

Chapter 2

Bacterial Mutagenicity Assays: Test Methods

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Abstract

The most widely used assays for detecting chemically induced gene mutations are those employing bacteria. The plate incorporation assay using various *Salmonella typhimurium* LT2 and *E. coli* WP2 strains is a short-term bacterial reverse mutation assay specifically designed to detect a wide range of chemical substances capable of causing DNA damage leading to gene mutations. The test is used worldwide as an initial screen to determine the mutagenic potential of new chemicals and drugs.

The test uses several strains of *S. typhimurium* which carry different mutations in various genes of the histidine operon, and *E. coli* which carry the same AT base pair at the critical mutation site within the *trpE* gene. These mutations act as hot spots for mutagens that cause DNA damage via different mechanisms. When these auxotrophic bacterial strains are grown on a minimal media agar plates containing a trace of the required amino-acid (histidine or tryptophan), only those bacteria that revert to amino-acid independence (His^+ or Tryp^+) will grow to form visible 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.

This chapter provides detailed procedures for performing the test in the presence and absence of a metabolic activation system (S9-mix), including advice on specific assay variations and any technical problems.

Key words: Plate-incorporation test, Pre-incubation test, *Salmonella typhimurium*, *E. coli*, S9-mix, Metabolic activation, Histidine auxotroph, Tryptophan auxotroph

1. Introduction

The most widely used assays for detecting chemically induced gene mutations are those employing bacteria. These assays feature in all test batteries for genotoxicity as it is relatively straight forward to use them as a sensitive indirect indicator of DNA damage. Bacteria can be grown in large numbers overnight, permitting the detection of rare mutational events. The extensive knowledge of bacterial genetics that was obtained during the twentieth century allowed the construction of special strains of bacteria with exquisite sensitivity

to a variety of genotoxins. An offshoot of the studies on genes concerned with amino acid biosynthesis led to the development of *Escherichia coli* and *Salmonella typhimurium* strains with relatively well defined mutations in known genes. The most commonly used bacteria are the *S. typhimurium* strains which contain defined mutations in the histidine operon. These were developed by Bruce Ames and form the basis of the “reverse” mutation assays (1). In these assays, bacteria which are already mutant at the histidine locus are treated with a range of concentrations of test chemical to determine whether the compound can induce a second mutation that directly reverses or suppresses the original mutations. Thus, for the *S. typhimurium* strains which are histidine auxotrophs, the original mutation resulted in the loss of ability to grow in the absence of histidine. The second mutation (induced by the chemical) restores prototrophy, i.e. the affected cell is now able to grow in the absence of histidine, if provided with inorganic salts and a carbon source. This simple concept underlines the great strength of these assays for it provides enormous selective power which can identify a small number of the chosen mutants from a population of millions of unmutated cells and cells mutated in other genes. Each of the *S. typhimurium* strains contains one of a number of possible mutations in the histidine operon, and each can be reverted by either base-change or frameshift mutations. The genotype of the most commonly used strains is shown in Table 1, together with the types of reversion events that each strain detects.

In order to make the bacteria more sensitive to mutation by chemical agents, several additional traits have been introduced. Ames and colleagues realised that many carcinogens (or their metabolites) are large molecules that are often unable to cross the protective cell wall of the bacteria. Wild-type cells produce a lipopolysaccharide that acts as a barrier to bulky hydrophobic molecules. Consequently, an *rfa* mutation was introduced into the *Salmonella* strains, which resulted in defective lipopolysaccharide and increased permeability.

Bacteria possess several major DNA repair pathways that appear to be error-free. The test strains were constructed, therefore, with a deletion removing the *uvrB* gene. This codes for the first enzyme in the error-free excision repair pathway, and so gene deletion renders the strains excision repair deficient, thus increasing their sensitivity to many genotoxins by several orders of magnitude. Lastly, some of the bacterial strains do not appear to possess classical error-prone repair as found in other members of the Enterobacteria such as *E. coli*. This results from a deficiency in *umuD* activity. This deficiency is overcome by insertion of a plasmid containing *umuDC* genes. Plasmid pKM101 is the most useful (2) conferring on the bacteria sensitivity to mutation without a concomitant increase in sensitivity to the lethal effects of test compounds. Further sensitivity is gained by the fact that the initial mutation responsible for the

Table 1**Genotype of commonly used *S. typhimurium* LT2 and *E. coli* WP2 strains**

Bacterial strains	Histidine or tryptophan mutation	Full genotype ^a	Reversion events
<i>S. typhimurium</i>			
TA1535	hisG46	Dgal chlD bio <i>uvrB</i> rfa	Sub-set of base pair substitution events, extragenic suppressors
TA100	hisG46	Dgal chlD bio <i>uvrB</i> rfa (pKM101)	
TA1538	hisD3052	rfa Dgal chlD bio <i>uvrB</i>	Frameshifts
TA98	hisD3052	rfa Dgal chlD bio <i>uvrB</i> (pKM101)	Frameshifts
TA1537	hisC3076	rfa Dgal chlD bio <i>uvrB</i>	Frameshifts
TA97	hisD6610	his O ₁₂₄₂ rfa Dgal chlD bio <i>uvrB</i> (pKM101)	Frameshifts
TA102	hisG428	his D (G) ₈₄₇₆ rfa galE (pAQ1) (pKM101)	All possible transitions and transversions, small deletions
<i>E. coli</i>			
WP2 <i>uvrA</i>	trpE	<i>uvrA</i>	All possible transitions and transversions, small deletions
WP2 <i>uvrA</i> (pKM101)	trp E	<i>uvrA</i> (pKM101)	

^a*rfa* Deep rough, *galE* UDP galactose 4-epimerase, *ChlD* nitrate reductase (resistance to chlorate), *bio* biotin, *uvrB* UV endonuclease component B, *D* deletion of genes following this symbol, *pAQ1* a plasmid containing the his G₄₂₈ gene, *pKM101* a plasmid carrying the *uvrA* and B genes that enhance error-prone repair

histidine growth requirement is situated at a site within the gene that is particularly sensitive to reversion by specific classes of genotoxin (i.e. hotspots). The incorporation of strain TA102 into the test battery was subsequently proposed, as the target mutation has an AT base pair at the critical site. This allows the detection of genotoxins not detected by the usual battery of *S. typhimurium* strains that possess mutations exclusively at GC base pairs. As an alternative many guidelines recommend the use of the *E. coli* WP2 trpE strains which contain a terminating ochre mutation in the *trpE* gene. The ochre mutation involves an AT base pair, and so reverse mutation can take place at the original site of mutation or in the relevant tRNA loci. A combination of *E. coli* WP2 *trpE* (pKM101) and *E. coli* WP2 *trpE uvrA* (pKM101) can be used as alternatives to *S. typhimurium* TA102 for the detection of point mutations at AT sites.

Consequently the following base set of bacterial test strains have been recommended in several guidelines (3–5):

S. typhimurium: TA98, TA100, TA1535

S. typhimurium: TA1537, or TA97, or TA97a

S. typhimurium: TA102, or *E. coli* WP2 *uvrA* or *E. coli* WP2 *uvrA* (pKM101).

The use of the repair-proficient *E. coli* strain WP2 (pKM101) allows the detection of cross-linking agents that require an intact excision repair pathway to generate mutations and this strain may also be selected.

In general, the most widely used protocol is the “plate incorporation assay”. In this method, the bacterial strain, test material and an in vitro metabolic activation system (S9 mix) are added to a small volume of molten agar containing a trace of histidine and biotin (or tryptophan alone in the case of the *E. coli* strains). The mixture is poured across the surface of a basal (minimal glucose) agar plate and allowed to set prior to incubation at 37°C for 48–72 h. The trace of histidine allows the growth of the auxotrophic bacteria in the presence of the test compound and/or any in vitro metabolites. The period of several cell divisions is essential to allow fixation of any premutagenic lesions that have been induced in the bacterial DNA. Exhaustion of the histidine halts growth of the auxotrophic cells. Only those cells that have been reverted to histidine independence will continue to grow and form discrete visible colonies (Fig. 1). The growth of non-reverted cells forms a visible background lawn, the thinning of which can be used as a non-quantitative indication of chemical related toxicity. Revertant colonies can be counted manually or by use of an Image analyser. Untreated (vehicle) and suitable positive controls are included. The test concentration range is determined by performance of a preliminary toxicity test, whilst for non-toxic compounds a maximum concentration of 5 mg per plate is generally recommended.

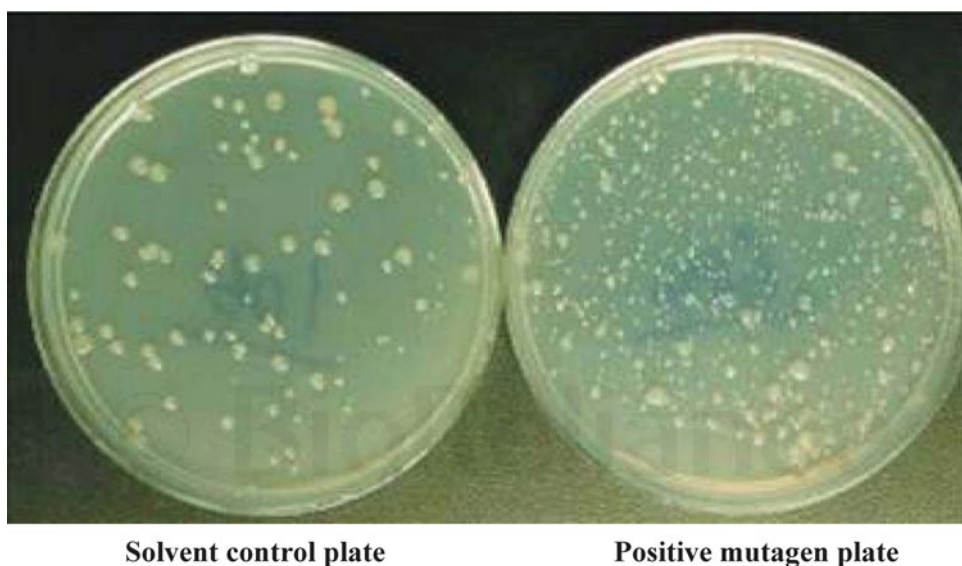


Fig. 1. Examples of a solvent control plate and a positive mutagen plate.

Bacterial mutation tests have been subjected to several large-scale trials over the years (6). These studies were primarily concerned with assessing the correlation between results obtained in the assays and the carcinogenic activity of chemicals. Most of the studies suggest that there is a good qualitative relationship between genotoxicity in the *Salmonella* assay and carcinogenicity for many, although not all, chemical classes. This figure varies between a sensitivity of 60 and 90% dependent upon chemical class. The bacterial assays seem to be particularly efficient in detecting trans-species, multi-organ animal carcinogens (7).

2. Materials

2.1. Vogel-Bonner Salts Medium E (50×)

Use: Salts for GM plates.

Ingredients	Per litre
Warm distilled water (45–50°C)	670 ml
Magnesium sulphate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$)	10 g
Citric acid monohydrate	100 g
Potassium phosphate dibasic anhydrous (K_2HPO_4)	500 g
Sodium ammonium phosphate ($\text{NaH}_2\text{N}_4\text{PO}_4 \cdot 4\text{H}_2\text{O}$)	175 g

Add salts in the order indicated to warm water in a 2-L beaker with stirring (magnetic stirrer) making sure each one is dissolved before adding next salt. Adjust volume to 1 L. Distribute into 20 ml aliquots and autoclave, loosely capped for 30 min at 121°C. When cooled tighten caps and store at room temperature in dark.

2.2. Glucose Solution (10% v/v)

Use: Carbon source for GM plates.

Ingredients	Per litre
Distilled water	700 ml
Dextrose	100 g

Add dextrose to water in 3 L flask. Stir until mixture is clear. Add more water to bring to 1,000 ml and then dispense in 50 ml aliquots and autoclave at 121°C for 20 min. When cooled tighten caps and store at 4°C.

2.3. Glucose Minimal Agar Plates

Use: Basal agar for mutagenicity assay.

Ingredient	Per litre
Agar	15 g
Distilled water	930 ml
VB Salts solution (50×)	20 ml
Glucose solution (10%v/v)	50 ml

Add agar to water in 3 L flask and autoclave for 30 min at 121°C. After cooling (to approx 65°C) add VB salts, mix thoroughly, then add glucose solution and swirl thoroughly. Dispense the medium into Petri dishes (approx 25 ml per dish). When solidified, plates can be stored at 4°C for several weeks in sealed plastic bags.

2.4. Histidine–Biotin Solution 0.5 mM (When Using *S. typhimurium* Strains)

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

Ingredient	Per litre
Distilled water	1,000 ml
d-Biotin (F.W. 247.3)	124 mg
l-Histidine. HCl (F.W. 191.7)	96 mg

Add biotin and histidine to boiling water. After cooling sterilise by filtration (0.45 µm) or by autoclaving at 121°C for 20 min. Dispense in 20 ml aliquots and store in glass bottle at 4°C.

2.5. Tryptophan Solution 0.25 mM (When Using *E. coli* Strains)

Use: To supplement top agar with a trace amount of tryptophan.

Ingredient	Per litre
Distilled water	1,000 ml
L-tryptophan HCl (F.W. 204.2)	51 mg

Dissolve tryptophan in water. Sterilise by filtration (0.45 µm) or by autoclaving at 121°C for 20 min. Dispense in 200 ml aliquots and store at 4°C.

2.6. Top Agar Supplemented with Histidine–Biotin or Tryptophan (Dependent Upon Test Strains)

Use: To deliver bacteria, chemical and buffer or S9-mix to the bottom agar.

Ingredient	Per litre
Distilled water	1,000 ml
Agar	6 g
Sodium chloride	5 g

Add agar and sodium chloride to water in 3 L flask. Agar may be dissolved in steam bath, microwave or by autoclaving briefly (10 min, liquid cycle). Dispense in 180 ml aliquots and autoclave at 121°C for 20 min. When ready to use, melt top agar in boiling water and add following to each 180 ml aliquot:

For <i>S.typhimurium</i> strains:	
Sterile histidine–biotin solution (0.5 mM)	20 ml
For <i>E. coli</i> strains:	
Sterile tryptophan solution (0.25 mM)	20 ml

2.7. Sodium Phosphate Buffer (0.1 mM, pH 7.4)

Use: For testing chemicals in the absence of metabolic activation (S9-mix).

Ingredients	Per litre
Sodium phosphate, monobasic (0.1 M): To 1 L water add 13.8 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	120 ml
Sodium phosphate dibasic (0.1 M): To 1 L water add 14.2 g $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$	880 ml

After mixing, adjust pH to 7.4 using 0.1 M dibasic sodium phosphate solution. Dispense in 100 ml aliquots and autoclave with loose caps at 121°C for 30 min. When cooled tighten caps and store at room temperature in the dark.

2.8. S9-mix

A factor of critical importance in bacterial mutagenicity screening is the need to include some form of in vitro metabolising system. This is because the bacterial indicator cells possess a very limited capacity for endogenous metabolism of xenobiotics. Many carcinogens and mutagens are unable to interact with DNA unless they have undergone some degree of metabolism. To improve the ability of the bacterial test systems to detect as many authentic in vivo mutagens and carcinogens as possible, extracts of mammalian liver (usually rat) are incorporated. The liver is a rich source of mixed-function oxygenases capable of converting carcinogens to reactive electrophiles. Crude homogenate such as the 9,000×g supernatant (S9 fraction) is used, which is composed of free endoplasmic reticulum, microsomes, soluble enzymes, and some cofactors. The oxygenases require the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), which is normally generated in situ by the action of glucose-6-phosphate dehydrogenase on glucose-6-phosphate and reducing NADP, both of which are normally supplied as cofactors. Normal uninduced S9 preparations are of limited value for screening as they are deficient in particular enzyme activities. In addition, species and tissue differences are most divergent in such preparations. These problems are reduced when

enzyme inducers are used, and most commonly preparations are made from rat livers after enzyme induction with Aroclor 1254, which is a mixture of polychlorinated biphenyls. Concern about the toxicity, carcinogenicity and persistence of this material in the environment has led to the introduction of alternatives, such as a combination of phenobarbitone and β -naphthoflavone. This combination induces a similar range of mono-oxygenases and has been recommended as a safer alternative to Aroclor (8).

It should be noted that this system is only a first approximation to the complex metabolic processes that occur in vivo, and in particular there is little account taken of the phase II detoxification reactions.

The S9-mix has the following composition:

Cofactors for S9-mix

Ingredient	Per litre
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 ($\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$)	12.8 g
Sodium phosphate monobasic ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$)	2.8 g

To 900 ml of water add each ingredient sequentially making sure all dissolve thoroughly (may take up to an hour). Filter sterilise (0.45 μm filter) and dispense into sterile glass bottles in aliquots of 7, 9 or 9.5 ml (or multiples of these volumes). This allows the convenient preparation of 30, 10, and 5% v/v S9, by addition of 3, 1, or 0.5 ml of S9 fraction, respectively, to produce the final S9-mix (10 ml volumes). Store the cofactor solution at -20°C .

3. Methods

3.1. Plate Incorporation Assay

The most widely used protocol is the “Plate Incorporation Assay” which is carried out as follows (see Fig. 2, Notes 1 and 2):

1. Each selected strain is grown for 10–15 h at 37°C in nutrient broth (Oxoid No 2) or supplemented media (Vogel-Bonner) on an orbital shaker, to a density of $1\text{--}2 \times 10^9$ colony forming units/ml. A timing device can be used to ensure that cultures are ready at the beginning of the working day.

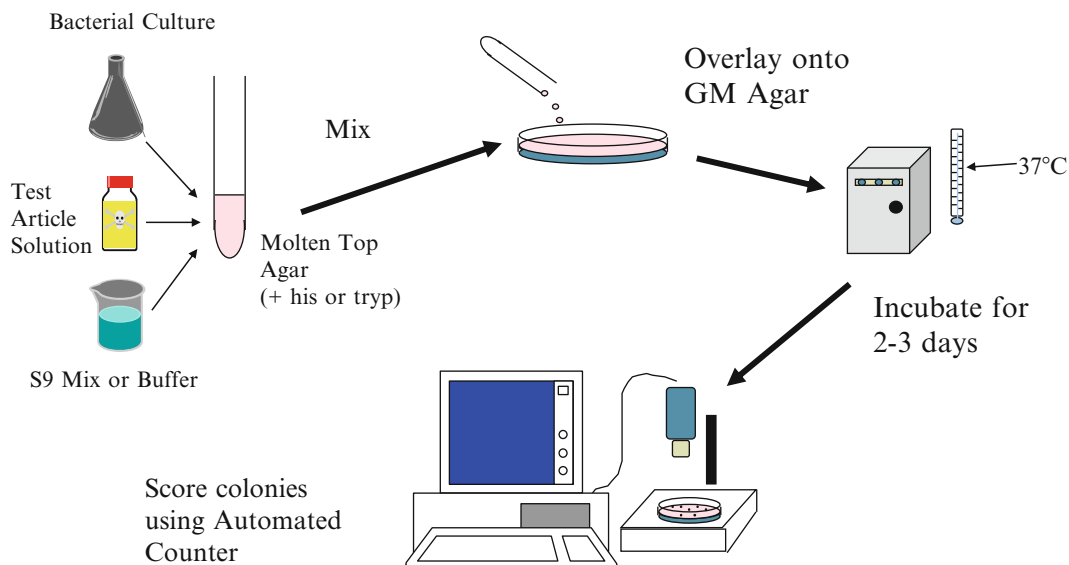


Fig. 2. Schematic representation of the conduct of the plate incorporation method.

2. Label an appropriate number of pre-dried GM agar plates and sterile test tubes for each test chemical and bacterial strain.
3. Prepare S9-mix and keep on ice until use.
4. Prepare chemical dilutions in a suitable solvent (see Note 3).
5. Melt top agar supplemented with either 9.6 µg/ml (0.05 mM) histidine and 12.4 µg/ml (0.05 mM) biotin (for *S. typhimurium* strains), or 5.1 µg/ml (0.025 mM) tryptophan alone (for *E. coli* WP2 test strains) (see Note 4). Dispense into tubes in 2 ml aliquots and keep semi-molten (43–48°C) by holding tubes in a thermostatically controlled aluminium block (see Note 5).
6. To each 2 ml volume of top agar, add in the following order (with thorough mixing/vortexing after each addition):
 - (a) Chemical dilution (10–200 µl) or equivalent volume of solvent (see Note 6) or positive control chemical for each test strain (see Note 7).
 - (b) S9-mix (500 µl) or equivalent volume of phosphate buffer.
 - (c) Overnight culture of required test strain (100 µl) to give approx 1×10^8 viable bacteria per tube.
7. The contents of the tubes are then quickly mixed and poured onto the surface of dried GM agar plates (see Note 8). There should be at least three replicate plates per treatment with at least five test concentrations (covering a range of three logs) and solvent/untreated controls. Duplicate plates are sufficient for positive controls and sterility controls (see Note 9).

8. When the top agar has hardened at room temperature (2–4 min), the plates are inverted and incubated (in the dark) at 37°C for 2–3 days.
9. Before counting the plates for revertant colonies, the presence of a light background lawn of growth (due to limited growth of non-revertant colonies before trace amounts of histidine or tryptophan is exhausted) should be confirmed for each test concentration by microscopic examination of the agar plates (see Note 10).
10. Revertant colonies can be counted manually or with an automatic counter (see Note 11). If colonies cannot be counted immediately after incubation, the plates can be stored in a refrigerator for up to 2 days. All plates must be removed from the incubator/refrigerator and counted at the same time.
11. The results are expressed as the mean number of revertants per plate for each test concentration and analysed in an appropriate manner (see Notes 12 and 13).
12. For any technical assay problems, see Notes 14–20.

4. Notes

1. Some genotoxins are poorly detected using the plate incorporation procedure, particularly those that are metabolised to short-lived reactive electrophiles (e.g. aliphatic nitrosamines). In these cases, a preincubation procedure should be used in which bacteria; test compound and S9-mix are incubated together in a small volume at 37°C for 30–60 min prior to agar addition (9). This maximises exposure to the reactive species and limits non-specific binding to agar. In addition, in the plate-incorporation method soluble enzymes in the S9-mix, cofactors, and the test chemical can diffuse into the basal agar. This can interfere with the detection of some mutagens.
2. Neither the plate incorporation assay nor pre-incubation assay is suitable for testing highly volatile substances. The use of a closed chamber is recommended for testing such chemicals in a vapour phase, as well as for gases (10). Procedures using plastic bags in lieu of desiccators have also been described (11).
3. Solvent of choice is sterile distilled water. Dimethylsulphoxide is often used for hydrophobic chemicals, although other solvents have been used (e.g. acetone, ethyl alcohol, dimethyl formamide, and tetrahydrofuran). It is essential to use the minimum amount of organic solvent (e.g. <2% w/w) compatible with adequate testing of the chemical, and to use fresh batches of solvent of the highest grade possible.

4. Because the bacteria stop growing when the amino-acid (histidine or tryptophan) is depleted, the final population of auxotrophic bacteria on the plate is dependant upon this concentration, which in turn affects the number of spontaneous revertant (prototrophic) colonies. It is therefore important that the utmost care is taken to accurately supplement the top agar with the correct amount of amino acid.
5. It is best to avoid water baths, as microbial contamination can cause problems.
6. The exact volume of test chemical or solvent may depend upon toxicity or solubility.
7. The diagnostic positive control chemicals validate each test run and help to confirm the nature of the bacterial test strain. Table 2 lists representative positive controls.
8. 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 S9-mix or buffer.
9. Checks should be made on the sterility of S9-mix, media and test chemicals to prevent unnecessary loss of valuable time and resources.
10. When plates are inspected after 48 h incubation and growth retardation is seen, as evidenced by smaller than anticipated colony sizes, the plates should be incubated for an additional 24 h.

Table 2
Representative positive control chemicals

Bacterial strain	Chemical (µg/plate ^a)	
	Without S9-mix	With S9-mix
<i>S. typhimurium</i>		
TA97/TA97a	9-Aminoacridine (50)	2-Aminoanthracene(1–5) ^b
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)
TA1535	Sodium azide (5)	2-Aminoanthracene(2–10)
TA1537	Neutral red (10)	2-Aminoanthracene(2–10)
TA1538	4-Nitro-o-phenylenediamine (2.5)	2-Aminoanthracene(2–10)
<i>E. coli</i>		
WP2 <i>uvrA</i>	Nifuroxime(5–15)	2-Aminoanthracene (1–10)
WP2 <i>uvrA</i> (pKM101)	Cumene hydroperoxide (75–200)	2-Aminoanthracene (1–10)

^a Concentration based upon 100× 15 mm Petri plate containing 20–25 ml GM agar

^b Some researchers have suggested that 2-aminoanthracene should not be used as the only positive control to evaluate S9-mix activity as it has been shown that the chemical may be activated by enzymes other than the microsomal cytochrome P₄₅₀ family (15)

This should be done for all plates from an experiment even if growth retardation is only seen at the higher test concentrations. At concentrations that are toxic to the test strains the lawn will be depleted and colonies may appear that are not true revertants but surviving, non-prototrophic cells. If necessary the phenotype of any questionable colonies (pseudo-revertants) should be checked by plating on histidine or tryptophan-free medium.

11. Automatic colony counters are relatively accurate in the range of colonies normally observed (although calibration against manual counts is a wise precaution). Manual counting may be needed where a potent mutagen is being tested and accurate quantitative colony counts are required. In addition, manual counting could be required if heavy precipitate and lack of contrast between the colonies is observed on the plates (especially at higher dose levels).
12. If the test chemical induces increases in revertant counts, these values are compared with the spontaneous revertant counts for each bacterial test strain. Each test strain has a characteristic spontaneous revertant 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 this value. It is recommended that each laboratory establish its own historical data base on which to base an acceptable spontaneous revertant rate. Table 3 presents a range of spontaneous revertant values per plate considered valid by many experienced test laboratories.

Table 3
Representative ranges for spontaneous revertant values

Bacterial strain	Number of revertants per plate	
	Without S9-mix	With S9-mix
<i>S. typhimurium</i>		
TA97/TA97a	75–200	100–200
TA98	20–50	20–50
TA100	75–200	75–200
TA102	100–300	200–400
TA1535	5–20	5–20
TA1537	5–20	5–20
TA1538	5–20	5–20
<i>E. coli</i>		
WP2 <i>uvrA</i>	15–50	15–50
WP2 <i>uvrA</i> (pKM101)	45–151	45–151

13. Several statistical approaches have been applied to the results of these assays and all have their strengths and weaknesses (12, 13). Another approach that has been widely used is to set a minimum fold increase, usually two- to threefold, in revertants (over the solvent control) as the cut-off between a mutagenic and non-mutagenic response (14). In general, positive results should be statistically significant, dose related and reproducible.
14. If the spontaneous revertant count is *too low* relative to the historical control range for an individual laboratory, this may be due to:
 - (a) Toxicity associated with new batch of agar.
 - (b) Too little histidine added to top agar.
15. If the spontaneous revertant count is *too high* relative to the historical control range for an individual laboratory, it may be due to the following:
 - (a) Too much histidine added to top agar.
 - (b) Initial inoculums from working culture contained unusually high number of His⁺ bacteria (jackpot effect).
 - (c) Petri plates have been sterilised with ethylene oxide with residual levels inducing mutations in *S. typhimurium* strains TA1535, TA100 and the *E. coli* strains.
 - (d) Contamination might be present.
16. If the positive control does not work, it may be due to the following:
 - (a) Use of incorrect chemical.
 - (b) Chemical may have deteriorated during storage.
 - (c) Omission of S9-mix if required.
 - (d) S9-mix has lost its activity.
 - (e) Incorrect bacterial test strain used.
17. If there are few, if any, revertant colonies on any plates, it may be due to the following:
 - (a) Test chemical is highly toxic.
 - (b) Temperature of top agar was too high.
 - (c) Solvent is toxic.
18. If most of colonies are concentrated on one half of plate, it is because the basal and/or top agar were allowed to solidify on a slant.
19. If the top agar does not solidify, it may be due to the following:
 - (a) Agar concentration was too low.
 - (b) Gelling is retarded by acidic pH of test material.

20. If top agar slips out of place, it is because surface of basal agar was too wet. If too much moisture is present, the GM plates should be dried by incubating plates at 37°C overnight before use.

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