	6.3	0.7	–
		1:10	5.25	0.7	1.05 mL
<i>E. coli</i> Combo	<i>uvrA</i>	1:14.3	6.3	0.49	–
	pKM101	1:33.3		0.21	
	<i>uvrA</i>	1:14.3	5.25	0.49	1.05 mL
	pKM101	1:33.3		0.21	

exposure time of 20 min for *E. coli* in the presence of S9. This clearly improves the relatively weak response after 90 min to the positive control 2-aminoanthracene (2-AA), as well as to benzo(a) pyrene (B(a)P), another indirect mutagen (Fig. 6). For other tested chemicals that are positive in the absence and presence of S9 (4-nitroquinoline-*N*-oxide, methyl methanesulfonate, streptonigrin, *N*⁺-aminocytidine, cumene hydroperoxide, and glutaraldehyde), the reduction of the exposure time in assays with metabolic activation results in an equal or slightly better sensitivity. For all *Salmonella* tester strains and for *E. coli* without S9, the exposure time is 90 min.

3.4.10 Transfer of the Exposed Cultures to 384-Well Plates and Incubation

The procedure is the same as for the Ames II (Sect. 2.5.9), except that 2.6 mL *E. coli* indicator medium is added to the *E. coli* Combo plates, and that the *E. coli* Combo with S9 should be processed after 20 min of exposure (*see above*).

3.4.11 Scoring, Calculation, and Evaluation Criteria

Plate scoring, toxicity assessment, and evaluation criteria for the Ames MPF are the same as described in detail for the Ames II under Sect. 2.5.10.

Preliminary results for the TA100, TA1537, and TA1535 cultures can be obtained already after 1 day of incubation. Revertant growth of TA100 and TA1537 cultures is almost completed after 28 h, whereas the number of positive wells of the TA1535 plates will further increase overnight. TA98 and *E. coli* plates can be scored reliably only after 2 days.

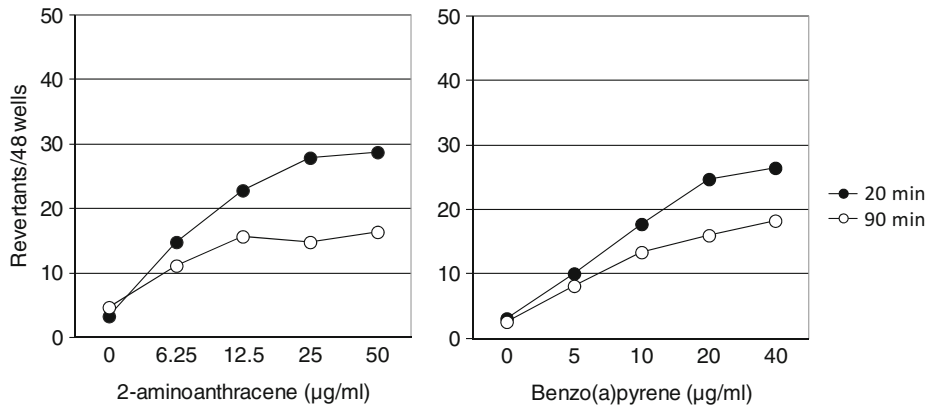


Fig. 6 Effect of exposure time on the 2-AA and B(a)P induction profile with *E. coli* strains in the presence of Aroclor 1254-induced S9

Table 7

Positive controls and acceptable range for solvent and positive controls in the Ames MPF Penta I assay mean of positive wells

	TA98	TA100	TA1535	TA1537	<i>E. coli</i> Combo
<i>Without S9</i>					
Solvent control	≤8	≤12	≤8	≤8	≤12
2-NF (2.0 µg/mL)	≥25				
4-NQO (0.1 µg/mL)		≥25			
N4-ACT (100 µg/mL)			≥25		
9-AAc (15 µg/mL)				≥25	
4-NQO (2 µg/mL)					≥25
<i>With S9</i>					
Solvent control	≤8	≤12	≤8	≤8	≤12
2-AA (0. 5–5.0 µg/mL) ^a	≥25	≥25	≥25	≥25	
2-AA (50 µg/mL)					>2-fold baseline ^b

2-*NF* 2-nitrofluorene, 4-*NQO* 4-nitroquinoline-*N*-oxide, *N4-ACT* *N*⁴-aminocytidine, 9-*AAC* 9 aminoacridine, 2-*AA* 2-aminoanthracene

^aDifferent concentrations of 2-AA may be used depending on the S9-inducing agent (Aroclor-1254 or phenobarbital/β-naphthoflavone), on the different *Salmonella* strains, and on the actual S9 batch. When using Aroclor-1254-induced S9, a 2-AA concentration of 2.5 µg/mL may be used for all *Salmonella* strains if the preparation of different concentrations seems to be too laborious. Since strain TA98 is considerably more sensitive to 2-AA than strains TA1535 and TA1537, the use of strain-specific 2-AA concentrations will better indicate eventual problems with the condition of a culture. The 2-AA dose-responses of all tester strains in the presence of S9 are given in the Xenometrix S9 Certificates of Analysis for each individual batch

^b20 min exposure

An Ames MPF assay is considered valid if the solvent control and the positive control values are within the acceptable range (Table 7).

An alternative way to evaluate the Ames MPF results is to use a detection limit based on the binomial formula employed by Smith et al. [24], since Ames test responses are not normally distributed, but follow a binomial distribution.

4 Testing of Non-concentrated Water Samples

The Ames microplate format has also been adapted to test native non-concentrated water samples [25]. The use of a tenfold concentrated exposure medium, which will be diluted to single strength by the aqueous sample, allows to decrease the dilution factor in the assay 18.5-fold.

5 Conclusion

The Ames II/Ames MPF procedure has several significant advantages over the standard Ames test: less test sample, less hands-on time, less S9 consumption, less hazardous waste, suitable for being automated, and easy colorimetric readout. Based on the many comparative studies, there is no evidence that the Ames II/Ames MPF is less capable than the standard Ames at detecting mutagens.

The Ames II test can be used for the screening of a large number of samples or samples available in limited quantities in early drug discovery. It contains the frameshift strain TA98 and covers all six possible base-pair mutations detecting strains in TAMix. However, it will not detect frameshift mutations that specifically revert TA1537. Furthermore, the six base-specific strains are combined to a single culture—the TAMix—and thus diluted sixfold. Alternatively, TAMix can be replaced by the traditional strain TA100 in the Ames MPF 98/100 assay, hence using a classic base-pair strain with comparable spectrum of responsiveness [16].

The liquid Ames MPF procedure (e.g., Ames MPF Penta I) with its substantial advantages can be used with the traditional *Salmonella* and *E. coli* tester strains and covers all the genetic endpoints recommended in the OECD 471. It is therefore a valid alternative to the standard Ames plate test for testing of chemicals, pharmaceuticals, cosmetics, and—with an appropriate combination of strains—environmental samples such as surface water, wastewater, air, or sediment.

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