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Mutation Research 455 (2000) 29–60

Fundamental and Molecular Mechanisms of Mutagenesis

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The Ames *Salmonella*/microsome mutagenicity assay

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Abstract

The Ames *Salmonella*/microsome mutagenicity assay (*Salmonella* test; Ames test) is a short-term bacterial reverse mutation assay specifically designed to detect a wide range of chemical substances that can produce genetic damage that leads to gene mutations. The test employs several histidine dependent *Salmonella* strains each carrying different mutations in various genes in the histidine operon. These mutations act as hot spots for mutagens that cause DNA damage via different mechanisms. When the *Salmonella* tester strains are grown on a minimal media agar plate containing a trace of histidine, only those bacteria that revert to histidine independence (*his*$^+$) are able to form colonies. The number of spontaneously induced revertant colonies per plate is relatively constant. However, when a mutagen is added to the plate, the number of revertant colonies per plate is increased, usually in a dose-related manner.

The Ames test is used world-wide as an initial screen to determine the mutagenic potential of new chemicals and drugs. The test is also used for submission of data to regulatory agencies for registration or acceptance of many chemicals, including drugs and biocides. International guidelines have been developed for use by corporations and testing laboratories to ensure uniformity of testing procedures.

This review provides historical aspects of how the Ames was developed and detailed procedures for performing the test, including the design and interpretation of results.

Keywords: Ames *Salmonella*/microsome mutagenicity assay; *Salmonella* strains; Gene mutation

1. Introduction

The identification of substances capable of inducing mutations has become an important procedure in safety assessment. Chemicals that can induce mutations can potentially damage the germ line leading to fertility problems and to mutations in future generations. Mutagenic chemicals are also capable of inducing cancer, and this concern has driven most of the mutagenicity testing programs. Mutations can occur as gene (point) mutations, where only a single base is modified, or one or a relatively few bases are inserted or deleted, as large deletions or rearrangements of DNA, as chromosome breaks or rearrangements, or as gain or loss of whole chromosomes.

Gene mutations are readily measured in bacteria and other cell systems when they cause a change in the growth requirements of the cell, whereas chromosome damage in mammalian cells is typically measured by observing the cell's chromosomes under magnification for breaks or rearrangements. The *Salmonella typhimurium*/microsome assay (*Salmonella* test; Ames test) is a widely accepted short-term bacterial assay for identifying substances that can produce genetic damage that leads to gene mutations. The test uses a number of *Salmonella* strains with preexisting muta-

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0027-5107/00/$ – see front matter
PII: S0027-5107(00)00064-6

tions that leave the bacteria unable to synthesize the required amino acid, histidine, and therefore unable to grow and form colonies in its absence. New mutations at the site of these preexisting mutations, or nearby in the genes, can restore the gene's function and allow the cells to synthesize histidine. These newly mutated cells can grow in the absence of histidine and form colonies. For this reason, the test is often referred to as a "reversion assay."

The *Salmonella* strains used in the test have different mutations in various genes in the histidine operon; each of these mutations is designed to be responsive to mutagens that act via different mechanisms. Additional mutations were engineered into these strains to make them more sensitive to a wide variety of substances.

The *Salmonella* mutagenicity test was specifically designed to detect chemically induced mutagenesis [7]. Over the years its value as such has been recognized by the scientific community, and by government agencies and corporations. The test is used world-wide as an initial screen to determine the mutagenic potential of new chemicals and drugs because there is a high predictive value for rodent carcinogenicity when a mutagenic response is obtained [49,71,85,92]. International guidelines have also been developed (e.g., Organisation for Economic Co-operation and Development (OECD); International Commission on Harmonization (ICH)) for use by corporations and testing laboratories to ensure uniformity of testing procedures prior to submission of data to regulatory agencies for registration or acceptance of many chemicals, including drugs and biocides.

2. Historical aspects

The Ames *Salmonella*/microsome mutagenicity assay evolved over the years from the initial screening of a number of histidine mutants which led to the selection of mutants that were highly sensitive to reversion by a variety of chemical mutagens [2,5,39,40,47]. Because bacteria are unable to metabolize chemicals via cytochromes P450, as in mammals and other vertebrates, a key component for making the bacterial mutagenicity test useful was the inclusion of an exogenous mammalian metabolic activation system [6,43]. At the same time, the development of the plate incorporation assay protocol to replace spot test or liquid suspension procedures was a major contributing factor to the success of the Ames test because it made the test easier to perform and reduced its cost.

2.1. Screening of histidine mutants and development of the plate incorporation assay

Studies performed to identify and map the genes responsible for histidine biosynthesis produced a large number of spontaneous, radiation-, and chemical-induced histidine mutants of *Salmonella typhimurium* LT-2 [27,82]. Some of the mutants contained single base changes (base-pair substitution mutants), and others contained additions or deletions of one or more bases (frameshift mutants). It was later realized that some of these mutant strains could be used to identify and characterize mutagenic chemicals by their ability to revert to wild-type (histidine-independence) in the presence of mutagens. In 1966, Ames and Whitfield [4] proposed a set of histidine mutant strains for screening chemicals for mutagens using a spot test procedure that was previously used by Szybalski [72] and Iyer and Szybalski [34] for mutagen screening with an *E. coli* strain. The spot test consists of applying a small amount of the test chemical directly to the center of a selective agar medium plate seeded with the test organism. As the chemical diffuses into the agar a concentration gradient is formed. A mutagenic chemical will give rise to a ring of revertant colonies surrounding the area where the chemical was applied. If the chemical is toxic, a zone of growth inhibition will also be observed [2]. Fig. 1 depicts a spot test with methylmethane sulfonate and strain *Salmonella* strain TA100.

In 1973, Ames et al. [5,6] developed the plate incorporation assay procedure which is more sensitive and quantitative than the spot test. The procedure consists of adding the buffer or S-9 mix, the histidine dependent bacteria (about 10^8) and test chemical to 2 ml of top agar containing biotin and a trace amount of histidine (0.05 mM each). The mixture is then gently mixed and poured on glucose minimal (GM) agar plates. When the top agar has solidified the plates are incubated in an inverted position in a 37°C incubator for 48 h at which time the histidine revertant colonies are counted. The assay procedure is depicted in Fig. 2.

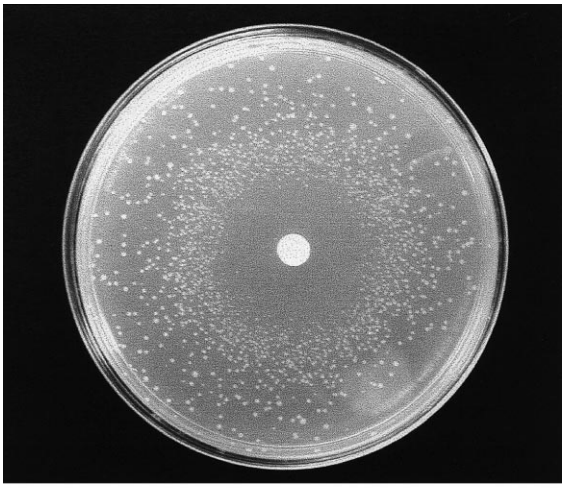

Fig. 1. Spot test with strain TA100 and methyl methanesulfonate (10 μl).

When the histidine dependent bacteria are grown on a glucose minimal (GM) agar plate containing a trace amount of histidine, only those cells that revert to histidine independence (His^+) are able to form colonies. The small amount of histidine allows all the plated bacteria to undergo a few cell divisions; in many cases, this growth is essential for mutagenesis to occur. The His^+ revertants are easily scored as colonies against the slight background growth. The number of spontaneously induced revertant colonies is relatively constant for each strain. However, when a mutagen is added to the plate, the number of revertant colonies per plate is increased, usually in a dose related manner as depicted in Fig. 3.

The plate incorporation test does not permit the enumeration of the total number of surviving cells because of bacterial growth on the plate and competing toxicity due to the chemical treatment. Also, because of the extra cell divisions that take place after adding the

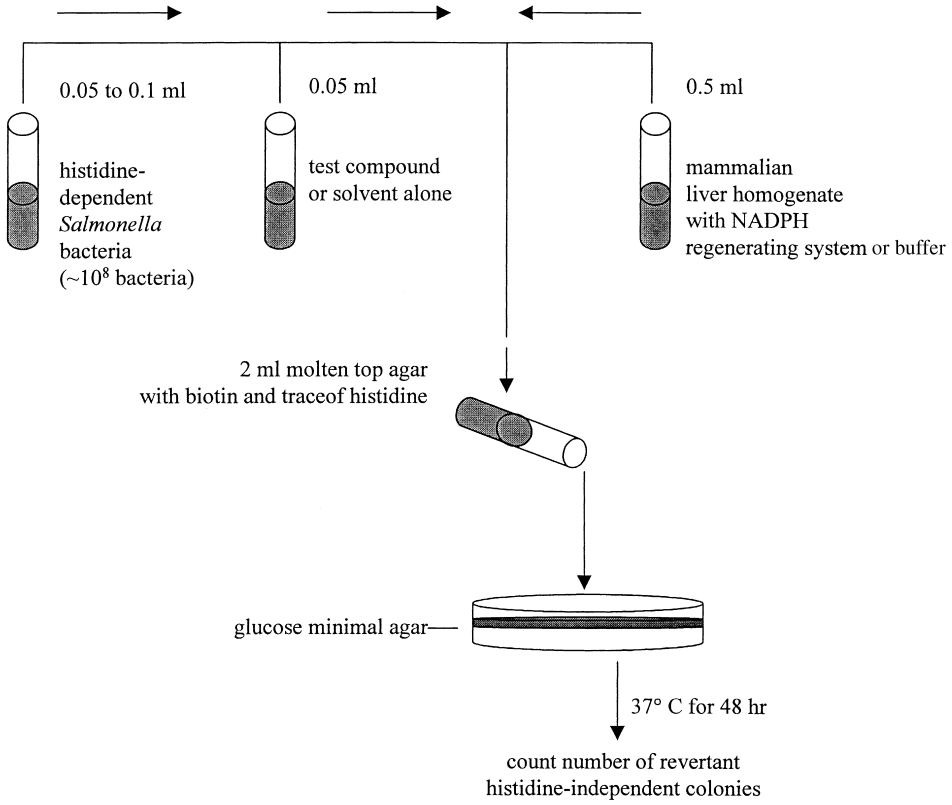

Fig. 2. Diagram depicting the steps involved in the plate incorporation assay.

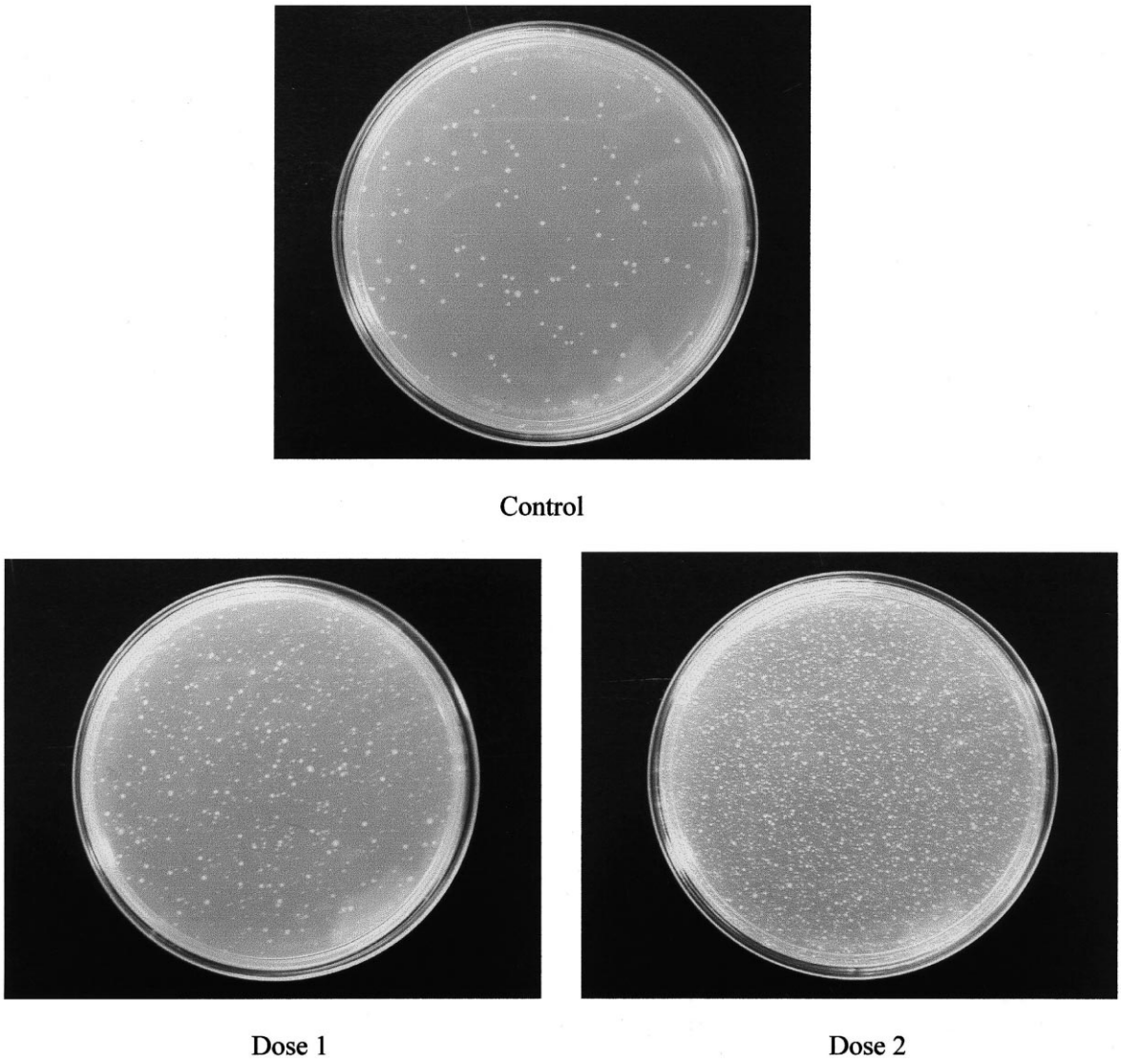

Fig. 3. Mutagenic dose response with strain TA100 and sodium azide. Control: spontaneous revertants; dose 1:2.5 μg/plate; dose 2:5 μg/plate.

bacteria to the plate, it is not possible to estimate the number of cells at risk for mutation. Therefore, the mutation values obtained can only be expressed as number of mutants/plate, or number of mutants/amount of chemical added.

A plate test that can provide quantitative toxicity information is the “treat-and-plate” suspension procedure but takes longer to perform than the plate incorporation assay. In this procedure, the bacteria are washed free of growth medium, resuspended in non-nutrient growth medium and treated with the test substance for various time intervals. Separate samples of the bacteria are then plated on selective medium for mutant determination and on complete medium for survival determination. The results establish the mutation frequency by calculating the number

of mutants per surviving fraction of bacteria [26]. However, the plate incorporation assay offers the advantages that limited steps are required to expose the bacteria to the test chemical with no need for washing or resuspending the bacteria prior to or after treatment. In addition, the bacteria are allowed to undergo a few cell divisions in the presence of the test substance, which increases their sensitivity to mutation induction.

2.2. Metabolic activation systems

2.2.1. Oxidative metabolism

Some carcinogenic chemicals, such aromatic amines or polycyclic aromatic hydrocarbons, are biologically inactive unless they are metabolized to active forms. In humans and lower animals, the cytochrome-based P450 metabolic oxidation system, which is present mainly in the liver and to a lesser extent in the lung and kidneys, is capable of metabolizing a large number of these chemicals to DNA-reactive, electrophilic forms. Some of the intermediate metabolites are potent mutagens in the Ames *Salmonella* assay. Since bacteria do not have this metabolic capability, an exogenous mammalian organ activation system needs to be added to the petri plate together with the test chemical and the bacteria. For this purpose, a rodent metabolic activation system was introduced into the test system [5,22,43,51,69]. The metabolic activation system usually consists of a $9000 \times g$ supernatant fraction of a rat liver homogenate (S-9 microsomal fraction), which is delivered to the test system in the presence of NADP and cofactors for NADPH-supported oxidation (S-9 mix) [47]. To increase the level of metabolizing enzymes, the animals are pretreated with the mixed-function oxidase inducer Aroclor 1254. Other inducers, such as phenobarbital and β-naphthoflavone, can also be used.

The mixed function oxidase enzymes (S-9 fraction) can also be obtained from animal species other than rat such as mouse, hamster, guinea-pig and monkey and organs other than liver such as kidney [1,31,48] and from human liver [66]. In comparative studies in which coded compounds were tested, induced and uninduced Syrian hamster or mouse liver S-9 offered no overall advantage over Aroclor-1254 induced rat liver S-9 for the induction of mutagenesis [17,18,29]. However, there are chemicals that may be more efficiently detected as mutagens with rat, mouse, or hamster liver S-9 [17,18,90].

2.2.2. Reductive metabolism

The metabolic activation system can also consist of a reductive enzyme system for classes of chemicals containing azo and diazo bonds. Reduction of chemical substances can occur in mammals, including humans, by anaerobic intestinal microflora, and very likely by mammalian reductases in the intestinal wall or in the liver. Two types of reductive in vitro metabolic activation systems have generally been used, those based on a liver homogenate supplemented with FMN [58,59], and those that are based on rat intestinal microflora preparations [62–64].

2.3. The Salmonella tester strains

2.3.1. Genotypes

The genotypes of the commonly used *Salmonella* tester strains are listed in Table 1. All strains are histi-

Table 1
Genotype of the most commonly used *Salmonella* tester strains

Mutation (strain)	*bio chlD uvrB gal*	LPS defect	Plasmid
hisG46			
TA1535	Deletion mutation	*rfa*	No plasmid
TA100	Deletion mutation	*rfa*	pKM101
hisD3052			
TA1538	Deletion mutation	*rfa*	No plasmid
TA98	Deletion mutation	*rfa*	pKM101
hisC3076			
TA1537	Deletion mutation	*rfa*	No plasmid
hisD6610	Deletion mutation	*rfa*	pKM101
hisO1242			
TA97			
hisG428			
TA104	Deletion mutation	*rfa*	No plasmid
TA102	Wild type	*rfa*	pKM101, pAQ1

dine dependent by virtue of a mutation in the histidine operon. Additional mutations/genetic alterations that have made the tester strains more sensitive to chemical mutagens are listed below.

- A deletion mutation through the *uvrB-bio* genes in all strains, except TA102. The *uvrB* deletion mutation eliminates the accurate excision repair mechanism, thereby allowing more DNA lesions to be repaired by the error-prone DNA repair mechanism. The deletion through the biotin gene makes the bacteria biotin dependent [5].
- A mutation (*rfa*) in all strains that leads to a defective lipolysaccharide (LPS) layer that coats the bacterial surface, making the bacteria more permeable to bulky chemicals [5].
- Introduction of plasmid pKM101 in strains TA1535 and TA1538 resulting in the corresponding isogenic strains TA100 and TA98 [7] and in strains TA97 [39] and TA102 and TA104 [40]. Plasmid pKM101 enhances chemical and UV-induced mutagenesis via an increase in the error-prone recombinational DNA repair pathway [50,65,80]. The plasmid confers ampicillin resistance, which is a convenient marker to detect the presence of the plasmid [53].
- Insertion of the mutation *hisG428* on the multicopy plasmid pAQ1 which was introduced in strain TA102 with the aim of amplifying the number of target sites. To enhance the ability of this strain to detect DNA cross-linking agents, the *uvrB* gene was retained making the bacterium DNA repair proficient [40].

2.3.2. Specific target DNA sequence

Table 2 lists the DNA sequences of the target mutations in the commonly used *Salmonella* tester strains. The *hisG46* marker in strains TA1535 and TA100 results from the substitution of a leucine (GAG/CTC) by a proline (GGG/CCC) [11]. This mutation is reverted to the wild-type state by mutagens that cause base-pair substitution mutations primarily at one of the GC pairs. The *hisD3052* mutation carried by strains TA1538 and TA98 is a -1 frameshift mutation which affects the reading frame of a nearby repetitive –C–G–C–G–C–G–C–G– sequence [33]. Reversion of the *hisD3052* mutation back to the wild-type state is induced by various frameshift mutagens such as 2-nitrofluorene and various aromatic nitroso derivatives of amine carcinogens. Strain TA1537 which carries the *hisC3076* mutation appears to have a +1 frameshift mutation

Table 2
DNA sequence specificity on the *Salmonella* tester strains

Allele/strain(s)	DNA target	Reversion event	Reference
hisG46 TA100 TA1535	–G–G–G–	Base-pair substitution	[11]
hisD3052 TA98 TA1538	–C–G–C–G–C–G–C–G–	Frameshifts	[33]
hisC3076 TA1537	+1 frameshift (near –C–C–C– run)	Frameshifts	[5]
hisD6610 TA97	–C–C–C–C–C–C– (+1 cytosine at run of C's)	Frameshifts	[39]
hisG428 TA102 TA104	TAA (ochre)	Transitions/transversions	[40]

near the site of a repetitive –C–C–C– sequence and is reverted to the wild-type level by frameshift mutagens that are not readily detected by the *hisD3052* marker, such as 9-aminoacridine [5]. The *hisD6610* mutation in strain TA97 also carries a +1 frameshift mutation (cytosine) resulting in a run of 6 cytosines (–C–C–C–C–C–C–). This strain is believed to be more sensitive than TA1537 to frameshift mutagens, and, unlike strain TA1537, is sensitive to some of the mutagens that revert strains TA1538 and TA98 [39].

It should be noted that the DNA target sites of the above described tester strains contain GC base pairs. In contrast, two additional strains TA102 and TA104 were developed that contain AT base pairs at the *hisG428* mutant site. The mutation is carried on the multi-copy plasmid pAQ1 in strain TA102 and on the chromosome in strain TA104. The plasmid confers tetracycline resistance, which is a convenient marker to detect the presence of the plasmid. The *hisG428* mutation is an ochre mutation, TAA, in the *hisG* gene which can be reverted by all six possible base-pair changes; both transitions and transversions. This mutation is also reverted by mutagens that cause oxidative damage. In addition, the DNA repair proficient strain TA102 detects cross-linking agents such as bleomycin and mitomycin C [40].

2.4. Construction of base-specific Salmonella tester strains

A set of 6 base-specific *Salmonella* tester strains was developed by Gee et al. [24], with the unique property that each strain can be reverted by a unique transition or transversion event thereby enabling the identification of specific base-pair substitutions. In addition to the *his* mutation all strains carry the following genetic markers:

- the *rfa* mutation that affects the permeability of the cell wall
- the *uvrB-bio* deletion that affects the accurate DNA repair pathway and which makes the cells biotin dependent
- the mutagenesis-enhancing plasmid pKM101.

Each strain carries a unique missense mutation with the base change indicated below in the histidine biosynthetic operon:

Strain	Operon	Mutation
TA7001	*hisG1775*	AT to GC
TA7002	*hisC9138*	TA to AT
TA7003	*hisG9074*	TA to GC
TA7004	*hisG9133*	GC to AT
TA7005	*hisG9130*	CG to AT
TA7006	*hisC9070*	CG to GC

The results of a validation study were published by Gee et al. [25].

2.4.1. Modifications of the standard plate incorporation assay

Over the years, modifications to the standard plate incorporation assay (Fig. 2) have been developed by different researchers that enhanced the sensitivity of the test and allowed the testing of a wider range of chemicals, including gases and volatile chemicals. The most commonly used modifications are described below.

2.4.2. The preincubation assay

In the preincubation assay, the tester strains are exposed to the chemical for a short period (20 to 30 min) in a small volume (0.5 ml) of either buffer or S-9 mix, prior to plating on glucose agar minimal medium (GM agar) supplemented with a trace amount of histidine. With few exceptions it is believed that this assay is more sensitive than the plate incorporation assay, because short-lived mutagenic metabolites may have a better chance reacting with the tester strains in the small volume of preincubation mixture, and the effective concentration of S-9 mix in the preincubation volume is higher than on the plate [29,55,84, 89,90].

2.4.3. The desiccator assay for volatile liquids and gases

The use of a closed chamber is recommended for testing highly volatile chemicals and gases [8,30,67,91].

2.4.4. The Kado Salmonella microsuspension assay for testing small sample volumes

This procedure was designed to detect mutagenic metabolites in urine samples obtained from animals treated with test chemicals [35] because of the relatively small sample volumes obtained in these stud-

ies. In this procedure, the overnight cultures are centrifuged to obtain a 10-fold higher than normal cell density (i.e., about 1 to 2×10^{10} bacteria per ml, which results in about 1 to 2×10^{9} bacteria per tube when delivered in a 0.1 ml inoculum) for use in the preincubation assay. The exposure of a higher number of bacteria to the urine samples is believed to enhance the detection of mutagenic metabolites present in urine samples. This procedure is also well suited for testing small quantities (about 20 mg) of samples where the sample quantities are limited, and for testing complex mixtures other than urine samples.

2.4.5. Testing of chemicals in a reduced oxygen atmosphere

Anaerobic environments, such as anaerobic chambers, have been used to study mutagenicity of chemicals and fecal samples under reduced oxygen levels [14,28,41,54,74,75]. Highly reduced oxygen levels may lead to a reduction in both spontaneous and induced revertant colonies and may interfere with the interpretation of the results. A complete absence of revertant colonies is observed when a strict anaerobic environment and prereduced and anaerobically sterilized (PRAS) media are used [54]. It is therefore important to perform preliminary experiments to determine whether the spontaneous reversion rate is affected by the intended reduced oxygen level.

2.5. Validation studies

Over the years, many validation studies have been performed to determine the reproducibility of test results on an intra- and inter-laboratory level [17,18,45,57,61,77,85]. In addition, many studies have been performed to determine the sensitivity and correlation of the Ames test with animal carcinogenicity studies. It has indeed been established that there is a high predictivity of a positive mutagenic response in the Ames test for rodent carcinogenicity [3,49,71,73,88], ranging from 90% [49] to 77% [88], the primary differences being the chemical composition of the compiled databases. The test therefore is in many instances used as a first screen to determine the mutagenic potential of new chemicals and drugs. In addition, data from the test is submitted to regulatory agencies in support of registration or acceptance of many chemicals, including drugs and biocides [9,21,38,68]. International guidelines have been developed for use by corporations and testing laboratories to ensure uniformity of testing procedures [32,56].

3. Long term storage and propagation of the tester strains

3.1. Preparation of frozen permanent cultures

For long-term preservation, the *Salmonella* tester strains should be kept frozen at −80°C (freezer or liquid nitrogen). Upon receipt of the new strains, up to five frozen permanent cultures should be prepared from one single colony isolate that has been purified and checked for its genotypic characteristics (*his*, *rfa*, *uvrB-bio*) and for the presence of plasmids pKM101 and pAQ1, when appropriate. These cultures should be considered the frozen permanent strains and should be accessed only for the preparation of new frozen working cultures or master agar plates.

If the new strain is received on a small sterile filter disk embedded in nutrient agar, first wipe the disk across the surface of a nutrient agar plate, and then transfer the disk to 5 ml of nutrient broth. If the strain comes as a lyophilized culture, aseptically add 1 ml of nutrient broth to rehydrate the culture (a process which should take up to 2 min), then transfer the rehydrated culture to 4 ml of nutrient broth. Keep a drop of the rehydrated culture for transfer to a nutrient agar plate and streak the inoculum for individual colonies across the surface of the plate. The broth culture serves as a back-up in case no growth is observed on the agar plate.

After overnight incubation at 37°C, inspect the agar plates for growth. If single colonies are observed, pick one healthy looking colony and restreak it for individual colonies on GM agar plate supplemented with an excess of biotin and histidine, and with ampicillin and tetracycline (for the strain(s) carrying plasmid pKM101and pAQ1, respectively). This purification step should be repeated. At least two purification steps are recommended to ensure that a pure culture will be used for propagation of the frozen permanent cultures.

Note: For purification purposes nutrient agar plates may be used instead of appropriately supplemented

GM agar plates; however, the use of minimal defined medium reduces the risk of contamination. It takes 2 days of incubation at 37°C to obtain good growth on GM agar plates. The supplemental nutrients and antibiotics can be applied directly to the surface of the GM plates by delivering two to three drops of the appropriate solutions (see the Section 15: Recipes for reagents and media) which are then evenly distributed over the agar surface by a sterile glass spreader. These solutions can also be incorporated in the agar when the plates are prepared.

Pick up to five single colonies from the second (or later) purification plate and transfer to a preassigned location on a GM agar plate supplemented with the appropriate nutrient/antibiotics which will serve as the master plates during the process of preparing the permanent frozen cultures. For convenience, label the locations, 1, 2, 3, 4, 5, on the backs of the plates. Incubate the master plates for 2 days at 37°C. When good growth is observed, inoculate 5 ml of nutrient broth with a small inoculum from each of the single isolated colonies on the master plate. After overnight incubation at 37°C confirm the genotypes (strain check) of the tester strains (see the section: Genetic analysis).

Upon completion of the strain check, select the colony from the master plate that has given the best overall results in terms of phenotypic characteristics, including the best overall spontaneous mutation induction. Transfer a small inoculum in 4.5 ml of nutrient broth and incubate overnight at 37°C with shaking till the cells reach a density of 1 to 2×10^9 CFU/ml ($O.D._{540}$ between 0.1 and 0.2). Add 0.5 ml of a sterile cryopreservative such as glycerol or DMSO to the culture (final concentration, 10%, v/v), mix thoroughly and dispense 1 ml aliquots in sterile cryogenic tubes prelabelled "PERMANENT CULTURES" with strain number and date of preparation. Quick freeze on dry ice and store at −80°C.

Note: If permanent frozen cultures are prepared of several tester strain, up to 25 clones can be placed on the master plate. Replica plating can then be performed for determination of histidine and biotin dependence and for the presence of plasmid pKM101 (ampicillin resistance) and plasmid pAQ1 (tetracycline resistance).

3.2. Preparation of frozen working cultures

New frozen working cultures should be prepared at least every 12 months with an expiration date of 1 year from the time the frozen working cultures were prepared. For preparation of the working cultures, scrape the surface of one frozen permanent culture with a sterile wooden applicator stick or inoculation loop. This procedure should be done quickly to prevent the frozen permanent culture from thawing which might result in decreased viability and loss of plasmid(s). Inoculate 5 ml of nutrient broth with the small inoculum. After overnight incubation at 37°C with shaking (100–120 rpm), streak a loopful of the culture for individual colonies on GM agar plates supplemented with an excess of biotin and histidine, and as needed with ampicillin and tetracycline, for the strain(s) carrying plasmid pKM101and pAQ1, respectively. Purify one healthy looking colony at least twice.

Pick up to five single colonies from the second purification plate and transfer to a preassigned location on a GM agar plate supplemented with the appropriate nutrient/antibiotics; this will be considered the master plate. All subsequent steps are identical to those described above for the preparation of the frozen permanent cultures except for the following. The cryogenic tubes should be prelabelled: WORKING CULTURES, with strain number, date of preparation and expiration date and more than 5 frozen cultures should be prepared (see Note below).

Note: It is recommended that one frozen working culture (one cryogenic tube) be used for each experiment and that the unused portion be discarded. Frequent rethawing and refreezing of the tester strains may result in reduced viability and loss of the plasmid(s). It is therefore important to prepare sufficient frozen working cultures for 1 year. However, if needed, frozen working cultures can be prepared several times a year. It is important that the final concentration of the cryopreservative, glycerol or DMSO, is at least 10%, v/v.

3.3. Preparation of working stock agar plates

In case there is a problem maintaining frozen working cultures, working stock agar plates can be prepared by streaking appropriately supplemented GM agar plates with the tester strains after a strain

check. These plates should be stored in the refrigerator wrapped in parafilm to prevent dehydration of the agar. For inoculation purposes, a small inoculum, preferably from an area of confluent growth instead of a single colony, should be used to inoculate the nutrient broth. The plates can be stored up to 2 months, except for the plate containing strain TA102 which can only be stored for 2 weeks because the number of spontaneous revertant colonies increases over time. This may be due to the generation of a higher copy number of plasmid pAQ1 and does not appear to be an indication of tetracycline mutagenicity [40,47]. Therefore, it is recommended that frozen working cultures, rather than working agar plates, be prepared for strain TA102.

3.4. Growing overnight cultures

For each experiment, the tester strain cultures are grown overnight in Oxoid nutrient broth No. 2 to a density of $1–2\times10^9$ colony forming units (CFU)/ml. The volume of the cultures will depend on the size of the experiment but is usually between 10 and 50 ml. Care should be taken that the size of the culture flask be at least three to five times the volume of the culture medium to ensure adequate aeration.

Individual culture flasks are inoculated with each strain. When using frozen stock cultures, allow the culture to thaw at room temperature. Aliquots between 0.1 and 0.5 ml are then transferred into nutrient broth with volumes between 10 and 50 ml, respectively, which gives an initial cell density between 10^6 and 10^7 CFU/ml (1:100 dilution). The remainder of the working culture should be discarded, because frequent thawing and refreezing of the frozen cultures results in permanent injury to the bacteria. Overnight cultures may also be inoculated with a small inoculum taken from a working stock agar plate.

The freshly inoculated cultures are placed on a shaker in the dark at room temperature for 4 h without shaking, then gently shaken (100 rpm) for 11–14 h at 37°C. The use of a timer is convenient for following this procedure. The next morning the cultures are removed from the incubator and kept at room temperature away from direct fluorescent light by wrapping them in aluminum foil. This precautionary procedure is not required if the laboratories are equipped with yellow or red overhead lights. Some investigators keep the cultures on ice to prevent loss of viability of the *rfa* strains which will occur if they are maintained for an extended period of time at room temperature in the nutrient broth. However, to prevent thermal shock to the bacteria when they are added to the 43°C to 48°C top agar, it is best to leave the cultures at room temperature during the course of the mutagenicity testing.

4. Genetic analysis

It is recommended that the tester strains be analyzed for their genetic integrity and spontaneous mutation rate when frozen cultures are prepared. A strain check should also be performed whenever an experiment is performed. The strain check is usually performed with the nutrient broth overnight cultures in the following way.

The following steps should be followed for a complete strain check.

- **Histidine dependence (*his*):** streak a loopful of the culture across a GM agar plate supplemented with an excess of biotin. Because all the *Salmonella* strains are histidine dependent, there should be no growth on the plates.
- **Biotin dependence (*bio*):** streak a loopful of the culture across a GM agar plate supplemented with an excess of histidine. There should be no growth on the plate except for strain TA102 which is biotin independent.
- **Biotin and histidine dependence (*bio*, *his*):** streak a loopful of the culture across a GM agar plate supplemented with an excess of biotin and histidine. Growth should be observed with all strains.
- ***rfa* marker:** streak a loopful of the culture across a GM agar plate supplemented with an excess of biotin and histidine. Place a sterile filter paper disk in the center of the streak and apply 10 μl of a sterile 0.1% crystal violet solution. All *Salmonella* strains should show a zone of growth inhibition surrounding the disk.

  Note: The crystal violet disks can be prepared in advance and stored at room temperature. Nutrient agar plates can be substituted for the GM agar plates.

- ***uvrB* deletion:** because the deletion mutation stretches across the *bio-uvrB* region of the chromosome and cannot revert to wild-type, it is sufficient to show that the tester strains are biotin dependent (see

above) to infer that they are also defective in the accurate DNA repair pathway due to the *uvrB* deletion.

• **Presence of plasmid pKM101 (ampicillin resistance)**: streak a loopful of the pKM101-carrying *Salmonella* culture across a GM agar plate supplemented with an excess of histidine and biotin and 24 μg/ml ampicillin. Alternatively, a sterile 6-mm filter paper disk containing 10 μg/ml ampicillin can be placed on a streak of the strain on the GM agar plate supplemented with histidine and biotin. As a control, a strain should be included that does not carry plasmid pKM101. Growth should be observed only with strains carrying pKM101.

Note: The ampicillin disks can be prepared in advance and stored in the refrigerator.

• **Presence of plasmid pAQ1 (Tetracycline resistance)**: streak a loopful of a TA102 overnight culture across a GM agar plate supplemented with an excess of histidine and biotin, and 2 μg/ml tetracycline. Alternatively, a sterile 6-mm filter paper disk containing 2 μg/ml tetracycline can be placed on a streak of the strain on the GM agar plate supplemented with histidine (biotin is not required for growth of TA102). However, there is no problem using GM agar plates supplemented with both histidine and biotin. As a control, a strain should be included that does not carry plasmid pAQ1 plasmid. Growth should be observed with strain TA102 which is the only strain that carries plasmid pAQ1.

Note: The tetracycline disks can be prepared in advance and stored in the refrigerator.

• **Spontaneous mutant frequency:** use the standard plate incorporation assay procedure (see below) without the inclusion of a solvent for determining the spontaneous mutant frequency (negative control) of each of the tester strains. When the spontaneous control values fall outside an acceptable historical range (see Table 3) the genetic integrity of the strain(s) is considered compromised, and a new culture should be isolated. There may be non-strain related reasons for a spontaneous value that is too high or too low (see Troubleshooting, below). These should be considered before a new culture is isolated.

If one or more of the above strain checks fails, the culture is considered unacceptable for use. If such a culture was used for performing an experiment, the results should be considered invalid and the experiment should be repeated. In case the strain check was performed when the strains were frozen (permanent or working cultures) and yielded unacceptable results, the strain(s) should be reisolated following the procedures outlined above.

Note: For routine genetic analysis when experiments are performed, the modified procedure described by Zeiger et al. [94] may be used instead of the more lengthy procedure described above.

5. Spontaneous control values

Each tester strain has a characteristic spontaneous mutant frequency. There is usually some day-to-day and laboratory-to-laboratory variation in the number of spontaneous revertant colonies. Choice of solvent may also affect the spontaneous mutant frequency [46]. Each laboratory has a characteristic range of revertant colonies for each strain which is referred to as "historical control values". The spontaneous mutant frequency obtained when the strain check is performed should be compared to the laboratory's historical control values. Table 3 presents a range of spontaneous histidine revertant (negative solvent) control values per plate with and without metabolic activation considered valid in the authors' laboratories. The values obtained in the presence of a metabolic activation system includes both rat and hamster liver S-9. The spontaneous values presented for S-9 were from 10% S-9 in the S-9 mix. Some of the strains (e.g., TA97, TA102, TA104) are highly sensitive to S-9 concentrations and their spontaneous reversion values will increase with the S-9 concentra-

Table 3
Spontaneous revertant control values

Strain	Number of revertants[a]	
	Without S-9	With S-9
TA97	75–200	100–200
TA98	20–50	20–50
TA100	75–200	75–200
TA102	100–300	200–400
TA104	200–300	300–400
TA1535	5–20	5–20
TA1537	5–20	5–20
TA1538	5–20	5–20

[a]Range considered valid in the authors' laboratories.

tion. Other acceptable ranges of background revertant counts have been published [7,37,47].

6. Toxicity determination

Toxicity determination in the Ames *Salmonella* test requires the evaluation of characteristics of the final population on the GM agar plate after the 48-h incubation instead of a quantitative survival determination. These characteristics are:

- thinning of the background lawn which may be accompanied by a decrease in the number of revertant colonies
- absence of background lawn (i.e., complete absence of growth)
- presence of pinpoint non-revertant colonies (generally in conjunction with an absence of background lawn)

The overnight *Salmonella* nutrient broth cultures largely consist of histidine-dependent bacteria with just a few pre-existing histidine-independent (His^+) bacteria that arose during the overnight incubation. The addition of a small amount of histidine to the top agar allows all the plated bacteria (approximately 1×10^8 cells) to undergo between six and eight cell division before the histidine is depleted. In many cases, this limited growth is essential for mutagenesis to occur by allowing fixation of the mutational lesions. The pre-existing His^+ bacteria as well as the His^+ revertants that arose on the plate (plate revertants) will continue to grow in the absence of histidine and will give rise to visible colonies. These His^+ colonies are easily scored against a slightly hazy looking background lawn which is made up of the microcolonies of the histidine-dependent bacteria.

Microscopic ($40\times$) examination of the background lawn in the absence of toxicity will reveal the presence of densely packed microcolonies which form a uniform, though somewhat granular thin film. In such cases, all the plated histidine-dependent bacteria were able to undergo six to eight cell divisions. However, when a chemical is toxic there may be "thinning" or complete absence of the background lawn compared to the negative or solvent control. Partial toxicity of the chemical will give rise to thinning since not all the plated bacteria were killed or had their growth inhibited. In this case, the surviving bacteria still form microcolonies but they are not densely packed and may appear as single sparsely spaced microcolonies which results in the "thinning" effect; these colonies will not be visible to the naked eye. A decrease in the number of revertant colonies to levels below the spontaneous reversion level may on occasion be seen with thinning. A complete absence of background lawn indicates a high level of toxicity with the inability of the bacteria to grow and form a lawn. Such a toxic dose should not be used.

Occasionally numerous small non-revertant colonies are present on the plate. The colonies are referred to as "pinpoint colonies" and consist of histidine-dependent bacteria that survived high chemical toxicity. These colonies are readily visible by the naked eye and may be mistaken for revertant colonies. Microscopic inspection of the plates will, however, reveal that there is a total absence of background lawn. The pinpoint colonies arise due to the fact that the high level of toxicity resulted in more histidine being available to the surviving His^- bacteria on a per cell basis. Therefore, these bacteria can undergo additional cell divisions until the depletion of the histidine. The histidine dependency can be readily checked by streaking a few pinpoint colonies on GM agar plates supplemented with biotin but without histidine in the absence of the test chemical. A preliminary toxicity experiment is usually performed to determine the top dose that can be tested (see Dose selection, below).

It is important that the same number of bacteria be used in the preliminary toxicity assay as well as in the definitive mutagenicity assay. Dilution of the tester strain to plate <1000 cells to enable a quantitative toxicity determination could lead to an erroneous result. On a per cell basis, more chemical would be available to each bacterium than would be available if $1–2\times10^8$ bacteria are exposed to the substance, as happens in the definitive mutagenicity test procedure [79].

7. Minimal defined agar medium

7.1. Top agar

The top agar, consisting of 0.6% agar and 0.6% NaCl, is one of the most critical medium components in the Ames test because it contains the trace amount of histidine (0.05 mM) for limited growth. It also contains

biotin at a concentration of 0.05 mM which is in excess of what is needed for the growth of the *Salmonella* strains. Because the His^- bacteria stop growing when the histidine is depleted, the final population of His^- bacteria is dependent on the histidine concentration. In turn, the final population on the plate will affect the number of spontaneous revertant colonies. It is therefore important that utmost care is taken to accurately supplement the top agar with 0.05 mM histidine. Too little histidine may result in the background lawn looking sparse, which might be taken as evidence that toxicity is present even on the solvent negative control plates. Too much histidine will cause heavy growth that may obscure the revertant colonies. For the same reason, it is also important to ensure that consistent techniques are used to deliver 2 ml of the top agar to the GM agar plates.

7.2. GM agar plates

The Ames test uses minimal defined agar medium consisting of Vogel-Bonner E medium (V/B salts) [78], supplemented with glucose (between 0.5% and 2%, w/v) and agar (1.5%, w/v). This medium is generally referred to as glucose minimal agar medium (GM agar). Plates usually contain between 20 and 30 ml of the medium and may be stored for several months in the refrigerator in sealed plastic bags to prevent dehydration. Ideally, each laboratory should work with plates that have a constant volume of agar medium. The plates should be used fairly soon after they have been poured before excessive dehydration of the agar medium occurs. Dehydration of the agar medium will increase the concentrations of the V/B salts, glucose and agar which may affect the spontaneous and induced mutagenesis process. As described above for the top agar, small variations in the medium composition may increase variability in test results.

8. Assay procedures

8.1. Standard plate incorporation assay

Concept: The standard plate incorporation assay consists of exposing the tester strain(s) to the test chemical directly on a minimal glucose agar plate (GM plate) usually in the presence and absence of a metabolic activation system (see Fig. 2). The different components are first added to sterile test tubes containing 2 ml of molten top agar supplemented with limited histidine and biotin. It is important to maintain the top agar at a temperature between 43°C and 48°C and to minimize prolonged exposure to avoid killing of the tester strains. The contents of the tubes are mixed and poured on glucose minimal agar plates. After the top agar has hardened the plates are inverted and incubated at 37°C for 48 h, at which time histidine-revertant colonies are counted on all plates. The number of colonies on the test plates are compared to those on the (negative) solvent control plates.

8.2. Experimental procedure

1. Steps taken prior to performing the experiment
   - Inoculate *Salmonella* cultures 15–18 h prior to performing the experiment.
   - Label an appropriate number of GM agar plates and sterile test tubes for each test chemical
   - Prepare metabolic activation system and keep on ice until use
   - Prepare chemical dilutions
   - Melt top agar supplemented with 0.05 mM histidine and biotin and maintain at 43°C to 48°C.
2. To the 13×100 mm sterile glass tubes maintained at 43°C, add in the following order with mixing (e.g., vortexing) after each addition.
   - 2 ml of molten top agar
   - 0.50 ml of metabolic activation (S-9) mix or buffer
   - 0.05 ml of the test chemical dilution
   - 0.05–0.10 ml overnight culture of the *Salmonella* strain (about $1–2\times10^8$ bacteria per tube).
3. The contents of the test tubes are then mixed and poured onto the surface of GM agar plates.
4. When the top agar has hardened (2–3 min), the plates are inverted and placed in a 37°C incubator for 48 h.
5. The colonies are then counted and the results are expressed as the number of revertant colonies per plate.

8.3. Preincubation assay

Concept: The preincubation assay is a modification of the standard plate incorporation assay and involves

exposing the tester strains for a short period (usually 20 min) in a small volume (usually 0.50 ml) containing the test agent with buffer or S-9 mix prior to plating on GM agar medium. It is believed that short-lived mutagenic metabolites have a better chance of reacting with the tester strains in the small volume of the preincubation mixture compared to when the incubation mixture is plated immediately on minimal defined agar plates [29,55,84,89–91]. In addition, the smaller preincubation volume results in a higher effective concentration of the S-9 and cofactors.

8.4. Experimental procedure

The steps taken prior to performing the experiment are the same as those for the standard incorporation assay described above. The preincubation assay is performed as follows:

1. To the 13×100 mm sterile glass tubes maintained at room temperature add in the following order with mild mixing after each addition
   - 0.50 ml of metabolic activation (S-9) mix or buffer
   - 0.05 ml of the test chemical dilution
   - 0.05 to 0.10 ml overnight culture of the *Salmonella* strain (about $1–2\times10^8$ bacteria per tube).
2. Incubate the mixture at 37°C for 20 min.
3. To each tube add 2 ml of molten top agar maintained at 43°C to 48°C. The contents of test tubes are then mixed and poured onto the surface of GM agar plates.
4. When the top agar has hardened (2–3 min), the plates are inverted and placed in a 37°C incubator for 48 h.
5. The colonies are then counted and the results are expressed as the number of revertant colonies per plate.

Note: If the test chemical is known or suspected to be volatile the tubes should be capped prior to the preincubation step.

8.5. Desiccator assay for volatile liquids

Concept: Neither the standard plate incorporation assay nor the preincubation assay is suitable for testing highly volatile substances. The use of a closed chamber is recommended for testing such chemicals in a vapor phase, as well as for gases [67,91]. Procedures using plastic bags in lieu of desiccators have also been described [8,30]. Fig. 4 depicts a desiccator assay procedure for volatile liquids. This procedure should be used only if it is known that the test chemical will volatilize at 37°C and disperse throughout the desiccator. A 9-l desiccator is recommended because up to 18 plates can be placed inside one desiccator.

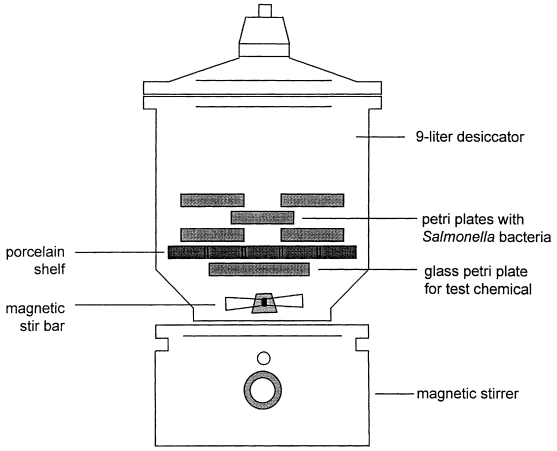

Fig. 4. Diagram depicting the desiccator assay set-up.

8.6. Experimental procedure

The steps taken prior to performing the experiment are the same as those for the standard incorporation assay described above with the exception that the chemical is omitted from the test tubes.

(1) To the 13×100 mm sterile glass tubes add in the following order with mild mixing after each addition:

- 2 ml of molten top agar
- 0.5 ml of metabolic activation (S-9) mix or buffer
- 0.05 to 0.1 ml overnight culture of the *Salmonella* strain (about $1–2\times10^8$ bacteria per tube).

(2) The contents of the test tubes are then mixed and poured onto the surface of GM agar plates.

(3) While the top agar is hardening, tape a sterile half of a glass petri dish, or a watchglass, to the bottom center of the perforated ceramic shelf of the desiccator. Place a magnetic stir bar in the bottom of the desiccator prior to replacing the ceramic shelf in the desiccator.

(4) Remove the lids of the petri plates containing the *Salmonella* bacteria and S-9 mix or buffer and loosely stack the plates in the inverted position on the ceramic shelf.

(5) Add a predetermined volume of the liquid test chemical to the glass dish taped to the bottom of the ceramic shelf.

Note: for highly toxic liquid chemicals, a suitable solvent is needed for diluting the test chemical. In such a case, the control desiccator (no test chemical) should contain the highest volume of solvent used.

(6) Seal each desiccator and place on a magnetic stirrer in a 37°C incubator or warm room for 24 h.

(7) The desiccators are then removed from the incubator and placed in a chemical safety hood and opened with caution; the plates are removed, their lids are put back on, and they are transferred to a 37°C incubator for an additional 24 h.

(8) The colonies are then counted and the results are expressed as number of revertant colonies per plate per volume of test chemical per desiccator.

8.7. Desiccator assay for gases

Concept: Like volatile liquids, the testing of gases requires the use of a closed chamber such as a 9-l desiccator [67,91]. Procedures using plastic bags in lieu of desiccators have also been described [8,30].

8.8. Experimental procedure

The steps taken prior to performing the experiment are the same as those described for the desiccator assay for liquid chemicals with the exception that the gas is introduced into the desiccator after the lid of the desiccator is replaced.

Note: It is essential that each desiccator lid has an inlet that can be connected to a stopcock for transfer of the gas.

The assay is performed as follows.

(1) To the 13×100 mm sterile glass tubes maintained at 43°C, add in the following order with mild mixing after each addition:

- 2 ml of molten top agar
- 0.5 ml of metabolic activation (S-9) mix or buffer
- 0.05 to 0.10 ml of overnight culture of the *Salmonella* strain (about $1–2\times10^8$ bacteria per tube).

(2) The contents of test tubes are then poured onto the surface of GM agar plates.

(3) While the top agar is hardening, remove the ceramic shelf from a 9-l desiccator and place a magnetic stir bar in the bottom of the desiccator; replace the ceramic shelf in the desiccator.

(4) Remove the lids of the petri plates containing the *Salmonella* bacteria and S-9 mix or buffer and loosely stack the plates in the inverted position on the ceramic shelf.

(5) Seal the desiccator and evacuate a predetermined volume of air by using a pressure gauge.

(6) Introduce the test gas until atmospheric pressure is reached.

(7) Place each desiccator on a magnetic stirrer in a 37°C incubator or 37°C warm room for 24 h.

(8) The desiccators are then removed from the incubator and placed in a chemical safety hood and opened with caution; the plates are removed, their lids are put back on, and they are transferred to a 37°C incubator for an additional 24 h.

(9) The colonies are then counted and the results are expressed as number of revertant colonies per plate based on moles of gas added to each desiccator. To calculate the moles of gas the following formula is used:

$$n = \frac{PV}{RT}$$

where n is the number of moles, P is 1 atm, V is volume in liters, R is the universal gas constant (0.0921 l atm/mol K) and T is temperature expressed in Kelvin.

8.9. Reductive metabolism assay

Concept: The procedure is a modification of the preincubation assay and employs a modified cofactor mix and the incubation mixture is shaken at 180 rpm for 90 min [58,59,62–64]. This assay is recommended for testing chemicals that contain azo bonds and other substances that may require enzymatic reduction.

8.10. Experimental procedure

The steps taken prior to performing the experiment are the same as those for the preincubation assay with the exception that a reductive metabolic activation system is used (see the section: Recipes for reagents and media). The assay is performed as follows:

1. To the 13×100 mm sterile glass tubes add in the following order with mild mixing after each addition
   - 0.5 ml of reductive metabolic activation (S-9) mix or buffer
   - 0.05 ml of each dilution of the test chemical
   - 0.05 to 0.10 ml of overnight culture of the *Salmonella* strain (about 1–2×10^8 bacteria per tube).
2. The mixture is incubated at 37°C with shaking (180 rpm) for 20 min.
3. The top agar (2 ml) is then added to the incubation mixture. After mixing, the contents of the tubes are mixed and poured onto the surface of GM agar plates
4. When the top agar has hardened (2–3 min), the plates are inverted and placed in a 37°C incubator for 48 h.
5. The colonies are then counted and the test results are expressed as the number of revertant colonies per plate.

8.11. Modified (Kado) Salmonella microsuspension assay

Concept: This is a highly sensitive assay for testing the mutagenicity of biological samples, such as urine, and pure chemicals that are available only in small amounts [35]. The procedure is a modification of the preincubation assay and involves using smaller test tubes (10×70 mm) and shaking of the incubation mixture for 90 min. When urine samples are tested, a deconjugating enzyme system is usually included to detect substances that are excreted as inactive β-glucuronides [19].

8.12. Experimental procedure

The steps taken prior to performing the experiment are the same as those for the preincubation assay with the exception that the overnight cultures of the *Salmonella* strains are washed two times in sodium phosphate buffer, 0.1 M, pH 7.4 and concentrated 10-fold. The assay is performed as follows:

1. To the 10×70 mm test tubes placed in an ice bath, add in the following order with mild mixing after each addition:
   - 0.1 ml of the 10-fold concentrated overnight *Salmonella* culture (about 1 to 2×10^9 bacteria per tube)
   - 0.1 ml of the metabolic activation system or buffer
   - 0.001, 0.0025, 0.005, or 0.01 ml of test article (delivered with micropipettes)
   - 0.1 ml of a deconjugating enzyme system (e.g., β-glucuronidase; when testing urine samples)
   - Additional solvent to adjust final volume to 0.25 ml or 0.35 ml when β-glucuronidase is used.
2. The mixture is allowed to incubate for 90 min at 37°C with shaking (120 rpm).
3. The top agar (2 ml) is then added to the incubation mixture. After mixing, the contents of the tubes are poured onto the surface of GM agar plates.
4. When the top agar has hardened (1–2 min), the plates are inverted and placed in a 37°C incubator for 48 h.
5. The colonies are then counted and the results are expressed as the number of revertant colonies per plate.

9. Important steps to follow for all assay procedures

- The cofactors for preparing the S-9 mix are the same for each of the assay protocols, with the exception of the reductive metabolism assay. The preparation of the cofactors is described in the section: Recipes for reagents and media.
- It is important to quickly swirl the plates after addition of the top agar to the surface of the GM agar plates to ensure an even distribution of the top agar which contains the bacteria, test chemical and S-9 mix or buffer.
- When the plates are inspected after 48 h of incubation and growth retardation is seen as evidenced by smaller than anticipated colony sizes, the plates should be incubated for an additional 12–24 h. All plates from an experiment should be incubated for the same length of time in the same incubator, so that all plates from an experiment that show retarded growth at only the higher test chemical concentrations are incubated for the additional time.
- If colonies cannot be counted immediately after the 48-h incubation, the plates can be stored in a re-

frigerator for up to 2 days. All plates must be removed from the incubator, and be counted, at the same time.

- Hand-counting may be needed if a potent mutagen is being tested or when strains TA102 and TA104 are used because the automatic colony counter may not be effective if the colony density on each plate is too high. In this case, quadrants of the agar plates may be hand counted with appropriate adjustment of the counts, provided the colonies are evenly distributed over the entire surface of the agar plate.
- If precipitate or lack of contrast between the colonies is observed on some plates, especially at the higher dose levels, hand-counting is required of all plates (including the control plates).
- A check for genetic integrity should be performed with every experiment. For this strain check, it is sufficient to monitor the *rfa* mutation and ampicillin and tetracycline resistance using disks impregnated with the appropriate concentration of each component [94].

10. Experimental design

The Ames *Salmonella* test is a versatile assay as evidenced by the different procedural variations that are available, in addition to the numbers of strains and types of metabolic activation systems that can be used. Before initiating an Ames test, a number of critical parameters will need to be evaluated.

10.1. Assay procedure

For liquid chemicals the assay of choice is the preincubation assay because many of these chemicals may be volatile. For most other chemicals the standard plate incorporation assay or the pre-incubation assay may be used. However, some researchers believe the pre-incubation assay to be somewhat more selective in detecting mutagens compared to the plate incorporation assay. The reason may be that short-lived mutagenic metabolites have a better chance of reacting with the tester strains in the small volume of the preincubation mixture. For general screening purposes the laboratory should adopt one testing procedure that will help in developing a historical database for the negative control, and the solvent and positive control chemicals.

The modified (Kado) *Salmonella* microsuspension assay is recommended for testing samples that are available in only small (i.e., mg) amounts or complex mixtures such as urine. The reductive metabolism assay is recommended for chemicals that require reduction for their activation such as azo dyes. The desiccator assays are recommended for volatile liquids and for gaseous substances.

10.2. Solvents

The solvent of choice is sterile distilled water. Chemicals that do not dissolve in water should be dissolved in dimethyl sulfoxide (DMSO). Other solvents that may be considered are: acetone, ethyl alcohol (95%), tetrahydrofuran, dimethylformamide and methyl ethyl ketone (MEK). These other solvents may be toxic to the bacteria at higher concentrations. Therefore, a toxicity assay with these solvents should be determined in a preliminary assay to determine the maximum concentration that can be used without interfering with bacterial growth and survival. Some of the solvents may also interfere with the metabolic activation system. In such case, the concentration of the S-9 fraction may have to be adjusted. For a comprehensive study on the compatibility of solvents with the Ames *Salmonella* test see Maron et al. [46].

10.3. Positive and negative (solvent) controls

Each experiment should include solvent controls and diagnostic positive control chemicals specific for each strain and for the metabolic activation system Table 4 lists the representative positive controls.

10.4. Dose selection

10.4.1. Preliminary toxicity determination

It is recommended that a preliminary toxic dose range experiment be performed to determine an appropriate dose range for the mutagenicity assay. If more than one tester strain will be used in the mutagenicity assay, it is sufficient to perform the toxicity assay with strain TA100 with and without metabolic activation. Otherwise, the toxicity assay should be performed with the strain that will be used in the definitive assay. The toxicity determination can also

Table 4
Representative positive control chemicals

Strain	Control chemical (μg/plate)[a]	
	Without activation	With activation
TA97	9-Aminoacridine (50)	2-Aminoanthracene (1–5)
TA98	4-Nitro-*o*-phenylenediamine (2.5)	2-Aminoanthracene (1–5)
TA100	Sodium azide (5)	2-Aminoanthracene (1–5)
TA102	Mitomycin C (0.5)	2-Aminoanthracene (5–10)
TA104	Methyl methane sulfonate (250)	2-Aminoanthracene (5–10)
TA1535	Sodium azide (5)	2-Aminoanthracene (2–10)
TA1537	9-aminoacridine (50)	2-Aminoanthracene (2–10)
TA1538	4-Nitro-*o*-phenylenediamine (2.5)	2-Aminoanthracene (2–10)

[a]Concentration based on 100×15-mm petri plate containing 20 to 25 ml of GM agar.

be performed using an alternate toxicity assay developed by Waleh et al. [79].

The chemical should be tested for toxicity over a wide range of concentrations with a total of eight concentrations, spaced in half-log intervals, with the highest dose limited by solubility, or by an arbitrary value (usually 5000 or 10,000 μg/plate). For volatile liquids that are tested in desiccators, 0.5 and 5 ml should be used as the low and high dose, respectively. For gases tested in desiccators, the low and high dose should be 0.5 and 4.5 l, respectively.

The preliminary toxicity assay should be performed in the absence and in the presence of the metabolic activation system that is to be used in the definitive mutagenicity assay. Positive and solvent control chemicals need to be included in the assay; one plate per dose level should be sufficient. As mentioned in the Toxicity determination section, it is important that the same number of bacteria be used in the preliminary toxicity assay as well as in the definitive mutagenicity assay. Exposing a diluted culture to the chemical could result in an erroneous higher toxicity level [79].

10.4.2. Doses for definitive assay

A minimum of five dose levels covering a range of at least three logs should be selected for the definitive test. Two or three plates should be used for each dose level and for the controls. For toxic chemicals, only the highest dose used should exhibit toxicity. For non-toxic chemicals, a high dose of 5000 or 10,000 μg/plate is acceptable. Non-soluble chemicals may be tested as a suspension up to a dose level that does not interfere with handling of the suspension with pipets or pipet tips. For non-toxic volatile liquids that are tested in desiccators, 5 ml should be used as the high dose. For non-toxic gases tested in desiccators, the high dose should be 4.5 l.

Note: Each test should be performed using a single batch of reagents, media, and agar. The use of different batches of bottom agar plates, top agar, buffer, solvents, cofactors, or S-9 batches within an experiment can lead to excess variation in the results, or clear demarcations of results between the different batches.

10.5. Strain selection and testing strategies

There has been no evidence to show that the specific tester strains, or the number of tester strains mutated by a chemical, is related to the chemical's potency in other test systems, or the predictivity of the test result for cancer induction or other effects. Therefore, it may not be necessary to test all chemicals with the full complement of strains. Although a single strain is sufficient to demonstrate a mutagenic response, it is generally accepted that a negative result can be defined using 4–5 tester strains. Strains TA98, TA100, and TA97 or TA1537 are always considered necessary. The other strains usually used are TA1535 and/or TA102.

When planning to test a large number of chemicals, it may be most time and cost efficient to perform the testing in stages. For example, it has been shown that testing with strains TA98 and TA100 without metabolic activation and with S-9 from rats or hamsters was sufficient to identify approximately 90% of the mutagens in a population containing about 35% mutagenic chemicals [95]. Using such a strategy, the chemicals not mutagenic or equivocal in these two

strains can be tested in additional strains. The higher the anticipated number of mutagens among the chemicals to be tested, the more cost effective the staged testing strategy will be.

If there is prior information about the chemical or class of chemicals to be tested that indicates the need for specific treatment conditions, or that the chemical is likely to induce a certain type of DNA damage, the testing strategy should be directed appropriately. For example, if the chemicals are of a structure that is known to require metabolic activation, the initial screen could be performed only with S-9 mix, or if the chemicals are structurally related to a class known to produce only base-pair substitutions, the initial testing could use only the appropriate target strains.

10.5.1. Tier approach

Unless required by specific guidelines, it is recommended that for general screening purposes a tier approach be used using strains TA98 and TA100 with and without metabolic activation

If a positive response is obtained the assay is repeated only using the condition(s) and strain(s) that elicited the positive response. If needed, the dose range may be adjusted to better clarify a dose response. If equivocal or weak positive responses are obtained, the corresponding plasmid-free strains, TA1538 and TA1535, respectively, should be used. Justification for the use of TA1535 comes from a number of reports about unique positive results in TA1535 [12,16,29,52,60,86]. Demonstration of a unique response in strain TA1538 is more challenging because few databases have data available compared to databases that have strain TA1535 included. However, specific examples of strain TA1538 giving a unique response exist.

If negative results are obtained in the initial mutagenicity test with strain TA98 and TA100, other strains such as TA1535 and TA97 are used with and without metabolic activation. A confirmation experiment is performed if a positive response is obtained using only the condition(s) and strain(s) in which the mutagenic response was observed. If equivocal results or a weak response are obtained in strain TA97, strain TA1537 can be used in an attempt to clarify the results.

Strains TA102 and TA104 may be used if it is suspected that the chemical may induce oxidative damage (i.e., free radical production), or be a DNA cross-linking agent. Strain TA102 is especially sensitive for detecting DNA cross-linking damage because it has an intact (wild-type) DNA excision repair mechanism which is required for the repair of such damage. Fig. 5 depicts the tier approach experimental design described above which is based on the procedure described by Zeiger et al. [89].

If prior to testing information is available about the potential mutagenicity of the chemical or chemical class, the initial testing is performed with the strain(s) most likely to yield a positive response.

10.5.2. Battery approach

A battery approach employs a fixed set of tester strains for testing a chemical in the presence and absence of a metabolic activation system. For general mutagenicity testing it is recommended that strains TA97, TA98, TA100 and TA102 be used. However, some laboratories use strain TA1535 instead of strain TA102 when a tier approach is used in the selection of tester strains [91]. As in the tier approach, strains TA1538 and TA1535 are used when equivocal results are obtained with their respective pKM101-carrying strains, TA98 and TA100. Strain TA1537 can detect mutagens not detected by strain TA97. Strain TA104, though in some laboratories tested in parallel with TA102, is recommended when negative results are obtained in strain TA102 especially if it is suspected that a chemical may cause oxidative DNA damage.

10.6. Metabolic activation

Prior to performing an experiment, the S-9 fraction is added to the enzymic cofactors containing NADP to make up the S-9 mix to be used in the assay. The S-9 fraction concentration is usually expressed as "% v/v". It is important to note that both the cofactors and S-9 fraction should be stored frozen. They should be thawed at room temperature and quickly transferred to ice. Once the S-9 mix is prepared it should be kept on ice for the duration of the experiment. Because the metabolic conversion of different chemicals to their mutagenic form may require different optimum levels of S-9 fraction, the amount of S-9 fraction used is variable; it can range from 4% to 30% (v/v) in the S-9 mix [7,47].

Aroclor 1254-induced rat liver is the most widely used source of activating enzymes [7]. In the ab-

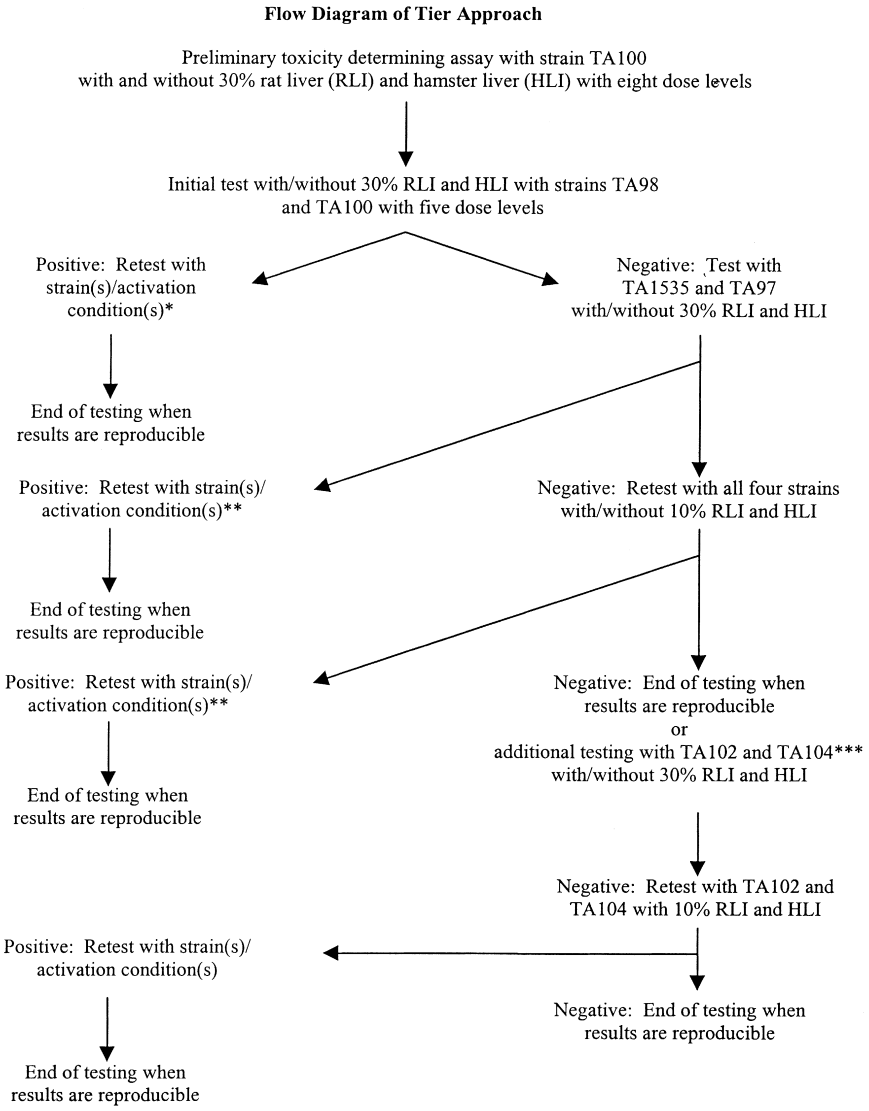

Fig. 5. Diagram of tier approach testing strategy.

sence of specific information about the optimizing S-9 concentration, it is recommended that between 5% and 30% (v/v in the cofactor mix) of the S-9 fraction be used. Some laboratories use a low S-9 concentration (e.g., 5% or 10%) in the first mutagenicity assay. If negative results are obtained, a subsequent test with a high S-9 level (30%) is performed. Other laboratories use the higher level of S-9 first, and use the low level in the second experiment if negative results are obtained in the initial experiment.

Procedures for preparing the S-9 fraction have been described in detail [7,47,93]. If the S-9 fraction is prepared in the testing laboratory, it is recommended that more than one animal be used and the tissue homogenate be pooled in order to minimize animal-to-animal variation among batches. Some laboratories standardize the S-9 concentration based

on measured protein content while other laboratories standardize the S-9 fraction on the assumption that 1 ml of the S-9 fraction contains microsomes from about 250 mg of wet livers with a protein concentration of 40 mg/ml. S-9 fractions may be stored frozen in small aliquots for up to 1 year at −80°C.

Note: When using live animals, it is required that all protocols be reviewed and approved first by an Institutional Animal Care and Use Committee (IACUC). Officially approved procedures must be followed for the care and use of the animals.

A number of commercial vendors are now providing S-9 preparations. This has the advantage that information is provided about enzyme activities and effectiveness against standard mutagens, and it may be more cost-effective than purchasing and maintaining animals for this purpose. In the United States, a supplier for rodent post mitochondrial supernatant (S-9) is Molecular Toxicology, 157 Industrial Park Drive, Boone, NC 28607, Phone: +1-828-264-9099.

10.7. Positive control chemicals

Diagnostic mutagens (positive control chemicals) must be included in each experiment to confirm the reversion properties and specificity of each tester strain, and the efficacy of the metabolic activation system. Table 4 lists the positive control chemicals with their respective concentrations/plate that have been routinely used in the authors' laboratories. Other chemicals can be selected as positive controls [7,37,47]. Where the test chemicals are members of a specific chemical class, a positive control chemical with a similar structure, or requiring similar metabolism, should be used. The optimum positive control concentration should be determined for each new batch of metabolic activation (S-9). Some researchers have suggested that 2-aminoanthracene should not be recommended as the only positive control to evaluate the metabolizing activity of the S-9 fraction [23], because it has been shown that 2-aminoanthracene may be activated by enzymes other than the microsomal cytochrome P450 family [10].

10.8. Number of replicates and repeat experiments

It is recommended that at least three plates per dose level are used with at least five dose levels and a solvent control. When positive results are obtained, the test should be repeated using the identical conditions that yielded the positive response in order to confirm the response. It is recommended that only the strain(s) and condition(s) that yielded the mutagenic response are used for the confirming assay. The dose levels may be changed to better establish the dose response. If negative results are obtained, a repeat assay is recommended in both the tier and battery approach which may include a slight modification of the procedure by using a different concentration of the S-9 fraction, or a different procedure (e.g., preincubation instead of plate incorporation).

11. Interpretation and reporting

11.1. Counting of histidine revertant colonies

After the plates are removed from the incubator, the colonies are counted and the results are expressed as revertant colonies per plate. For this purpose, an electronic counter is a convenient way to count the colonies, especially for strains TA100 and TA97 which usually have a spontaneous background above 100 colonies/plate. However, hand-counting is required when strains TA102 and TA104 are used because of the high number of spontaneous revertant colonies, usually above 200 colonies/plate. Hand-counting is also required when precipitate is present on the plate, when there is poor contrast between the colony and the agar, or when a test chemical discolors the agar which prevents sufficient light from passing through the agar. If precipitate is present on the plates at the higher dose levels, all plates, including the solvent control plates, should be hand-counted. Also, all plates from a single experiment, including positive and negative control plates, should be counted using the same method at the same time, because the cells on the plate will continue dividing and produce more mutant colonies with increased storage time.

At the time the plates are scored, their background lawns should be examined by eye and by using a 40× dissecting microscope for thinning, which indicates toxicity, and for the presence of precipitate. Slight toxicity or precipitate will not invalidate the experiment, or the doses at which they were found, but they may

affect the interpretation of the results and the design of follow-up experiments.

11.2. Data reporting

The results are generally reported as mean revertant colonies per plate±the S.D. or S.E.M. for the test chemical and the controls. The concentration of the test chemical is expressed as mg or μg/plate, or milliliter or mole in the desiccator procedure for volatile liquids and gases, respectively. Information regarding toxicity and/or precipitation of the test chemical should also be included. The results can also be expressed as the fold-increase over the solvent control value, but this must be done in addition to the mean plate count. Fold-increase, alone, may not be sufficient to allow an objective evaluation of the data, or of the effectiveness of the experiment.

11.3. Data evaluation

11.3.1. Statistical procedures

A number of statistical programs have been developed for analyzing *Salmonella* mutagenicity data, and all have their strengths and weaknesses [13,20,36,44,70,76,81]. Another approach that has been widely used is to set a minimum fold increase, usually 2–3 fold, in revertants (over the solvent control) as the cut-off between a mutagenic and nonmutagenic response [42]. This approach also has its weaknesses, for example, if a two-fold increase is required, a change in the average plate count by one revertant out of one or a few hundred can be the difference between calling a chemical mutagenic or nonmutagenic. Also, it has been shown [15] that the two-fold rule may be too insensitive for *Salmonella* strains with relatively high reversion frequencies, such as TA100, TA97, and TA102, and too sensitive for chemicals with low reversion frequencies, such as TA1535 and TA1537.

11.3.2. Non-statistical procedure

A non-statistical procedure has been established to evaluate the results of *Salmonella* experiments [91]. Using this procedure the following criteria are used to interpret results.

- ***Positive***: A compound is considered a mutagen if it produces a reproducible, dose-related increase in the number of revertant colonies in one or more strains. A compound is considered a weak mutagen if it produces a reproducible, dose-related increase in the number of revertant colonies in one or more strains but the number of revertants is not double the background number of colonies.
- ***Negative***: A compound is considered a nonmutagen if no dose-related increase in the number of revertant colonies is observed in at least two independent experiments. The maximum dose level tested for nontoxic compounds is 5 or 10 mg/plate (unless otherwise dictated by solubility). For volatile liquids and gases, the maximum dose level tested in desiccators is 5 ml and 4.5 l, respectively. For toxic compounds, ideally only the highest dose level should exhibit evidence of toxicity (e.g., thinning or complete absence of background lawn with or without the presence of pinpoint colonies.
- ***Inconclusive***: If a compound cannot be identified clearly as a mutagen or a nonmutagen, the results are classified as inconclusive (e.g., if there is a scattering of slightly elevated revertant counts or one elevated count).

Regardless of whether a statistical or non-statistical approach is used for data evaluation, it should be adequately described and consistent across strains and activation procedures. Responses that are judged to be weakly positive or equivocal should be considered for additional testing, using a modification of the exposure protocol, or a change in the dose range tested or S-9 mix concentration used.

Because control ranges vary among laboratories, each laboratory should establish its own solvent and positive control ranges for each tester strain, with and without S-9. This range should be used to develop acceptance criteria for individual experiments, and experiments should be rejected if solvent controls are outside the acceptable range, or if positive control chemicals do not respond appropriately.

12. Safety considerations

12.1. Aseptic technique

It is important that basic bacteriological laboratory procedures be used to minimize exposure to the *Salmonella* tester strains. Surface areas must be

properly disinfected before and after use. Though wild-type *S. typhimurium* can cause diarrhea and food poisoning, the *gal* and *rfa* (deep rough) mutations that are present in all the *Salmonella* tester strains described here eliminate to different levels, the polysaccharide side chain of the LPS layer that coats the bacterial surface which makes the bacteria non-pathogenic [5,83]. It is nevertheless prudent to use caution at all times and to practice standard laboratory safety procedures such as using plugged pipettes and autoclaving all contaminated material. Mouth-pipetting as a general rule should never be practiced. Aseptic techniques, being part of basic bacteriological laboratory procedures, are also essential to prevent contamination of the overnight cultures, GM agar plates, and the solutions and reagents. Contaminated frozen permanent and working cultures of the *Salmonella* tester strains will be useless and should be discarded.

12.2. Safety procedures

As a general rule, it is prudent to consider all chemicals as if they were mutagens and carcinogens. All handling of chemicals, as well as the test itself, should be performed in a chemical safety cabinet. Workers should protect themselves from chemical exposure by wearing gowns, eye glasses and gloves. Wearers of contact lenses should wear regular eye glasses since some volatile chemicals might react with the contact lens. All contaminated material (e.g., test tubes, pipets and pipet tips, gowns and gloves) should be properly disposed of as well as the unused chemical dilutions and stock solutions of the test chemical, and positive control chemicals.

13. Mutagenicity information

13.1. Available databases

There are a couple of databases publicly available through the internet. The Environmental Mutagen Information Center (EMIC) database can be entered through http://toxnet.nlm.nih.gov/servlets/simple-search?1.25.0.3876. In this database, information can be retrieved regarding the test organisms used and endpoints examined.

The other database is that of the U.S. National Toxicology Program's genetic toxicology testing program. Results have been published on more than 1500 chemicals, which are summarized and referenced in Zeiger [87]. The summary test results for specific chemicals, published and unpublished, can be retrieved from http://ntp-server.niehs.nih.gov/cgi/iH_Indexes/Res_Stat/iH_Res_Stat_Frames.html.

14. Troubleshooting

Following are some problems that are frequently encountered and a brief description of their probable causes and suggested solutions.

PROBLEM: The spontaneous revertant count is too low

Cause: (1) Toxicity is associated with a new batch of agar
(2) The wrong tester strain may have been used
(3) Too little histidine was added to the top agar

Solution: (1) Each new batch of agar should be checked for toxicity prior to using it on a routine basis
(2) Double check the identity of the strain, and if needed, reisolate the strain selecting a clone that gives rise to a spontaneous reversion rate in the proper range
(3) Prepare new top agar ensuring that the proper concentration of histidine is used

PROBLEM: The spontaneous revertant count is too high

Cause: (1) Too much histidine was added to the top agar
(2) The initial inoculum, from the frozen working culture, contained an unusually high number of His^+ bacteria (jack-pot effect)
(3) Petri plates may have been sterilized with ethylene oxide with residual levels

inducing mutations in strains TA1535 and TA100
(4) Contamination may be present

Solutions: (1) Inspect the background lawn. A heavier than normal background lawn indicates that too much histidine was present in the top agar. Prepare new top agar ensuring that the proper concentration of histidine is used. It is also possible that the test sample may contain histidine. This is often the case when biological fluids such as urine, or food-related items, such as proteins and meat extracts, have been tested
(2) Check a few frozen working stock ampoules for spontaneous reversion. If the problem persists, reisolate the strain selecting a clone that gives rise to a spontaneous reversion rate in the proper range
(3) Use radiation-sterilized plates, or allow plates to off-gas for one day in a chemical or biological fume hood
(4) Reisolate strain

PROBLEM: The positive control does not work

Cause: (1) The wrong positive control chemical may have been used
(2) The chemical may have deteriorated during storage
(3) The metabolic activation system may have been omitted
(4) The metabolic activation system has lost its activity
(5) The wrong tester strain was used

Solution: (1) Double check the positive control chemical ensuring that it is tested with the proper strain
(2) Prepare a new batch of the positive control chemical
(3) Retest the positive control ensuring that the metabolic activation system is included. If the problem persists, prepare a fresh solution of the positive control and retest. If the problem persists, it is possible that the S-9 fraction or cofactors may have lost activity
(4) Prepare a new batch of cofactors and test with the S-9 fraction against the positive control. If the problem persists obtain a fresh batch of S-9 fraction
(5) Confirm the identity of the strain

PROBLEM: There are few if any revertant colonies on the plate

Cause: (1) The chemical is highly toxic
(2) The temperature of the top agar was too high
(3) The solvent is toxic

Solution: (1) When a chemical is highly toxic, most if not all of the plated bacteria will be killed and not enough survivors will be present to revert to histidine independence. If this is the case, an inspection of the plate under 40× magnification should reveal thinning or an absence of background lawn. A lower dose range of the chemical should be used
(2) Care should be taken that the temperature of the top agar does not exceed 48°C. Ideally the top agar should be maintained at 43°C
(3) If the solvent is toxic and the preincubation assay was used, repeat the assay using the plate incorporation assay. If the problem persists, try another solvent, or dilute the solvent with one that is less toxic

PROBLEM: Most of the colonies are concentrated on one half of the plate

Cause: The bottom and/or top agar were allowed to solidify on a slant

Solution: The petri plates should be level whenever the plates are poured and when the top agar is added to the plates

PROBLEM: The top agar does not solidify properly

Cause: (1) The top agar concentration was too low

(2) Gelling of the agar is retarded by acid pH

Solution: (1) The recommended concentration of the agar is 0.6% (w/v)
(2) Additional buffering capacity should be added to the agar, or the pH of the chemical should be adjusted

PROBLEM: The top agar slips out of place

Cause: The surface of the bottom agar was too wet

Solution: Prior to use, inspect the surface of the bottom agar plate. If moisture is present, do not use the plate. If there is a recurring problem with too much moisture being present on the GM plates, place the plates in a 37°C incubator overnight prior to use

PROBLEM: The bottom agar slips out of the plate

Cause: Insufficient agar was used when the GM plates were prepared

Solution: The recommended concentration for the bottom agar is 1.5%, w/v

15. Recipes for reagents and media

15.1. Vogel–Bonner (VB salts) medium E (50×)

Use: salts for the GM agar plates

Ingredients	Per liter
Warm distilled water (about 50°C)	650 ml
Magnesium sulfate ($MgSO_4 \cdot H_2O$)	10 g
Citric acid monohydrate	100 g
Potassium phosphate, dibasic, anhydrous (K_2HPO_4)	500 g
Sodium ammonium phosphate ($Na_2NH_2PO_4 \cdot 4H_2O$)	175 g

Add the above ingredients in the order indicated to warm water in a 2-l flask making sure that each salt is dissolved thoroughly by stirring on a magnetic stirrer before adding the next salt. It takes about 1 h to dissolve all ingredients. Adjust volume to 1 l. Distribute in 20-ml aliquots and autoclave, loosely capped, for 30 min at 121°C. When the solutions have cooled, tighten the caps and store at room temperature in the dark.

15.2. Glucose solution (10% v/v)

Use: as carbon source for the GM agar plates

Ingredients	Per liter
Distilled water	700 ml
Dextrose	100 g

Add the dextrose to the water in a 3-l flask. Stir on a magnetic stirrer until mixture is clear. Add additional water to bring the final volume to 1000 ml. Dispense 50-ml aliquots into 250 ml screw-cap bottles. Label as 10% glucose with date. Autoclave 121°C for 20 min making sure the caps are on loosely. When cooled, tighten the caps and store at 4°C.

15.3. GM agar plates

Use: bottom agar for mutagenicity assay

Ingredients	Per liter
Distilled water	900 ml
Agar	15 g
VB salt solution (50×)	20 ml
Glucose solution (10% v/v)	50 ml

Add the agar to the water in a 3-l flask. Autoclave for 30 min at 121°C. Let cool for about 45 min to about 65°C. Add 20-ml of sterile VB salts and mix thoroughly, then add the 50 ml of a sterile glucose (10% v/v) solution; again swirl thoroughly. Dispense the agar medium in 100×15 mm petri dishes (approximately 25 ml/plate). When solidified, the plates can be stored at 4°C for several weeks when placed in sealed plastic bags. Before use, the plates should be warmed up to room temperature and examined for excess moisture. If too much moisture is present, incubate the plates overnight at 37°C prior to use. It takes about 12 l of agar medium to prepare one case of petri plates (500 plates).

Note: A precipitate may form when the VB salts are added. However, thorough mixing will solubi-

lize the salts. The agar should never be autoclaved together with the VB salts and glucose. The GM plates prepared this way will not fully support the growth of the *Salmonella* tester strains.

15.4. Histidine/biotin solution (0.5 mM)

Use: to supplement top agar with excess biotin and a trace amount of histidine

Ingredients	Per liter
Distilled water	1000 ml
D-biotin (F.W. 247.)	124 mg
L-Histidine·HCl (F.W. 191.7)	96 mg

Bring water to a boil and add the biotin and histidine. It may take the biotin a little while to completely dissolve; histidine is readily dissolved. If used immediately, follow the procedure for making top agar. If not used immediately sterilize the solution either by filtration through a 0.45 μm membrane filter or by autoclaving for 20 min at 121°C. Store at 4°C in a glass bottle.

15.5. Top agar supplemented with histidine/biotin

Use: to deliver the bacteria, chemical and buffer or S-9 mix to the bottom agar

Ingredients	Per liter
Distilled water	900 ml
Agar	6 g
Sodium chloride	6 g
Histidine/biotin solution (0.5 mM)	100 ml

Add the agar and sodium chloride to a 3-l flask containing 900 ml of distilled water. Heat for 10 min in an autoclave, liquid cycle, to melt the agar. Then, add 100 ml of limited histidine and biotion solution (0.5 mM). Dispense 200-ml aliquots in 500-ml screw-cap bottles. Label as TA with date with the date of preparation. Autoclave for 30°C and store at room temperature in the dark. When ready to use, melt the top agar in a microwave oven or in boiling water.

15.6. Nutrient broth

Use: to grow the tester strains overnight

Ingredients	Per liter
Distilled water	1000 ml
Oxoid nutrient broth #2	25 g

Add the nutrient broth powder to the water and stir to dissolve. Dispense 50 ml in 125 ml Erlenmeyer flasks or 5 ml in 100×16 mm test tubes. Autoclave for 20 min. When cooled, store in the dark at room temperature.

Note: Difco nutrient broth can also be used but the overnight cultures are usually less dense compared to when Oxoid nutrient broth is used. Follow the manufacturer's directions for preparing the Difco nutrient broth.

15.7. Sodium phosphate buffer, 0.1 mM, pH 7.4

Use: for testing chemicals in the absence of metabolic activation

Ingredients	Per liter
Sodium phosphate, monobasic (0.1 M) To a 1 l water add 13.8 g $NaH_2PO_4.H_2O$	120 ml
Sodium phosphate, dibasic (0.1 M) To a 1 l water add 14.2 g $Na_2HPO_4.H_2O$	880 ml

After mixing the two ingredients, mix well. Adjust pH to 7.4 using 0.1 M dibasic sodium phosphate solution. Dispense 100 ml aliquots in 250 ml screw-cap bottles and label as "Buffer, 0.1 M, pH 7.4" with date. Autoclave for 30 min at 121°C. Make sure the caps fit loosely during autoclaving. When cooled, tighten the caps and store the bottles at room temperature in the dark.

15.8. Co-factors for S-9 mix

Use: to provide the NADH regenerating system

Ingredients	Per liter
Distilled water	900 ml
D-Glucose-6-phosphate	1.6 g
Nicotinamide adenine dinucleotide phosphate (NADP)	3.5 g
Magnesium chloride (MgCl)	1.8 g
Potassium chloride (KCl)	2.7 g
Sodium phosphate, dibasic ($Na_2HPO_4·H_2O$)	12.8 g
Sodium phosphate, monobasic ($NaH_2PO_4·H_2O$)	2.8 g

To 900 ml of water, add each ingredient sequentially making sure that each ingredient is dissolved before adding the next one. This process may take up to 1 h. When all ingredients are dissolved, filter sterilize the cofactors (0.45 μm filter). Dispense in sterile glass bottles in aliquots of 7, 9 and 9.5 ml, or multiples of these volumes, for convenient use when in need of 30%, 10% or 5% S-9, respectively, in the final S-9 mix (10 ml volumes). Label "Cofactors" with date and store at −20°C.

Note: Prior to each experiment thaw sufficient co-factors and S-9 fraction and keep on ice as soon as the S-9 mix is prepared. A volume of 0.5 ml of the S-9 mix is usually added per plate.

15.9. Co-factors for reductive metabolism

Use: to provide a reductive environment for the metabolic activation system

Ingredients	Per 100 ml
Standard co-factors for S-9 mix	63 ml
D-glucose-6-phosphate	423 mg
Nicotine adenine dinucleotide (NADH) disodium salt (Reduced form)	142 mg
Glucose-5-phosphate dehydrogenase	280 U (2.8 U/ml final)
Liver S-9 fraction (preferably hamster liver)	30 ml
Flavin mononucleotide (FMN)	96 mg in 7 ml of water (filter sterilized and kept on ice)

To a 250 ml sterile Erlenmeyer flask, add the co-factors that have been allowed to thaw at room temperature. Add the D-glucose-6-phosphate and NADH. When dissolved, filter sterilize through a 0.45 μm filter. Aseptically add the glucose-6-phosphate dehydrogenase to the co-factor mix followed by 30 ml of liver S-9 fraction. Just prior to performing the experiment add the FMN solution. The final S-9 mix should be kept on ice for the duration of the experiment.

15.10. Enriched GM agar plates

Use: to provide medium supplemented with essential nutrients and antibiotics for the strain check or propagation of the strains and preparation of stock culture master plates.

Prior to preparing the 1-l GM agar plates (see recipe above) add the following item(s) as required to the flasks containing the GM agar medium; mix well before dispensing.

- Biotin (B) plates: 8 ml of 0.01% solution.
- Histidine (H) plates (excess histidine): 8 ml of 0.5% solution.
- Biotin/Histidine (BH) plates: 8 ml of 0.01% biotin and 0.5% histidine solution.
- Biotin/Histidine/Ampicillin (BHA) plates: same as BH plates but add 3 ml of ampicillin solution (8 mg/ml) to give a final concentration of ampicillin of 24 μg/ml.
- Biotin/Histidine/Tetracycline (BHT) plates: same as BH plates but add 0.25 ml of tetracycline solution (8 mg/ml) which will give a final concentration of 2 μg/ml.
- Biotin/Histidine/Ampicillin/Tetracycline (BHAT) plates: same as BH plates but add 3 ml of ampicillin solution (8 mg/ml) and 0.25 ml of tetracycline (8 mg/ml), to give a final concentration of 24 and 2 μg/ml, respectively.

15.11. Biotin solution (0.01%, w/v)

Use: to enrich GM agar plates for strain check, reisolation of strain and stock culture master plate.

Ingredients	Per 100 ml
Distilled water	100 ml
D-biotin	10 mg

Heat water to a boil and add the biotin; stir until dissolved. Filter sterilize using a 0.45 μm filter. Store at 4°C.

15.12. Histidine solution (0.5%, w/v)

Use: to enrich GM agar plates for strain check, reisolation of strain and stock culture master plate

Ingredients	Per 100 ml
Distilled water	100 ml
L-histidine	500 mg

Dissolve the histidine in the water. Autoclave for 15 min at 121°C. Store at 4°C.

15.13. Ampicillin solution (0.8%, w/v)

Use: to confirm the presence of plasmid pKM101 in strain TA97, TA98, TA100 and TA102 and preparation of master plates for plasmid-carrying strains.

Ingredients	Per 100 ml
Distilled water	100 ml
Ampicillin	8 mg

Dissolve the ampicillin in warm (65°C) water. Filter sterilize using a 0.45 μm filter. Store at 4°C.

15.14. Tetracycline solution (0.8%, w/v)

Use: to confirm the presence of plasmid pAQ1 in strain TA102 and preparing master plate for strain TA102.

Note: the stock culture master plate for strain TA102 should contain both ampicillin and tetracycline.

Ingredients	Per 100 ml
Hydrochloric acid (0.02 N)	100 ml
Tetracycline	8 mg

Dissolve the tetracycline in the 0.02 N HCl. Filter sterilize using a 0.45 μm filter. Dispense in 10 ml aliquots in sterile test tubes. Store at 4°C in the dark to protect against light (tetracycline is light sensitive).

15.15. Crystal violet solution (0.1%, w/v)

Use: to confirm the presence of the *rfa* mutation in all the tester strains

Ingredients	Per 100 ml
Distilled water	100 ml
Crystal violet	100 mg

Dissolve the crystal violet in the 100 ml of water. Mix well and store at 4°C in a brown glass bottle to protect against light.

15.16. Nutrient agar plates

Use: (1) to streak newly received cultures for single colonies, (2) to test for crystal violet sensitivity (*rfa*) in lieu of using GM agar plates supplemented with excess histidine and biotin, (3) to test for viability of bacteria

Ingredients	Per 100 ml
Distilled water	1000 ml
Agar	15 g
Oxoid Nutrient Broth #2	25 g

Add the agar to the water in a 3-l flask and heat to dissolve. Add the nutrient broth powder and stir until dissolved. Autoclave for 20 min at 121°C. Let the agar cool to about 65°C. Dispense 20 to 25 ml in sterile petri plates. Store upside down in sealed plastic bags at 4°C.

16. Equipment and supplies

The following items are needed for performing the mutagenicity protocols.

16.1. Supplies

- Petri plates (100×15-mm)
- Sterile glass tubes (100×13-mm and 150×16-mm)
- Test tube racks
- General laboratory glassware (flasks, bottles, graduated cylinders)
- Pipets (1, 2, 5 and 10 ml)
- Pipettors (adjustable volumes up to 200 and 500 μl)
- Pipet tips
- Dispensers for delivering top agar, buffer and S-9 mix to the test tubes

- Cryogenic tubes for freezing down permanent and working cultures
- Sterile glycerol
- Magnetic stir bars
- Reagents and media (as described in section 15)
- Mammalian tissue homogenate (S-9 fraction)
- Dimethylsulfoxide and other solvents
- Positive control chemicals
- Gloves
- Gowns
- Goggles or protective eye wear
- Biohazard bags
- Protective covers for work space

16.2. Equipment

- Colony counter (manual or electronic)
- Autoclave
- Shaking incubator set at 120 rpm and 37°C for growing the overnight cultures
- Incubator for incubating the GM agar plates equipped with electrical outlet for Desiccator assays
- Oven, heating or water bath set at 43°C to 48°C to maintain temperature of top agar
- Gas line for keeping aseptic techniques while inoculating cultures
- Boiling water bath or microwave oven for melting top agar
- Desiccator (9 l)
- Magnetic stirrers
- Balances
- Water purification system to generate distilled water
- Spectrophotometer
- Centrifuge (up to 8000 rpm)
- Revco freezer or liquid nitrogen tank
- Refrigerator/freezer
- Chemical/biological safety hood
- Liquid waste disposal
- Vacuum pump
- Solid waste disposal

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