CHAPTER 4

The Bacterial Reverse Mutation Test

Annie Hamel[1], Marise Roy[1] and Ray Proudlock[2]

[1]*Department of Genetic Toxicology, Charles River Laboratories, Montreal, ULC, Canada*
[2]*Boone, North Carolina, USA*

Chapter Outline

Genetic Toxicology Testing.
DOI: http://dx.doi.org/10.1016/B978-0-12-800764-8.00004-5

4.1 Introduction

Bacterial genetic toxicity tests fall into three main categories: back/reverse mutation singular, forward mutation, and DNA repair deficiency systems. Those that detect back mutations (reversion of a point mutation) are the only ones in widespread use and that are generally acceptable for regulatory submissions.

4.2 History

Originally, studies used mutagenic compounds to study the biochemical basis of mutation and to elucidate the structure and organization of the gene. The first demonstration of chemical induction of mutation was described by Auerbach and Robson in 1944 [1] and

involved production of mutations and chromosomal aberrations in *Drosophila* using mustard gas and related compounds. Demerec [2] showed that various carcinogenic polyaromatic hydrocarbons also induced mutations in *Drosophila*. Perhaps realizing the simplicity, enhanced sensitivity, and rapidity (based on population size and shorter generation time) of microbial systems, Witkin began work on chemically induced mutation to phage resistance in *Escherichia coli* [3]. Soon afterward, Demerec et al. developed a more practical system quantifying back-mutation from streptomycin dependence to independence in *E. coli* [4,5]. Dependent cells could divide a few times in the absence of streptomycin, allowing a chance for back-mutation (reversion) to streptomycin independence, consequently allowing their growth into visible colonies. Szybalski [6] screened more than 400 chemicals using a variation of Demerec's plate-test method in which the chemical was applied on a filter-paper disk (i.e. a spot test). Ironically, Szybalski was screening for potential antitumor agents as opposed to chemicals that might cause cancer; the test was not particularly successful, probably because it relied on a very specific base-pair reversion at a particular location of the gene, required diffusion of the test substance through the agar, and did not allow for mammalian metabolism.

The *E. coli* mutation system commonly in use today, requiring an auxotrophic trpA mutation to L-tryptophan independence, was originally conceived by Yanofsky et al. [7], and use of the WP2 strain to quantify mutagenesis was described by Hill [8]. The trpA gene is a part of the trp operon and codes for the tryptophan synthetase α chain. The strains used in routine mutation tests generally detect base-pair but not frameshift mutations. These can occur at the site of the original mutation or at a more distant site, which suppresses the original defect. The strains currently used are derived from *E. coli* B and have an incomplete lipopolysaccharide cell wall making them permeable to larger molecules [9]. To some extent, the *E. coli* strains may be considered complementary to the *Salmonella* tester strains described here and are generally used in conjunction with them [10].

The strains constructed by Ames and his colleagues at the University of California in Berkeley were derived from *Salmonella typhimurium* (the causative agent in mouse typhoid fever and a variant of the species *Salmonella enterica*) strain LT2 and were originally used to study genetic aspects of L-histidine synthesis. Mutants were selected based on sensitivity to chemically induced mutation and their relatively low spontaneous mutation frequencies. The mutations in the histidine operon are situated at hotspots that are particularly sensitive to reversion by certain classes of genotoxins, allowing detection of a wide range of chemically induced base-pair substitution and frameshift mutations. The first use of *Salmonella* histidine−requiring mutants to test for mutagenicity involved the carcinogenic methylating agent, cycasin (methylazoxymethanol glucoside), a carcinogen found in some cycad species [11]. The sensitivity of the strains subsequently selected for routine testing was enhanced by deletion of the enzyme responsible for the first step in error-free excision repair (uvrB) and, in the case of the R-factor (pKM101 plasmid) strains, incorporation of

the SOS mutagenesis gene umuD (coding for a subunit of DNA polymerase V), which promotes error-prone translesion synthesis. The uptake of large and hydrophobic molecules was enhanced by selection of strains with a deep rough (rfa) mutation that leads to incomplete formation of the smooth outer membrane and associated capsule coating the surface of the bacterium [9]. These strains may be supplemented by a strain with intact excision repair systems (most commonly TA102) so that cross-linking agents such as mitomycin C (which is lethal to excision repair−deficient strains) can be detected.

Xenobiotic metabolic systems that are present in mammals, but not bacteria, are often required for conversion of mammalian mutagens to their ultimate active form. These indirect mutagens could be detected in host-mediated assays in which the test organism was injected into the animal (often intraperitoneally in mouse), recovered, and plated a few hours after treatment of the animal with the chemical [12]. However, recovery was variable and the system was laborious and not very sensitive. Malling [13] used a mouse liver microsomal fraction to convert dimethylnitrosamine to a bacterial mutagen. Bruce Ames successfully adapted Malling's metabolic system using human and phenobarbital or methylcholanthrene-induced rat liver S9 (named after the supernatant postmitochondrial fraction after centrifugation at 9000 g) microsomal preparation with cofactors [14]. In this case, the chemical was mixed with the top agar and bacteria prior to plating to establish the standard plate incorporation bacterial reverse mutation test still in general use today. An important variation of this system for detection of short-lived reactive metabolites involves preincubation of the growing test organisms in the liquid phase with the test material and S9 while shaking before plating. Bartsch et al. [15] used the preincubation system to demonstrate mutagenicity of the dialkylnitrosamines. *Salmonella* strain TA1530 was plated in parallel in selective and survival media, so that mutation frequency could be estimated as in the treat and plate method described later; subsequently, mutagenicity was demonstrated in the same system with strain TA1535. Yahagi et al. [16] used TA98 and TA100 to demonstrate mutagenicity of a range of N-nitrosamines using a preincubation method; interestingly, DMSO (dimethyl sulfoxide, the most widely used organic solvent in mutagenicity testing) inhibited the activity of dimethylnitrosamine and diethylnitrosamine. The preincubation method described by Yahagi et al. and the plate incorporation method versions of the pour plate method are generally the only systems considered acceptable for general regulatory submission. A variation of the preincubation method referred to as "treat and plate" involves removal of the test agent after the preincubation period, and is used where the test agent shows very strong antibacterial activity in preliminary testing. Most regulatory bodies require adherence of testing to OECD guideline 471 [17], which implies that confirmation of "negative results" (i.e., apparent absence of mutagenicity of the test material) is expected. Therefore, the plate incorporation and preincubation methods are often used in tandem or in sequence to provide a complete study. In contrast, a recent revision of ICH guidance that covers testing of pharmaceuticals in the United States, Europe, Japan, and

Canada indicates that a single method and occasion of testing is acceptable when a clear result is obtained [18]. Other bacterial mutation and related methods are available (e.g., liquid fluctuation, spot and spiral plate, differential killing/repair tests), but these do not necessarily meet ICH or OECD criteria and are not recommended for regulatory submission or routine use except when required by specific regulations.

4.3 Fundamentals

The bacterial reverse mutation test detects point mutations, which are the cause of many human genetic diseases and play an important role in tumor initiation and development. The strains have various mutations that inactivate a gene involved in the synthesis of an essential amino acid, either histidine (*Salmonella*) or tryptophan (*E. coli*), so they can only grow in culture medium that is supplemented with that amino acid. When the bacteria are exposed to a mutagen, mutation(s) occur that may restore/reverse the ability of the bacteria to synthesize the amino acid and to continue growth once the limited amount of the amino acid in the top agar is depleted. Relevant mutations involve substitution of individual base pairs or frameshift mutations caused by addition or deletion of a stretch of DNA.

In the plate incorporation method, top agar, the test article formulation, the metabolic activation system or equivalent buffer, and the bacteria are mixed together before pouring onto semi-solid minimal medium containing glucose in a Petri dish/plate. The metabolic activation system usually consists of liver fraction (obtained from rats pretreated/induced by treatment with a chemical) supplemented with appropriate cofactors and is referred to as S9 mix or, more often, simply as S9. The top agar is allowed to set, then the plates are inverted and incubated at 37°C for approximately 65 h before examination. The top agar contains enough of the required amino acid to allow a few bacterial divisions before the amino acid is exhausted, giving rise to millions of microscopic bacterial colonies that give a hazy appearance to the medium referred to as a background lawn. Any preexisting revertants and those bacteria that mutate back (revert) to histidine or tryptophan independence continue to grow, forming macroscopic revertant colonies. A test material formulation that causes a substantial increase in the revertant colony count is regarded as a bacterial mutagen. Usually, increases are dose-proportionate (dose-related; Figure 4.1), but precipitation, toxicity, and limitations on metabolic conversion can have profound effects on the shape of the dose-response curve. The response can be generally linear at low doses, but some compounds such as the interchelating agent 9-aminoacridine show a very steep dose-response curve leading a narrow mutagenic window.

A common variation of the plate incorporation method, the preincubation method, involves incubation of a mixture of the liquid bacterial culture with test article and buffer or S9 mix with shaking for a period of usually 20 or 30 min prior to plating. Because the bacteria are beginning

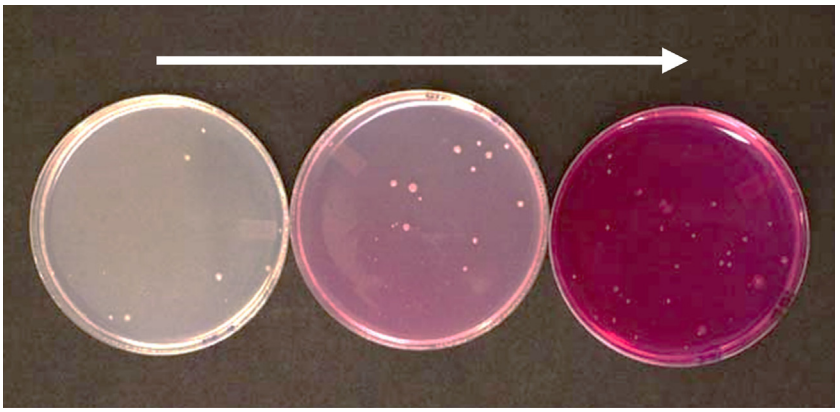

Figure 4.1
Dose-related increase in revertants.

to grow during this relatively high exposure period, and because the test chemical is present at a relatively high concentration, this method can be more sensitive to some classes of mutagen, such as those with short-lived metabolites and volatile agents. In particular, it is usually more appropriate to test short-chain aliphatic nitrosamines, divalent metals, aldehydes, azo-dyes and diazo-compounds, pyrollizidine alkaloids, allyl compounds, and nitro compounds using the preincubation method. However, the preincubation method can be more prone to toxic effects because of the higher test chemical concentration and therefore less sensitive to some chemicals [19]. Both methods use standard 90−100 mm diameter Petri dishes; either and sometimes both methods are generally used in regulatory submissions.

4.4 Equipment

The following is a list of specialized equipment expected to be found in a laboratory performing tests on a routine basis:

- Agar dispensing pump + foot switch + sterile silicon tubing
- Autoclave
- Automatic colony counter with direct data capture system
- Bacterial counting chamber
- Biological containment cabinet class II (externally vented)
- Boiling water bath or microwave
- General laboratory equipment: balances, refrigerator, purified water
- Heating block 45°C
- Incubator 37°C
- Liquid nitrogen cell store or ultralow (≤70°C) freezer

- Microscope(s), ideally a phase contrast for counting bacteria and an inverted for examination of bacterial growth on plates (background lawn)
- Micropipettes (adjustable, repeating, and positive displacement types; range, 2−1000 μL)
- Pipette aids
- Shaker (platform type) or shaking incubator
- Spectrometer or nephelometer
- UV lamp
- Water bath 45°C

4.5 Consumables

Consumables typically used in a testing laboratory (sterile where appropriate) include:

- Bacterial tester strains
- Bacteriological plastic loops
- Crystal violet, ampicillin, and tetracycline in discs or as stock solutions
- Culture flasks
- Disposable bacteriological swabs
- Membrane filters, a range of 0.2 μm filters for aqueous solutions and organic solvents
- Minimal glucose agar (MGA) (plates) Minimal glucose master plates with appropriate antibiotics
- Nutrient agar (plates)
- Nutrient broth
- Phenotype test plates
- Phosphate buffer 0.2 M, pH 7.4
- Pipette tips
- Positive control and diagnostic mutagens
- S9 fraction and cofactors
- Solvents including appropriate anhydrous organic solvents; DMSO in particular is hygroscopic and can develop mutagenic impurities in the presence of small amounts of water. Pure organic solvents should be maintained in an anhydrous condition by addition of a small quantity of a compatible predried molecular sieve (type 4A in the case of DMSO) and stored well-sealed over anhydrous silica gel.
- Spectrometer cuvettes, 1 mL disposable
- Top agar (with and without histidine, biotin, and tryptophan)
- Tubes (disposable glass sample tubes, 13 mm diameter).

Radiation-sterilized standard polystyrene Petri dishes (nominal diameter of 90−100 mm) should be used because ethylene oxide sterilization can leave mutagenic residues. These are used to prepare the bottom agar (MGA) plates. These or six-well MGA plates can also be used to prepare plates for phenotypic testing.

4.6 Reagents and Recipes

The following reagents can be purchased from commercial suppliers such as Moltox or manufactured in-house, in which case we suggest that each recipe should be prepared using a form (an appendix to the Standard Operating Procedure) so that appropriate details including supplier, batch number, and amounts of components can be maintained. It is convenient to create a template for each class of reagent (e.g., solution, plated medium).

In the following recipes, water refers to deionized reverse-osmosis purified water; other forms of purified water including distilled water may be used. Autoclaving is generally for 15 min at 121°C, but large volumes of liquid require a longer time to ensure sterility. Filter-sterilization normally involves the use of a 0.22 μm filter. Reagents should be labeled with identity, preparation date (or batch number), and expiration date.

A calibrated peristaltic pump can be used to dispense media including bottom and top agar into dishes. Laboratories using large numbers of plates should consider purchasing an automatic plate pourer (e.g., Eppendorf, Integra Biosciences AG, Microbiology International), which can be directly linked to sterilizing and plate-labeling systems for sequentially numbering plates. Pre-poured plates can also be purchased from commercial suppliers. Plates should be labeled on the side (e.g., using an indelible marker pen) using a code that corresponds to the study design document to avoid mix-ups or interference with automatic colony counters when labeled elsewhere. Petri dishes/plates have numerous variations, including venting (present or absent), size (internal diameter), ridging around the edge of the plate (stackable vs. slippable), and tabs on the base that can interfere with automatic colony counters; it is a good idea to qualify a specific source and type of plate and then consistently use that to avoid problems. After cooling and a drying period to minimize condensation, plated media can be stored in the plastic bags and boxes in which the original empty plates arrived; care should be taken when using other plastic bags because of potential transfer of toxic impurities into the agar. Pourite (Aurical Company, San Mateo, CA) can be added to semi-solid (agar) media at 100 μL/L to disperse bubbles, which can be a problem with an automatic dispenser. If the poured plates are obtained from a commercial supplier, they can become contaminated during shipment, especially when condensation is present; within 1 or 2 days of arrival, check for and discard any showing microbial growth.

To make the required volume of the reagents listed here, multiply each component by an appropriate constant proportion (e.g., 5 if the recipe describes how to make 1 liter and you need 5 liters). Expiration dates are based on the date of preparation and should take into account the expiration dates of individual components. Expiration dates can be extended provided that results are available in the laboratory to prove the reagent is still fit for its purpose.

Filter paper discs used to test sensitivity/resistance to antibiotics can be purchased or cut from Whatman filter paper No. 1 using a 7-mm hole punch. The absolute amount added to

the disc should be optimized so that an appropriate ring of inhibition is formed around the disc in the sensitive strains under the conditions used in the laboratory—amounts indicated herein are for guidance.

Note that some laboratories routinely place antibiotic-resistant strains (e.g., TA98, TA100, TA102) in medium containing appropriate antibiotics, but this does not seem to serve any practical purpose because the bacteria are checked for resistance when they are isolated or subcultured.

4.6.1 Ampicillin 2 μg/disc

Ampicillin sodium salt is dissolved in water at 5 mg/mL and then filter-sterilized. The solution can be stored in a refrigerator for up to 1 year. This solution is diluted to 200 μg/mL with sterile water and then spotted at 10 μL per filter disc to check the strains for the R-factor plasmid pKM101 that confers resistance. The ampicillin discs can be stored in the refrigerator or freezer under desiccating conditions for up to 1 year.

4.6.2 Biotin 0.37 mg/mL

Add 1 liter of water to an appropriate container and heat to boiling point with stirring. Add 370 mg of D-biotin and stir until it is dissolved. The solution should be sterilized by autoclaving or filtration (0.22 μm) and stored at room temperature in ambient light for up to 1 year.

4.6.3 Crystal Violet 5 μg/disc

Crystal violet is dissolved in water at 5 mg/mL and then filter-sterilized. The solution can be stored in darkness in a refrigerator for up to 1 year. The solution is diluted to 500 μg/mL with sterile water and then spotted at 10 μL per filter disc to check strains for the rfa deep rough mutation conferring sensitivity in all *Salmonella* strains. The discs can be stored in the refrigerator for at least 1 year [20].

4.6.4 Glucose 0.4 g/mL

Add approximately 700 mL of water to an appropriate container. Add a magnetic stir bar and 400 g D-glucose in increments while continuously stirring, allowing the sugar to dissolve between additions. Once all the glucose is in the solution, make up to the final volume with water and then filter-sterilize into sterile containers. The solution can also be sterilized by autoclaving, but this tends to cause carmelization, which leads to a slight increase in spontaneous revertant counts. The solution can be stored in a refrigerator for up to 6 months.

4.6.5 G6P 1M: *Glucose-6-Phosphate*

The solution is prepared by dissolving G6P at 260 mg/mL of water; if the sodium salt is used, then it should be dissolved at the rate of 282 mg/mL. Filter-sterilize and then store in a freezer. This expires after 1 year.

4.6.6 HBT: *500 μM Histidine, 500 μM Biotin, 500 μM Tryptophan Solution*

Combine the following volumes of solutions in a measuring cylinder:

- Histidine HCl.H_2O 5 mg/mL, 21 mL
- Biotin 0.37 mg/mL, 333 mL
- Tryptophan 5 mg/mL, 20 mL
- Make up to 1 liter with water

Autoclave or filter-sterilize. The solution is stored at room temperature in ambient light and expires after 1 year.

4.6.7 *Histidine HCl.H_2O 5 mg/mL*

Add 1 liter of water in an appropriate container; then, add 5 g of L-Histidine.HCl.H_2O and stir until dissolved. The solution should be sterilized by autoclaving or filtration and stored in a refrigerator for up to 1 year.

4.6.8 *KMg*

- Potassium chloride (KCl, formula weight 75), 124 mg
- Magnesium chloride ($MgCl_2.6H_2O$, formula weight 203), 81 mg

Each mL of solution contains KCl and $MgCl_2.6H_2O$ in the proportions shown here. Dissolve the salts in water (80% of the final volume) and then make up to full volume with water. Autoclave or filter-sterilize the solution and then store at room temperature in ambient light for up to 1 year.

4.6.9 *MGA Plates*

This agar contains glucose at a final level of 0.4% and is suitable for all tester strains; higher glucose levels may inhibit the growth of TA97a [21]. Other types of agar may be suitable but, because the type of agar can affect the negative/vehicle control count, it is best to use only one type.

Add 15 g Bacto™ agar (BD) and a magnet stir bar to 920 mL water in a 2 L glass Erlenmeyer flask, cover with aluminum foil, and then autoclave. The remaining procedures should be performed using aseptic technique in a laminar flow cabinet. When the solution has cooled to approximately 65°C, gradually add 20 mL VB salts 50 × in increments while stirring, allowing the salts to dissolve completely between additions, and then add 10 mL glucose 0.4 g/mL and stir for at least 1 min. Maintain molten at a temperature of approximately 45°C on a hotplate and dispense 25 mL aliquots into 90−100 mm diameter plastic Petri dishes. Leave the plates on a level surface at room temperature while the agar gels. Allow the plates to cool overnight and then store them inverted (agar side uppermost) in the plastic bags in which the plates were supplied in a refrigerator for up to 6 months (or until they show signs of drying if earlier). Allow the plates to dry at room temperature for 3 days prior to bagging them if excessive condensation is found after overnight cooling/drying.

4.6.10 Minimal Glucose Master (MGM, MGMA and MGMAT) Plates

This agar is similar to MGA but is supplemented with excess histidine, tryptophan and biotin at 333, 333 and 3 μM respectively to allow growth of all the standard (auxotrophic) tester strains. To select for bacteria with appropriate plasmids, ampicillin should be added for the pKM101 strains (including WP2 uvrA pKM101, TA97, TA98, TA100 and TA102); in addition, tetracycline should be added for the pAQ1 plasmid containing strain TA102 − refer to Figure 4.8.1 for plasmid details.

Add 15 g Bacto™ agar (BD), 920 mL purified water, 1.4 mL histidine 5 mg/mL, 2 mL biotin 0.37 mg/mL, 13.6 mL tryptophan 5 mg/mL, 920 mL purified water and a stir bar to a 2 L glass Erlenmeyer flask, cover with aluminum foil, and then autoclave. The remaining procedures should be performed using aseptic technique in a laminar flow cabinet. When the solution has cooled to approximately 65°C, gradually add 20 mL VB salts 50× in increments while stirring, allowing the salts to dissolve completely between additions, and then add 10 mL glucose 0.4 g/mL and stir until homogenous. While the medium is still warm and before the agar starts gelling, dispense 100 mL aliquots into sterile labelled glass bottles. This medium expires after 6 months.

To use for non-plasmid strains, melt the MGM medium in a boiling water bath or microwave then, once cool enough to handle, dispense 25 mL aliquots into standard sterile petri dishes. For the pKM101 strains, melt the medium then, once it has cool enough to handle, add 5 mL ampicillin 5 mg/mL and (for TA102) 333 μL tetracycline 6 mg/mL before dispensing as above. Allow the plates to gel on the level surface of the laminar flow cabinet until cool then replace the lids. Label the plates then store them inverted (agar side up) in the plastic bags in which the dishes came with a small perforation to avoid sweating then store them refrigerated for up to 3 months (MGM) or 1 month (MGMA and MGMAT).

4.6.11 NADP 0.1 M

Dissolve β-nicotinamide adenine dinucleotide phosphate and sodium salt (formula weight 765) at 76.5 mg/mL of water. Filter-sterilize and then store refrigerated in the dark and use on the day of preparation.

4.6.12 Nutrient Agar Plates

The manufacturer of the agar and nutrient broth is not critical; therefore, other equivalent types may be substituted.

Add 15 g Bacto™ Agar (BD), 25 g Oxoid nutrient broth no. 2 powder, and a magnet stir bar to 1000 mL water in a 2-L glass Erlenmeyer flask. Cover with aluminum foil. Stir for a few minutes and then autoclave. Stir to ensure homogeneity and then dispense 25 mL of the mixture into each Petri dish. Leave the plates on a level surface at room temperature while the agar gels. Allow the plates to cool overnight and then store them inverted (agar side uppermost) in the plastic bags in which the plates were supplied in a refrigerator for up to 6 months (or until they show signs of drying if earlier).

4.6.13 Nutrient Broth

Put 1000 mL of purified water into a 2-L Erlenmeyer flask. Add 25 grams of Oxoid nutrient broth no. 2 powder while stirring. Once dissolved, dispense aliquots of the solution into appropriate glass bottle(s) and autoclave at 121°C for 15 min. The solution can be kept at room temperature for 1 month.

4.6.14 Phosphate Buffer 0.2 M pH 7.4

This solution is used to make the S9 mix. It is diluted with an equal volume of sterile water for use as the 0S9 buffer for those plates treated in the absence of S9 mix.

Mix the following two solutions in the proportions shown:

- Sodium dihydrogen phosphate (NaH_2PO_4) 0.2 M, 146 mL
- Disodium hydrogen phosphate (Na_2HPO_4) 0.2 M, 854 mL

Confirm that the pH is in the range of 7.3−7.5 and then autoclave or filter-sterilize it. Store at room temperature in ambient light. This expires after 1 year.

4.6.15 Positive Control and Diagnostic Mutagen Solutions

Stock solutions of 2AA, 2AF, 2NF, 9AC, BaP, DAN, DMBA, MC, MMC, NaAz, and NQO can all be prepared in DMSO in advance of use and then aliquoted in convenient amounts

before storing in the freezer for up to 18 months [22] – see section 4.8.6 *Diagnostic Mutagen Test* for a list of chemical names and suggested concentrations. Alternatively, stocks of MMC and NaAz can also be prepared in water. Other positive controls may be substituted when convenient. Volatile positive controls should be avoided because of potential contamination of the incubator.

4.6.16 S9 Fraction

Rats (usually Sprague-Dawley outbred) are pretreated with Aroclor 1254 or phenobarbital plus β-naphthoflavone to promote the levels of xenobiotic metabolizing enzymes [23–25]. S9 fraction is conventionally prepared by homogenization of liver in isotonic 0.15 M potassium chloride at a rate of 1 g wet tissue per 3 mL; after separation by centrifugation, the S9 fraction may be standardized based on protein content by further dilution in potassium chloride solution. Rarely, S9 preparations from other species (e.g., pooled human liver when testing compounds with known human-specific metabolism) or even other tissues may be included when appropriate and justified [26,27]. For benzidine/azo-dyes and diazo-compounds, a reductive metabolic activation system using hamster liver S9 should be included [28–30]. When preparing S9, it is critical for all solutions and labware to be sterile for the liver to be removed under clean conditions by a trained technician.

Most laboratories purchase precertified S9 fraction from a commercial source to avoid issues with handling animals, Aroclor (polychlorinated biphenyls are banned by some countries and some individual companies) and additional biochemical assays. Commercial S9 fraction can be obtained in frozen or lyophilized form; in addition, lyophilized preformulated S9 mix is available from Moltox. Frozen S9 should be thawed entirely immediately before use and mixed well. Thawed S9 degrades fairly rapidly so any excess should be discarded and should not be refrozen for later use.

4.6.17 S9 Mix

The final concentration of S9 fraction in the S9 mix is usually 10% v/v (termed 10% S9); other percentages of S9 may occasionally be appropriate, in which case the volumes of S9 fraction and water should be adjusted. S9 mix also contains the following "cofactors": 8 mM $MgCl_2$, 33 mM KCl, 100 mM sodium phosphate buffer pH 7.4, 5 mM glucose-6-phosphate, and 4 mM NADP [23]; therefore, each mL of 10% S9 mix contains:

- Water, 0.335 mL
- Phosphate buffer 0.2 M pH 7.4, 0.500 mL
- NADP 0.1 M, 0.040 mL
- G6P, 0.005 mL
- KMg, 0.020 mL
- S9 fraction, 0.100 mL

All components should be sterile and added aseptically in the proportions and order listed to a sterile container on ice, kept on ice or refrigerated, and used on the day of preparation.

Unused S9 mix should be discarded and not frozen for future use.

4.6.18 Tetracycline 1 μg/disc

Tetracycline hydrochloride is dissolved in water at 6 mg/mL and then filter-sterilized. The solution is stable when stored in darkness in a refrigerator for up to 1 year. The solution is diluted to 0.1 mg/mL with sterile water and then spotted at 10 μL per filter disc to check for the presence of the pAQ1 plasmid, which confers resistance in *Salmonella* strain TA102.

4.6.19 Top Agar Incomplete: TAI

This is used to make the TAI and, with appropriate supplementation, may be used for phenotype testing.

Add 6 g agar directly to a glass bottle (the bottle size should be $\sim 2\times$ the solution volume), followed by 1 liter of 0.5% w/v sodium chloride solution. Autoclave and, before the agar sets, mix the contents well by swirling.

Store at room temperature in ambient light. Solution expires after 6 months. Before use, the top agar should be melted in a boiling water bath or microwave and then mixed well by swirling.

4.6.20 Top Agar Complete: TAC

This is the top agar used in a routine bacterial mutation system; it contains histidine, biotin, and tryptophan all at levels of 4.5 μM. Although *Salmonella* strains do not require exogenous tryptophan and *E. coli* strains do not require exogenous histidine or biotin, there is less chance of an error if this type of top agar is used for all routine tests.

If necessary, melt the TAI in a boiling water bath or microwave. Add 100 mL HBT per liter of TAI. Normally, the agar is kept molten and used on the same day, but it can be stored at room temperature in ambient light for up to 3 months. Before use, you should ensure that the medium is uniform and completely molten (with no waves/Schlieren pattern) by swirling the bottle. If necessary, continue heating and re-mix until homogenous. Equilibrate in a 45°C water bath before dispensing.

4.6.21 Tryptophan 5 mg/mL

Add 1 liter of water to an appropriate container. Add 5 g of L-tryptophan and mix until it dissolves. The solution should be sterilized by autoclaving or filtration (0.22 μm) and stored in a refrigerator for up to 1 year.

4.6.22 VB Salts 50 ×: Vogel-Bonner Salts

For each liter add approximately 700 mL of water in an appropriate glass container. Heat the water to 45°C using a heated stirring plate and then add salts to the water in the following order while stirring, allowing each salt to dissolve completely before adding the next:

- 10 g magnesium sulfate ($MgSO_4.7H_2O$)
- 100 g citric acid monohydrate ($C_6H_8O_7.H_2O$)
- 500 g potassium phosphate dibasic (K_2HPO_4)
- 175 g sodium ammonium phosphate ($NaNH_4HPO_4.4H_2O$).

Bring the solution to volume with water, mix thoroughly, and then aliquot into appropriate glass bottles. Sterilize the solution by autoclaving and store at room temperature for up to 1 year.

4.7 Suggested Phases in Development of the Test

For laboratories that have not worked on a particular version of the bacterial mutation test before, development may be conveniently divided into four phases:

1. Research: reading literature associated with the assay, particularly the originators of the method, regulatory guidelines, and the associated formative guidance/papers.
2. Setup: optimizing experimental conditions so that high-quality and reproducible results can be obtained. This phase is necessarily non-GLP because the methodology is in the process of being standardized and involves building up stocks of purified and characterized strains, optimizing conditions of bacterial growth, organizing dosing and plating procedures, establishing dose response to routine positive controls, and preparing documentation including instructions used to perform various parts of the test and to record important details so that (in the event of any unexpected results) the effect of potential variables can be assessed. The results of any set-up work should be recorded directly in the raw data, together with any conclusions and recommendations. It is useful to archive the files of this and the subsequent internal validation electronically for potential reference.

3. Internal validation: providing adequate proof that reliable and reproducible results can be produced in the laboratory using the procedures refined during the set-up stage. In a laboratory where subsequent studies will be used for regulatory submission, this phase is expected to be fairly extensive and will include several experiments performed under GLP (with formal protocols and reports) to help generate adequate control databases. Negative (as well as vehicle) control treatments should be included in every experiment to confirm the absence of solvent effects and to rapidly develop a meaningful laboratory control database. Dose-response curves for positive controls and diagnostic mutagens should be generated and limits of toxicity (maximum nontoxic dose volume) should be established for common vehicles/solvents in plate incorporation and preincubation versions of the test. Chemicals from representative classes of mutagen with different physical properties (e.g., poorly soluble, volatile) should be examined. Any paperwork generated including reports should be formally reviewed by the responsible scientist. Then, in the case of GLP facilities and after auditing, it should be archived and readily available for potential inspection by sponsors and regulatory agencies.
4. Routine maintenance and testing: all aspects should follow established SOPs. All checked/audited control results generated (except those from invalid experiments) should be added to the laboratory historical control database.

4.8 The Bacterial Strains

Although the bacterial mutation test is often referred to as a rapid method for evaluation of mutagenicity, it is not necessarily as straightforward as it first appears, partly because of the ongoing effort needed to maintain, monitor, and assess multiple strains for routine testing. Strains obtained externally or that have been rederived must be purified, characterized for appropriate phenotypic characteristics, must have sensitivity to selective (diagnostic) mutagens confirmed, and must be maintained appropriately to provide reliable and reproducible results. The growth characteristics of each strain should be established so that appropriate density working cultures can be prepared from frozen stocks for routine testing using convenient standardized conditions. The frozen stocks should be divided into working cultures (i.e., those used to prepare suspensions for routine testing) and master stock cultures (these are sometimes referred to as master permanents). Master stocks are only used to generate fresh working and master stocks once these become depleted. Details of the suggested procedures are given in the subsequent sections and summarized graphically.

The strains listed here are those mentioned by international guidelines and are those most commonly used for routine testing.

4.8.1 Genotypes of Routinely Used Strains

See Table 4.1.

Table 4.1

Strain Designation	Target Mutation	Repair Deficiency	LPS	Plasmids	Main Mechanism, Sensitivity
S. typhimurium					
TA1535	hisG46	uvrB	rfa		Most base pair substitutions
TA100	hisG46	uvrB	rfa	pKM101	
TA1537	hisC3076	uvrB	rfa		Frameshift, intercalation
TA97a	hisD6610	uvrB	rfa	pKM101	
TA1538	hisD3052	uvrB	rfa		Frameshift
TA98	hisD3052	uvrB	rfa	pKM101	
TA102	hisG428		rfa	pKM101 pAQ1	Base substitution, small deletions, cross-linking, and oxidizing agents
E. coli					
WP2 uvrA	trpE	uvrA			Base substitution
WP2 uvrA pKM101	trpE	uvrA		pKM101	

The uvrB deletion extends through the gal, nitrate reductase (chlorate resistance), and biotin (bio) genes and, like the uvrA mutation in *E. coli*, prevents (relatively error-free) nucleotide excision repair. The pKM101 gene enhances mutability by coding for umuD in an error-prone repair pathway that is otherwise absent in *Salmonella* strains. TA102 was constructed by deletion of the hisG gene and introduction of the pAQ1 plasmid carrying the mutant hisG428 gene; each bacterium contains approximately 30 copies of the plasmid, making the strain much more sensitive to mutagens because a back-mutation of just one copy of the gene will restore the his^+ phenotype [31,89].

Although multiple modes of reversion occur in all strains, each strain has a particular mutagen-susceptible sequence (hotspot); hotspots involve repeat sequences in hisD3052, hisD6610, and the A/T-containing codon of hisG428 and the G/C-containing codon of hisG46. The two *Salmonella* hisG46 strains in combination with TA102 or a WP2 strain are reverted by all six possible base substitution mutations [11].

4.8.2 Obtaining the Tester Strains

The *Salmonella* strains could originally be obtained directly from Dr. Ames' laboratory at the University of California at Berkeley; however, that laboratory has not maintained them for many years. Tester strains can be obtained from commercial suppliers or repositories such as: Molecular Toxicology Inc. (Moltox); ATCC; the National Collection of Industrial, Food, and Marine Bacteria (www.ncimb.com); Aberdeen, Scotland, UK (*E. coli* strains and WP2

derivatives); BCCM (Belgian Co-Ordinated Collections of Micro-Organisms); or from a reputable laboratory routinely performing tests in a GLP environment. The bacteria may be classified as potentially hazardous etiologic organisms (e.g., UN No. 3373 biological substance category B); therefore, even though they are attenuated, they may need an import license depending on the country.

Whatever the source, the provenance of the organisms is unlikely to be assured. These strains have been maintained and subcultured over an extended period of time during which they may have become mixed with other strains, mutated, lost or gained plasmids, or have undergone genetic drift. Therefore, any newly arrived strain should be purified and then thoroughly checked for appropriate characteristics as described later. If the organisms have been obtained from a reputable source, then they will have already performed these procedures themselves on a regular basis. If available, details of provenance, passage number, historical control counts, and quality control statements should be obtained from the supplier. These records and subsequent details relating to strain purification, maintenance, storage, subculturing, characterization, and utilization should be maintained in a Laboratory Bacterial Strain Maintenance Log.

Five complementary strains of bacteria are used in a routine study:

1. *S. typhimurium* TA1535
2. *S. typhimurium* TA1537 or TA97a (repurified/rederived form of TA97)
3. *S. typhimurium* TA98
4. *S. typhimurium* TA100
5. *E. coli* WP2 uvrA, *E. coli* WP2 uvrA pKM101, or *S. typhimurium* TA102.

When testing suspect cross-linking mutagens, it is preferable to include a DNA repair-proficient strain such as *S. typhimurium* TA102 or *E. coli* (WP2 or WP2 pKM101) if they are not routinely included. Other strains not mentioned here have been developed with increased sensitivity or to test for specific mutations, or have been engineered with mammalian metabolic enzymes [32,89]; these strains are not generally used for routine screening or regulatory studies.

4.8.3 Receipt of Bacterial Strains

Bacterial cultures may be obtained in freeze-dried (lyophilized), frozen, or liquid suspension forms or on semi-solid agar medium. Shortly after receipt bacteria should be grown up on supplemented minimal glucose master plates containing appropriate antibiotics (for the plasmid-containg strains). This and the isolation procedures described below ensure enventual healthy growth of the bacteria on MGA plates, the presence of the appropriate plasmid and the purity of the cultures. Lyophilized cultures should be reconstituted in nutrient broth and then streaked out onto (minimal glucose) master plates, i.e. MGM, MGMA

or MGMAT depending on the plasmid content of the strain — see section 4.6. If unopened, frozen and lyophilized cultures can be stored for up to 2 years when stored at ≤−70°C and 4°C, respectively. When convenient, frozen suspensions should be thawed rapidly (e.g., by shaking gently in a 37°C water bath) and then immediately streaked out on appropriate master plates (MGM, MGMA or MGMAT). Liquid cultures should be streaked out upon receipt. Cultures on agar can be stored in a refrigerator for a few weeks or even months if the culture medium does not dry out; however, viability/recovery of organisms stored at 4°C is expected to decline relatively quickly and mutations may accumulate. Bacteria obtained as colonies on semi-solid medium (i.e., as streak, stab, or slopes/slants) should be streaked out as soon as practical; the original material can be kept refrigerated until appropriate stocks of the strains have been built up. Streaks to isolate and purify bacteria prior to characterization may be prepared using a printout of the streak template shown in Figure 4.2 as a guide.

Place the master plate on the template and then load a small amount of the bacterial culture/suspension onto a sterile plastic loop; use this to prepare the streaks in section 1 (1A, then 1B, etc.), use a fresh loop to prepare streaks in section 2, and then use the other side of the loop for streaks in section 3; repeat with a fresh loop for sections 4 and 5. Alternately, a wire loop may be used; the loop should be sterilized before use and prior to streaking each of sections 2 to 5. Wire loops can be dry heat−sterilized using a gas or spirit burner or by infrared using a Bacti-Cinerator and then cooled by touching the surface of the agar before each use.

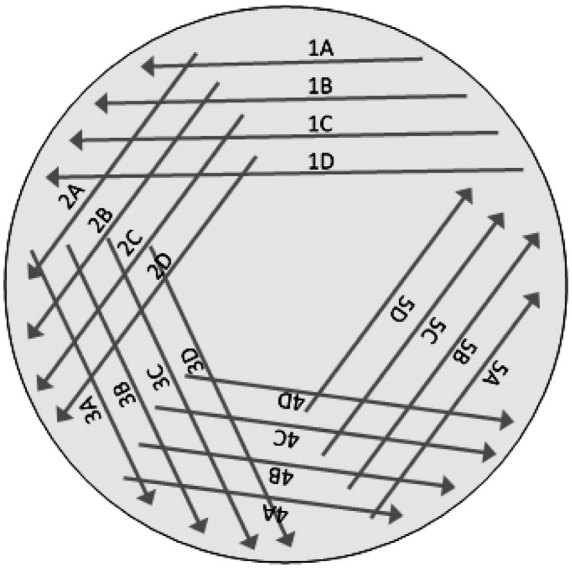

Figure 4.2
Streak template.

The procedure and source of each strain should be recorded in the Maintenance Log; plates should be labeled with strain, date, and study number, which cross-references the paper records. The streaked agar plates should be inverted, incubated for 48–72 h at 37°C, and then stored at 4°C in plastic bags so the medium does not dry out or develop excessive condensation. Discrete isolated colonies will appear in lower number sections of the plate depending on the number of bacteria transferred to section 1 and the type of loop used (wire or plastic).

Take 10 isolated cultures from each strain and streak out each on a master plate labeled with strain, date, study reference, and isolate serial number (1–10). Incubate these master plates at 37°C for 2 to 3 days; then, if not used immediately, store them refrigerated for up to an additional 2 days.

4.8.4 Phenotyping of New Isolates

Routinely, all 10 isolates of each required strain should be characterized. Subsequently, two will be selected for freezing and diagnostic mutagen testing to confirm their suitability.

1. First thing in the morning, pick off a whole isolated colony from the master plate of each of the 10 isolates, use it to inoculate a standard 25 or 30 mL suspension culture, incubate it for approximately 5 hours until there is a density of at least 1×10^9 viable bacteria per mL, as described in Appendix 1, and then keep it at room temperature. Meanwhile, return the master plates to the refrigerator.
2. Label seven plates per isolate *A* to *G* together with strain, isolate number, and date. These plates will be used to test the following characteristics:

Plate Label	Test
A	Crystal violet, ampicillin, and tetracycline resistance
B	UV sensitivity
C	Histidine requirement
D	Tryptophan requirement
E, F, G	Spontaneous reversion rate

3. For each culture, proceed as follows:
4. Label six disposable sterile glass 13 × 100 mm tubes with the letters *A* to *G*. Place the tubes in a 45°C heating block.
5. For tubes *A* to *D*, add 2 mL molten TAI (see Section 4.6) followed by 100 μL 0.5 mM biotin. Add 100 μL 50 mM histidine to tubes *A*, *B*, and *C*, and then add 100 μL 50 mM tryptophan to tubes *A*, *B*, and *D*. *Note that these additives are in excess of the limiting requirement.*
6. Dispense 2-mL aliquots of TAC into tubes labeled *E*, *F*, and *G*.

7. Ensure that the bacterial culture is uniform by swirling, dispense 100 μL into each tube of top agar, vortex briefly, and then pour the mixture onto the bottom agar in the corresponding MGA plate. Ensure the top agar is evenly distributed by tilting/rotating the plate and then leave the plate on a level surface to set.
8. Once all the plates for all the isolates have been poured, use sterile forceps to add one of each type (crystal violet, ampicillin, and tetracycline) of filter paper discs to plate *A*. The discs should be approximately equidistant from each other and from the edge of the plate.
9. Mark plate *B* into five zones of approximately equal width with four vertical lines using a marker pen. Place an opaque barrier over the plate, remove the lid, and place it agar-side up under a low-intensity UV source at a standardized height (15 W at 33 cm); alternatively, the lamp in the biological containment cabinet can be used. Turn the UV lamp on, expose the first sector for 2 s, withdraw the barrier to the next mark, and expose for an additional 2 s, repeating for the third and fourth zones before turning the lamp off. In this way zone 1 will have been exposed for 8 s while zone 5 will remain unexposed. Replace the lid. *UV is particularly damaging to the eyes; therefore, UV-blocking glasses should be worn and exposure of personnel should be minimized. The emission intensity of UV lamps reduces over their working life, so the lamp should be replaced when appropriate. Mitomycin C (0.2 μg/disc) sensitivity can be used as an alternate marker for uvr deficiency.*
10. Once all the isolates have been plated and the agar has set, invert the plates and then incubate them at 37°C for 24 h (plates *A*−*D*) or 65 h (plates *E*−*G*).
11. After incubation, the plates can be examined immediately or stored at 4°C for a few days.
12. Assess the growth in plates labeled *A* and record the diameter of any zone of inhibition of growth around the antibiotic discs.
13. For the UV-exposed plates labeled *B*, record the time taken to inhibit growth (inhibition for repair-proficient strains such as TA102 should take much longer).
14. For the histidine- and tryptophan-deficient plates, labeled *C* and *D*, respectively, record the presence or absence of growth.
15. For plates labeled *D*−*F*, check the background lawn using a microscope, where necessary, and record the number of revertants for each plate and calculate mean values. These values are referred to as the spontaneous revertant colony counts and reflect the uninduced rate of reversion. The spontaneous count is the phenotypic characteristic most likely to vary from expected.
16. Compare the results with the expected values based on the following chart; isolates that have values outside of expected ranges should be rejected and discarded. We suggest that each laboratory should take into account its own experience with the acceptance criteria and modify them as appropriate in a formal SOP (Table 4.2).

Table 4.2: Expected results for phenotype plates

Strain	Zone of Inhibition mm			UV Kill Time	Count on MGA Plates
	C	A	T	Seconds	Mean[a]
TA1535	>12	>12	>12	2	5–20
TA1537	>12	>12	>12	2	4–15
TA1538	>12	>12	>12	2	6–24
TA97a	>12	<10	>12	2	70–180
TA98	>12	<10	>12	2	20–45
TA100	>12	<10	>12	2	75–140
TA102	>12	<10	<10	>8	190–400
WP2	<10	>12	>12	>8	20–50
WP2 uvrA	<10	>12	>12	2	20–58
WP2 pKM101	<10	<10	>12	>8	50–130
WP2 uvrA pKM101	<10	<10	>12	2	100–200

[a]These values are based on Charles River, Montreal's control range and those summarized in various review articles [32–35] and depend not only on the strain but also on the glucose content of the plate, the volume of the agar, the growth phase and density of the bacterial suspension used and other factors [36–38]. Ranges within an individual laboratory may vary somewhat from these values and may be narrower than those shown here. Isolates with values close to the middle of the acceptable range should be selected for storage and future use to avoid genetic drift.

Note that, in routine testing experiments, values obtained for vehicle control plates with S9 tend to be slightly higher than those without S9.

4.8.5 Freezing of Selected Isolates

Based on the results of the phenotype tests, at least two appropriate isolates of each strain should be grown. Most of each culture will be frozen while the remainder is used to confirm suitability by checking the vehicle or negative control revertant colony count and responses to diagnostic mutagens.

The suggested procedure for growing selected isolates and freezing is listed here.

1. At least 2 days prior to the anticipated diagnostic mutagen test, restreak the selected isolates on nutrient agar plates using the template as a guide.
2. Two days later (first thing in the morning), take one isolated colony from each streak of the restreaked isolates and resuspend in nutrient broth and culture as described in Appendix 1.
3. Once the culture density has achieved $\geq 1.1 \times 10^9$ bacteria/mL, dilute it to 1.1×10^9 bacteria/mL with nutrient broth in a total volume of 30 mL in a sterile container (e.g., a 50 mL centrifuge tube). Discard any surplus culture material.
4. Dispense one 4 mL aliquot of each isolate into a sterile labeled tube and set aside at room temperature for testing as described in the diagnostic mutagen test in Section 4.8.6.

5. To the remaining 26 mL of each bacterial suspension, add 2.3 mL DMSO while agitating by hand. Dispense in 1 mL aliquots into cryovials labeled with strain, isolate, serial number, and date. Transfer the vials to labeled cartons (cryoboxes or similar) and place in the ultralow freezer (≤70°C) or in labeled canes and then store them in the vapor phase of the liquid nitrogen cell store. When using liquid nitrogen, bacteria are stored separately from cell lines in the vapor phase because the seal of cryovials shrinks at low temperatures, which can allow liquid nitrogen to enter, leading to potential contamination.
6. The cultures should be stored in at least two locations to insure against failure (e.g., running out of liquid nitrogen).

4.8.6 Diagnostic Mutagen Test

Using the 4 mL of liquid culture that was set aside prior to freezing, retest each isolate for phenotypic characteristics as described earlier and plate with selected diagnostic mutagens plus S9 mix or buffer as appropriate in a standard plate incorporation test. Although single or duplicate plates are adequate for testing the mutagenic agents, the DMSO should be tested in triplicate (both with buffer and S9 mix) to give a good idea of the spontaneous rate of reversion in comparison with historical control values and in case of loss of an individual plate (Table 4.3).

Table 4.3: Expected results diagnostic mutagens

		Without S9 Mix				With S9 Mix			
Compound	**DMSO[a]**	**9AC**	**MMC**	**2NF**	**NQO**	**2AA**	**2AA**	**2AF**	**DMBA**
μg/plate	–	50	0.5	1	0.5	1	20	5	20
TA1535	5–25	–	–	–	+	++	++	–	–
TA1537	5–15	++	–	–/+	+/++	+	++	–/+	+/++
TA1538	6–30	–	–	+/++	+/++	++	++	++	+/++
TA97a	70–130	+/++	–	+	–/+	++	++	+/++	+/++
TA98	20–45	–	–	+/++	+/++	++	++	++	+/++
TA100	75–140	–	–	–/+	++	+/++	++	+/++	+/++
TA102	191–400	–	+	–	+	+	+	–	–
WP2 uvrA	25–65	–	–	–	+/++	–	+	–/+	–/+
WP2 uvrA pKM101	100–200	–	–	–	+/++	–	+/++	–/+	+

9AC = 9-aminoacridine; MMC = mitomycin C; 2NF = 2-nitrofluorene; NQO = 4-nitroquinoline-1-oxide;
2AA = 2-aminoanthracene; 2AF = 2-aminofluorene; DMBA = 7,12-dimethylbenzanthracene
– no substantial increase
+ moderate response
++ strong response
[a]Vehicle control tested in the absence and presence of S9.

As with any mutation test, a study design spreadsheet should be generated to specify the contents of each numbered plate and to facilitate calculation and interpretation of results.

The design should include sterility checks of the reagents involved including the top agar, buffer, S9, DMSO, and diagnostic mutagen solutions, i.e., the first 12 plates in the study design. The study design will also indicate the number of MGA plates needed. The rows corresponding to plates 13 to 42 in the example study design are repeated for each isolate tested; the entry in the strain column should indicate the strain and isolate number used. The spreadsheet may also be used to capture the results by direct input or by linking to a data capture system. Depending on the SOPs of the laboratory, the electronic version of the spreadsheet or a printout of the study design should be used to document the procedures and raw data (i.e., the original observations and counts). The bold letters A, B, and C in the spreadsheet example represent the order in which these components are added; the 0 value in the S9 column indicates that buffer is added and the + symbol indicates that S9 mix is used. The abbreviations used in the treatment column are explained later in this section. The spreadsheet should be authorized (signed and dated) by the scientist responsible for the study before use (Table 4.4).

Table 4.4: Study design: diagnostic mutagen test

Plate No.	A Treatment	Dose No.	Dose Vol. μL	Dose μg/Plate	B S9	C Strain	Count	Observations (If Any)
1	–	–	–	–	–	–		
2	Buffer	–	–	–	0	–		
3	S9	–	–	–	+	–		
4	DMSO	0	100	0	–	–		
5	9AC	1	100	50	–	–		
6	MMC	1	100	0.5	–	–		
7	2NF	1	100	1	–	–		
8	NQO	1	100	0.5	–	–		
9	2AA	1	100	1	–	–		
10	2AA	2	100	20	–	–		
11	2AF	1	100	5	–	–		
12	DMBA	1	100	20	–	–		
13	DMSO	0	100	0	0	strain+I		
14	DMSO	0	100	0	0	strain+I		
15	DMSO	0	100	0	0	strain+I		
16	9AC	1	100	50	0	strain+I		
17	9AC	1	100	50	0	strain+I		
18	9AC	1	100	50	0	strain+I		
19	MMC	1	100	0.5	0	strain+I		
20	MMC	1	100	0.5	0	strain+I		
21	MMC	1	100	0.5	0	strain+I		
22	2NF	1	100	0.1	0	strain+I		
23	2NF	1	100	0.1	0	strain+I		
24	2NF	1	100	0.1	0	strain+I		
25	NQO	1	100	1	0	strain+I		

(*Continued*)

Table 4.4: (Continued)

Plate No.	A Treatment	Dose No.	Dose Vol. μL	Dose μg/Plate	B S9	C Strain	Count	Observations (If Any)
26	NQO	1	100	1	0	strain+I		
27	NQO	1	100	1	0	strain+I		
28	DMSO	0	100	0	+	strain+I		
29	DMSO	0	100	0	+	strain+I		
30	DMSO	0	100	0	+	strain+I		
31	2AA	1	100	1	+	strain+I		
32	2AA	1	100	1	+	strain+I		
33	2AA	1	100	1	+	strain+I		
34	2AA	2	100	20	+	strain+I		
35	2AA	2	100	20	+	strain+I		
36	2AA	2	100	20	+	strain+I		
37	2AF	1	100	5	+	strain+I		
38	2AF	1	100	5	+	strain+I		
39	2AF	1	100	5	+	strain+I		
40	DMBA	1	100	20	+	strain+I		
41	DMBA	1	100	20	+	strain+I		
42	DMBA	1	100	20	+	strain+I		

Where strain + I indicates strain plus isolate number (e.g., TA1535.1).

The suggested procedures for the diagnostic mutagen test are outlined as follows:

1. Label the MGA plates with a unique code for the study number (e.g., a single letter identified in the study design) and with the plate number. The plates should be numbered on the side using an indelible marker pen in case of mix-up of the lids.
2. Calculate the required volumes of each reagent including top agar, buffer, S9, and positive controls based on the study design sheet.
3. Store phosphate buffer 0.1 M pH 7.4 in a refrigerator and keep on ice during use.
4. Prepare an adequate volume of Aroclor 1254 or phenobarbital/benzoflavone-induced 10% rat liver S9 mix. Store refrigerated and keep on ice during use.
5. Melt TAC and then place in a water bath set at 50°C to equilibrate.
6. Set aside an aliquot of DMSO to use as the vehicle control for the test. Prepare formulations of the positive control chemicals (diagnostic mutagens) in DMSO and label them as shown here.

   Note that for laboratories performing tests regularly, it is generally convenient to prepare positive control and diagnostic mutagen solutions in bulk and store in appropriate aliquots deep frozen until use. Alternately, positive controls can be purchased in convenient premeasured aliquots.
7. Label individual test tube racks with treatment: DMSO, 9AC, MMC, 2NF, NQO, 2AA 1, 2AA 2, 2AF, and DMBA. For the DMSO rack, add one sterile

disposable 13 × 100-mm glass tube for the sterility check plus six tubes per isolate; for the remaining racks, add one tube for the sterility check plus three tubes per isolate.

8. Dose each tube with 100 μL of the appropriate control (DMSO or positive as per the rack) using a repeating micropipette (if available). Avoid touching pipette tips against sides of tubes.
9. Arrange the set of 12 sterility-testing tubes in a 45°C heating block as shown here.

9 2AA 1	10 2AA 2	11 2AF	12 DMBA	
4 DMSO	5 9AC	6 MMC	7 2NF	8 NQO
1 Blank	2 Buffer	3 S9		

10. Add 2 mL molten TAC to all tubes in the block, vortex each tube in turn, and pour the contents onto the bottom agar of the appropriate MGA plate. Discard the tube and replace the lid on the plate while ensuring the top agar is evenly distributed by tilting/rotating the plate. Leave the plate on a level surface in a clean area while the top agar gels.
11. Arrange the first set of 30 tubes for testing the first isolate in the 45°C heating block as shown here.

DMSO	**2AA 1**	**2AA 2**	**2AF**	**DMBA**
DMSO	**2AA 1**	**2AA 2**	**2AF**	**DMBA**
DMSO	**2AA 1**	**2AA 2**	**2AF**	**DMBA**
DMSO	*9AC*	*MMC*	*2NF*	*NQO*
DMSO	*9AC*	*MMC*	*2NF*	*NQO*
DMSO	*9AC*	*MMC*	*2NF*	*NQO*

12. Add 500 μL buffer to the front three rows of tubes (indicated in italics) and S9 to the back three rows of tubes (indicated in bold).
13. Immediately after, add 2 mL molten TAC to all tubes in the block, then add 100 μL of the appropriate bacterial suspension to all tubes in the block using a repeater pipette, vortex each tube, and pour the contents onto the appropriate plate. Discard the tube and replace the lid on the plate while ensuring the top agar is evenly distributed by tilting/rotating the plate. Leave the plate on a level surface in a clean area while the top agar gels.

    Note that if two technicians are available to perform the assay, it may be more convenient and quicker to add the top agar to the tube using a calibrated dispensing

peristaltic pump operated by a foot switch. The operator then immediately adds the bacterial suspension using a multidose pipette before passing the tube to the second technician who mixes the contents of the tube and then pours and distributes it on the surface of the plate. In this case, the bulk TAC is held at 45°C in a water bath and a hot block is not required.

14. Invert and then place the plates in an incubator set at 37°C.
15. Remove the plates from the incubator after 65 h (three overnight incubations). *The OECD guideline indicates that an incubation time of 48–72 h is suitable; however, we recommend that you use a standard incubation time in your laboratory to allow a more direct comparison with historical control counts. The 65 h period is convenient and allows time for growth of most revertant colonies even in the presence of slight toxicity. Note that the colony count for strains with high spontaneous counts particularly tends to drift up with incubation time.*
16. If necessary, store plates refrigerated up to 3 days before examination.
17. Read and record the results of the phenotype confirmation test as before.
18. For MGA plates, where necessary, check the background lawn using a microscope with a total magnification of approximately 100× (10× objective). *Treatment of the excision repair-deficient strains with mitomycin C will usually result in absence of a background lawn.* Record any relevant comments about the plates directly onto a paper or electronic copy of the study design sheet.
19. Count the number of revertants for each plate using the automatic colony counter and record results in the study design sheet electronically or on a printout. Save the file and, if an electronic signature system is not in place, print the results sheet and sign (raw data).
20. Transfer all documents to the Bacterial Strain Maintenance Log file.
21. The responsible scientist should review the results and authorize rejection and disposal of strains that do not meet acceptance criteria.

After confirmation of suitability the frozen cultures are designated as either test batch frozen permanents (set aside to inoculate working cultures for routine experiments) or master permanents (set aside for long-term storage and subsequently used to generate fresh-frozen permanents).

4.9 Routine Testing

4.9.1 Designing a Study

The most widely used study design for routine assessment of chemicals is outlined in OECD guideline 471. Studies for regulatory submission generally follow this guidance and are performed under GLP conditions, in which case any planned deviation from these practices should be described and scientifically justified in the protocol and report. The potential impact of any unplanned deviation should also be addressed in the report.

The guidelines published by US FDA in the FDA Redbook 2000 [39] and by the US EPA [40] are identical to the international guideline in terms of testing requirements and differ only in terms of layout and, in the case of the FDA, in some aspects of the descriptive information and in the following:

- FDA indicates that no toxicity should be evident at three or more doses in each assay, in each bacterial strain, both with and without metabolic activation. Both FDA and OECD indicate that at least five different analyzable concentrations of the test substance should be used without any indication of what is meant by analyzable. In practice, it is extremely unlikely that a mutagen will be missed if three nontoxic levels are assessed, so it is advisable to include this criterion in any formal protocol.
- FDA indicates that the S9 fraction concentration in the S9 mix should be 10–30% v/v, whereas OECD indicates 5–30%. In practice, most laboratories use 10% S9 by default.
- FDA indicates that detailed information on formulation preparation, storage, and confirmation (where available) should be reported. In practice, this is a GLP requirement and is recommended in any case.

Although the 1997 OECD guideline implies that a confirmatory test is appropriate (in the event of negative results), ICH guidance, which applies to pharmaceuticals to be registered in the United States, Canada, Europe, and Japan and is followed by most other countries, was revised in 2011 and indicates that confirmatory testing is not appropriate in the event of a clear result. Therefore, confirmatory testing is not normally required for submissions involving pharmaceuticals, pharmaceutical impurities, or medical device extracts, except in the case of borderline results. In practice, when an unexpected positive result occurs in a bacterial mutation test, it is usually good practice to investigate the result using appropriate methodology and to preclude the possibility of an error.

4.9.1.1 Metabolic activation system

The S9 mix, used as a model of intact mammalian metabolism, usually contains 10% v/v induced rat liver S9 fraction, although that can be varied between 4 or 5 and 30% depending on the chemical class and guideline being followed.

4.9.2 Test Article Considerations

The bacterial mutation test is used to evaluate a wide range of materials, including organic and inorganic compounds, medical devices, complex mixtures, environmental contamination, pharmaceuticals, household chemicals, impurities, and biological materials. Medical devices are usually extracted and tested as per ISO standards series 10993 (Part 3 Tests for genotoxicity, carcinogenicity and reproductive toxicity and Part 12 Sample preparation and reference materials). The reader should refer to the ISO web page for details of the most recent version of these documents (see https://www.iso.org).

It is important to gather relevant physical and chemical information on the nature of the test article in advance so that appropriate methods of sample preparation and testing are used. At the same time, the chemist involved in the project may be able to give you useful information about potential solvents. This is discussed in part in the Formulation section of this book; therefore, only the test-specific aspects are described here. In addition, despite the efforts of ICH, OECD, ISO, and others, there are national variations and preferences in test requirements, so it is useful to consider the final use of the test article and which regulatory bodies will be involved when designing the study.

For certain chemicals (e.g., pharmaceuticals that have specific effects on mammalian enzymes) it could be argued that the bacterial mutation test is not the most appropriate genotoxicity test; in which case, a mammalian cell mutation study report may be submitted along with appropriate justification for its use. Even so, we suggest that the bacterial test should also be performed because it is generally an expected part of the submission and it is often the most useful test for detection of minor mutagenic components and impurities. Although the test is generally regarded as predictive of long-term adverse effects in humans, the utility and relevance of results obtained with inorganic compounds including metal salts are questionable [41,42].

Regulatory authorities still expect assessment of antibacterial agents even though they may be highly toxic toward the test organisms. In this case it may be appropriate to perform a "Treat and Plate" modification of the preincubation test in addition to the standard test. Using this method, actively growing bacteria are exposed to the test article in suspension for a specified period (often 1 h), centrifuged, washed free of the test material, and plated out in top agar as per the standard method. At the end of the suspension exposure period, the number of viable surviving bacteria is quantified by diluting and plating them in complete medium with excess histidine/tryptophan. In the absence of any effect of the material on bacterial survival, mutagenicity will be evident as an absolute increase in revertant colonies in the normal way. However, at higher dose levels close to the limit of toxicity, the number of induced revertants per billion bacteria (i.e., the induced mutation frequency as opposed to the absolute mutation frequency) must be calculated to determine presence or absence of mutagenicity. The induced mutation frequency is therefore calculated as:

Treated count $-$ vehicle control count $\div 10^9$ survivors per plate.

Note the importance of using actively growing (log phase) bacteria to ensure fixation of mutations and, therefore, sensitivity, as well as the importance of performing accurate dilutions to quantify survival. Because the treat and plate method involves substantially more work than a standard test and may be less sensitive in some cases, it is generally prudent to run a standard plate incorporation test over an extended dose range

(based on available information) to justify following up with this method. For details of the method, refer to Green and Muriel [43] and Mitchell et al. [44].

Biological materials often need special consideration in terms of microbial load: filter-sterilization of formulated material may be appropriate if it is not likely to remove active components. Biological materials that contain amino acids or precursors for histidine or tryptophan can be problematic. In particular, S9 can degrade peptides yielding individual amino acids or oligomers that can be utilized by bacteria in place of histidine and tryptophan. Resulting excessive growth of nonrevertant bacteria leads to a corresponding increase in the number of spontaneous revertant colonies [45]. The increases are typically small, and the problem can usually be readily identified by the increased density of the background lawn. However, particulate material can release high levels of amino acids locally, leading to a densely overgrown area in the immediate area of the particle—such "colonies" can appear identical to normal revertant colonies. When this feeding effect is expected or known to occur, the treat and wash method [46] can be used. This is a minor modification of the treat and plate method mentioned that includes supplementation of bacteria with nutrient broth during the exposure period to enhance growth and sensitivity; it does not require plating for viability because toxic effects are not usually expected with this type of material.

Once bacteria are plated in the top agar, an initial lag occurs before bacteria start multiplying [47]; therefore, the preincubation method is generally preferred for labile/unstable or volatile test agents to ensure exposure of actively dividing organisms. For example, much greater increases in revertant colony counts are obtained with formaldehyde using this method compared with the plate incorporation method.

If the test article belongs to a specific chemical class that gives rise to concern for mutagenicity, it may be appropriate to modify the methodology or the strains tested appropriately; oxidizing or cross-linking agents and hydrazines may justify inclusion of *S. typhimurium* strain TA102, whereas azo-dyes and diazo-compounds, gases, and volatile chemicals and glycosides may require modified methods or metabolic activation preparations. Open source programs (e.g., ToxTree, VEGA, OECD) are available to identify structural alerts in organic chemicals (www.vega-qsar.eu, http://toxtree.sourceforge.net, http://www.oecd.org/chemicalsafety/risk-assessment/theoecdqsartoolbox.htm#Guidance_Documents_and_Training_Materials_for_Using_the_Toolbox). Structural alerts for bacterial mutagenicity are summarized in the Benigni-Bossa rulebase [48]. The VEGA program also identifies specific mutagens with similar alerts that can help determine the appropriate methodology and related positive controls.

A few chemicals are photosensitive (degrade) or are photomutagenic, causing bacterial mutations in the presence of light; laboratories that conduct bacterial mutation tests

routinely should consider incorporating ancillary gold/yellow lighting for routine use during formulation and dosing.

4.9.2.1 Solvent selection

As in all toxicity tests, the test article must be prepared in an appropriate form for dosing, taking into account chemical stability and compatibility of the vehicle with the test system (refer to the Formulations chapter for more details). Aqueous solvents such as water and saline are preferred and can be used at levels up to approximately 1 mL per plate before they interfere with gelling of the top agar. If the test article has low aqueous solubility (i.e., less than 5 mg/mL), then organic solvents are often used at a maximal dose of 100 μL per plate. Relatively nontoxic organic solvents include dimethyl sulfoxide, dimethlylformamide, ethanol, methanol, propanone (acetone), and acetonitrile (see also Maron et al. [49] and Vedmaurthy et al. [50]). These solvents should be used in the anhydrous form to maximize solubility and decrease accumulation of potentially mutagenic degradants. Some organic solvents (especially those that are not water-miscible such as toluene and those used in preincubation versions of the test) are more toxic and the dose volume must be reduced below 100 μL/plate. In such cases, it may be more practical to solubilize the test article in the primary solvent and then prepare dilutions in a less toxic organic solvent for dosing. Appropriate solvents are not expected to affect the spontaneous revertant colony rate substantially at nontoxic dose volumes; nevertheless, inclusion of an untreated control group is advisable if a novel solvent is used. When working with novel solvents, it may be appropriate to perform a preliminary compatibility test with one or two of the strains ahead of the study. We suggest you evaluate a range of likely solvents during the validation phase of any assay that is new to the laboratory.

Although OECD recommends three plates per experimental point in a standard assay, it may be desirable to increase this for the vehicle (e.g., a set of triplicate control plates at the start and end of the assay) if the laboratory has limited historical control data or, as mentioned, if it has not used that solvent previously.

4.9.2.2 Dose volumes

For the assays using standard Petri dishes, usually 100 μL of test solution, 100 μL bacterial suspension (containing approximately $1-2 \times 10^8$ viable organisms), and 0.5 mL of sodium phosphate buffer or S9 mix (the metabolic activation system) are mixed with 2.0 mL of overlay/top agar immediately before spreading on the plate. The volume of the test solution can be adjusted when appropriate as described in the previous section.

4.9.2.3 Dose levels

Typically, the test material is assessed at five highest concentrations up to the limit of toxicity or, if nontoxic, at five concentrations up to the standard limit of 5000 μg/plate;

concurrent solvent and strain-specific positive control groups are always required except in the case of a dose-range–finding toxicity test. Usually, the test material is dissolved or extracted or occasionally suspended in the chosen vehicle and a range of dilutions is prepared in the vehicle so that a standard dose volume (typically 100 μL per plate) can be administered throughout. When, for practical reasons, the dose volume is variable and the solvent is not expected to have a significant effect on the spontaneous revertant count, it is justifiable to use only the maximal dose volume for the concurrent vehicle control. It is acceptable to use a different solvent and dose volume for the positive control articles; they are generally dissolved in DMSO or, occasionally, for a few specific positive controls, water, and administered at 100 μL per plate. There is no need to include a separate vehicle control for the positive control; results for this group are compared to the corresponding test material vehicle control because appropriate vehicles are not expected to have any substantial effect on the spontaneous revertant colony count.

Toxic effects of the test material are normally indicated by the partial (Figure 4.3b) or complete absence of a background lawn (in which case colony counts, if any, should not be reported) or a substantial concentration-dependent reduction in revertant colony counts compared with lower concentrations and the concurrent vehicle control. The interval between each concentration should be approximately half log ($\sqrt{10}$). A smaller interval may be used where scientifically justified; this is often appropriate in the case of confirmatory testing and may be justified when the test article is not expected to be toxic. Results should be recorded and reported for at least the five highest nontoxic levels (where available) along with values for the concurrent vehicle and positive controls. Details of toxic effects should be recorded and reported; details for the lowest toxic level can be included in results tables to justify reported levels.

OECD guideline 471 indicates "If precipitation (insoluble material) is observed, at least one or more concentrations can be assessed," which can be interpreted in several ways. In practice, it is extremely unlikely that dosing at levels above the limit of solubility will lead to a false-positive result, and exposure to soluble metabolites and material impurities can be increased at these levels. Therefore, we recommend that all levels including those showing obvious precipitate should be scored. When precipitate interferes with automatic colony counting, revertant colonies should be counted by eye and that detail should be noted in the raw data. Often, precipitation will obscure the background lawn—this detail should be recorded in the raw data. In such cases, it is reasonable to assume that the background lawn is normal and intact if the colony counts are close to expected based on results for adjacent dose levels and the concurrent vehicle control. A phase contrast microscope should be available in the laboratory to facilitate examination for precipitate and to confirm the condition of the background lawn. Sometimes the peak dose for mutagenic and toxic effects can occur at a level slightly higher than the limit of solubility in the agar when an organic

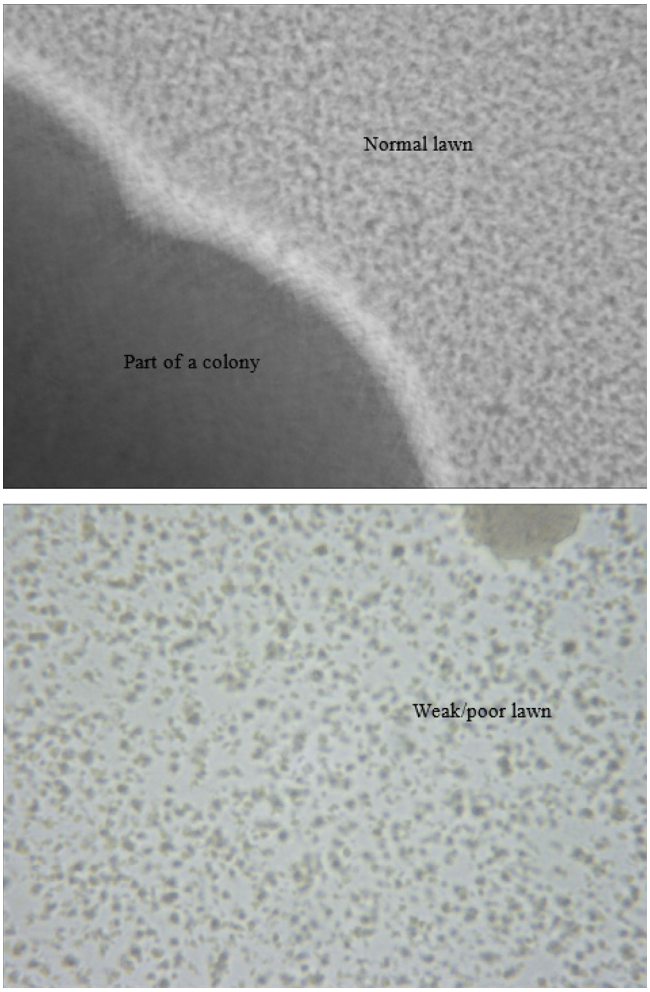

Figure 4.3
Background lawns. The top figure shows a healthy background lawn with part of a normal revertant colony while the bottom figure shows a weak/unhealthy lawn as viwed at low power under a microscope.

solvent is used because the test agent can be present in solution at supersaturated levels during the initial exposure period.

When the test material is in limited supply or when bactericidal effects are suspected, a preliminary toxicity test using one *Salmonella* (often TA100) and one *E. coli* strain (where appropriate) can be used to set dose levels for the subsequent definitive test. In this case, dose levels are usually separated by a factor of 10 and duplicate rather than triplicate plates may be used, although the test material should still be evaluated in the presence and

absence of S9 mix because, even in the absence of significant metabolism of the test article, the S9 often acts as a protective agent raising the limit of toxicity. The top dose for the subsequent definitive phase of testing should be one expected to show some toxic effect; six or seven lower dose levels should be included so the target number of five accessible dose levels is achieved.

In the unlikely event that results are not available for an adequate number of dose levels due to toxicity, a supplementary test should be performed (Table 4.5).

Table 4.5: Suggested standard study design: main test

Dose Level/Treatment	Final Conc. (μg/Plate)	Number of Replicates		Number of Strains
		0S9	+S9	
Vehicle	–	3	3	5
1/Test material	1.58	3	3	5
2/Test material	5.0	3	3	5
3/Test material	15.8	3	3	5
4/Test material	50	3	3	5
5/Test material	158	3	3	5
6/Test material	500	3	3	5
7/Test material	1581	3	3	5
8/Test material	5000[a]	3	3	5
Positive control	[b]	3	3	5

0S9 with buffer in place of S9
+S9 with S9
[a]Or 5 μL/plate, maximum dose recommended by OECD.
[b]Dose depends on the test organism, the positive control chemicals, and methodology used.

Based on this study design, you would expect the study to consist of a total of:

10 (treatments) × 2(S9 conditions) × 5 (strains) × 3 (replicate plates) = 300 plates plus appropriate sterility controls. It is a good idea to check that your study design spreadsheet (see later) results in the same number of plates as calculated in the example shown here to minimize the chance of errors. Although the maximum recommended dose for routine testing is 5000 μg per plate, occasionally this may not be achievable for practical reasons (e.g., solubility in the chosen solvent and limitations on dosing volume). Sometimes, a maximum dose above these standard limits may be appropriate (e.g., when testing mixtures or when qualifying a potentially mutagenic impurity in a drug substance). Justification for solvent and dose level selection should be presented in the report.

In the case of non-pharmaceuticals, confirmation of a negative result using a modified methodology is generally required for a regulatory study. The OECD guideline suggests that concentration spacing, the method of treatment (plate incorporation or liquid preincubation), or metabolic activation conditions may be considered appropriate modifications. In practice, it reduces the overall amount of work done if the confirmatory

test is performed alongside the original test; the standard plate incorporation method supplies the initial test result while the preincubation method provides confirmatory results. The preincubation method has the added advantage that the concentration of the S9 is effectively increased during the initial exposure and there is direct exposure of bacteria to the test article. In this way, an entire regulatory study can be performed in less than 1 week from the date of test article receipt with only one occasion of formulation.

The work involved in both plate incorporation and preincubation versions of the pour plate test is approximately equivalent. For non-GLP-screening tests, we recommend you use the same pour-plate methodology and S9 conditions that will be used in any eventual regulatory study. The preincubation method can be more sensitive to some chemical classes, which may justify its selection if only one methodology is to be used.

4.9.3 Positive Controls

Positive controls are used to confirm the sensitivity of the test system and the metabolic activity of the S9 mix. Because 2-aminoanthracene is activated by cytostolic enzymes [51], and because its activity can be enhanced using low S9 concentrations, it should not be the only indirect (i.e., metabolically activated) positive control used for routine tests. Example positive controls are listed by OECD and elsewhere; note, however, that mitomycin C is not an appropriate control for *E. coli* WP2 uvrA. For routine testing, each strain is evaluated against a single dose level of an appropriate positive control in both the absence and presence of S9. The dose of positive control should elicit a moderate response as established during the set-up stage of the assay in the laboratory. Intercalating agents such as 9-aminoacridine can have a steep dose-response curve with a narrow mutagenic window between no effect and toxicity, whereas others including the poorly soluble polyaromatic hydrocarbons requiring metabolic activation such as benz[a]pyrene can have a relatively flat dose-response.

Suggested routine positive controls for pour-plate methods and guidance dose levels for the commonly used strains are listed in Table 4.6.

Table 4.6

Strain	Compound	Abbreviation	Conc. μg/Plate	S9
TA1535	Sodium azide	NaAz	0.5	0
TA1537	9-Aminoacridine	9AC	50[a]	0
TA97a	9-Aminoacridine	9AC	50[a]	0
TA98	2-Nitrofluorene or	2NF	1	0
	4-Nitro-*o*-phenylenediamine	NOPD	2.5	0
TA100	Sodium azide	NaAz	0.5	0
TA102	Mitomycin C	MC	0.5	0
WP2 uvrA	4-Nitroquinoline-1-oxide	NQO	0.5	0
WP2 uvrA pKM101	4-Nitroquinoline-1-oxide	NQO	0.2	0

(*Continued*)

Table 4.6 (Continued)

Strain	Compound	Abbreviation	Conc. μg/Plate	S9
TA1535	2-Aminoanthracene	2AA	2	+
TA1537	Benz[a]pyrene	BaP	5	+
TA97	2-Aminoanthracene	2AA	2	+
TA98	Benz[a]pyrene	BaP	5	+
TA100	Benz[a]pyrene	BaP	5	+
TA102	Danthron	DAN	25	+
WP2 uvrA	2-Aminoanthracene	2AA	20	+
WP2 uvrA pKM101	2-Aminoanthracene	2AA	20	1

[a]A lower dose of 9AC may be appropriate if a preincubation method is used.

4.10 Standard Test Procedures

The procedures used for routine testing largely follow those described in the DIAGNOSTIC MUTAGEN TEST section. Note that we recommend that laboratories new to bacterial mutation testing should use:

Top agar supplemented with biotin plus limited histidine and tryptophan (TAC)
Bottom agar with low 0.4% glucose (MGA)
Nutrient broth without antibiotics

In this way, the media are suitable for use with any of the tester strains and there is much less danger of a mix-up.

The phenotype and diagnostic mutagen checks, spontaneous revertant colony counts (assuming adequate laboratory data are available for the selected vehicle and dose volume), and viability of the bacteria do not need to be confirmed as part of a routine study provided that these characteristics have been established in advance of the experiment. Instead, vehicle and positive control colony counts and the condition of the background lawn are generally considered adequate internal checks.

Fresh bacterial suspension cultures should be inoculated so that they are in the active growth phase with a density of $1-2 \times 10^9$ bacteria/mL at the time of use as described in Appendix 1 using a combination of OD (optical density) measurement and direct counting. Plating for assessing viability is not particularly useful because results are not available to confirm suitability of the culture until later; therefore, it is only considered necessary when the treat and plate modification of the test is used (refer to Modifications of Standard Methods section later).

4.10.1 Plate Incorporation Method

The general procedures are as detailed in the diagnostic mutagen test. A 0.5 mL aliquot of S9 mix (+S9) or phosphate buffer (0S9) is combined with a standard volume

(typically 100 μL) of the test solution in sterile tubes in a rack. At least the first and last tubes in the rack should be labeled with a number corresponding to the plate number in the experimental design. The tubes can be stored briefly in the refrigerator, and then 2.0 mL of molten TAC followed by 100 μL bacterial suspension are added to each tube in turn; the tube is vortex-mixed briefly and its contents are immediately poured onto the surface of the corresponding MGA plate. The plates are stacked on a level surface.

Although the order of addition may not be critical, adding the components in this order may help minimize toxicity of the test substance and reduce potential carry-over of bacteria—care should be taken to avoid carry-over of chemicals from one tube to another on the pipette tip. Laboratories with high throughput should purchase a metered peristaltic pump (Wheaton, Cole-Parmer) that is set to dispense 2.0 mL top agar every time a foot switch is depressed. Other components can be conveniently added using air-displacement multidose micropipettes unless a volatile or viscous vehicle is used, in which case a positive displacement pipette system should be used. In this way, all the tubes can be dosed with S9 or buffer and test article in the morning. Subsequently, one operator doses each tube with top agar and bacteria before passing it to a second operative who mixes the contents and pours and stacks the plates. With practice using this type of setup, a team of two technicians can handle an experiment involving formulation and approximately 1200 plates in 1 day. Alternately, when only one technician is available, the tubes with test article and buffer/S9 mix can be placed in a heat block set to 45°C and one block of tubes can be treated and plated at a time.

Once all the plates have been poured and the agar has gelled, they are inverted and incubated at 37°C. Although OECD indicates an incubation period of 48−72 h is acceptable, toxicity of the test solution can reduce the growth rate of the colonies, making them more difficult to detect, especially for strains with higher mutation rates that have more, but smaller, revertant colonies [52]. In addition, even for untreated controls, the colony size tends to increase even at the late stage of incubation. Therefore, we recommend standardizing the incubation period toward the end of this range to facilitate detection of all revertant colonies and to minimize variation in historical control counts. A standard incubation period of 65 h is convenient because it allows the plates to be placed in the incubator at 4:00 PM then removed at 9:00 AM 3 days later.

4.10.2 Preincubation Method

Both versions of the pour-plate (plate incorporation and preincubation) method are generally equally acceptable to regulatory authorities and, when testing non-pharmaceuticals, a combination of the methods is expected to capture nearly all bacterial mutagens. The study design for the two methods is identical and the procedure is very similar.

Using the preincubation method, the test solution is added to each tube, and then S9 mix/buffer followed by the bacterial suspension are added. The tubes are incubated at 37°C for a standard period of at least 20 (usually 30) min on the platform of an orbital shaker set to a speed just below that causing foaming (typically 180 rpm) in an incubator set to 37°C. It is convenient to incubate the tubes in batches soon after addition of the bacteria. After preincubation, 2.0 mL of molten top agar supplemented with histidine, biotin, and tryptophan is added to each tube in turn, and the contents are mixed briefly by vortexing and then overlaid onto a minimal glucose plate. After the overlay gels, the plates are inverted and then incubated as in the plate incorporation method.

Sometimes it is necessary to modify the method due to the nature of the test article. Studies using these methods may be fully valid and acceptable for regulatory submission provided that appropriate scientific justification for the modification is presented in the report.

4.10.3 Standard Study Design

The Study Design spreadsheet should be generated from a standard template file with plates numbered sequentially prior to the study to specify the contents of each plate and, in the case of GLP studies, who did what and when. The example in Table 4.7 for a single test article covers the sterility control checks, positive controls, and dose levels 0 (vehicle control), 1, and 2 for the first strains. Dose levels 3−8 follow the same layout as doses 1 and 2 at final concentrations of 15.8, 50, 158, 500, 1580, and 5000 μg/plate (typical dose levels for routine testing); this is repeated with each strain in turn. In this case, the final row of the spreadsheet is used to document the staff involved and dates. The preincubation time column is used only in the preincubation version of the test. Blank cells indicate that the additives are the same as in the cell above. The design can be broken down into sections:

1. The first section includes the appropriate sterility controls
2. Positive controls for all strains (typically 30 plates)
3. Vehicle control and the eight dose levels of test material with and without S9 (54 plates) with the first tester strain repeated for each of the other four strains
4. This is repeated for each additional test material tested concurrently—each additional compound tested adds 270 plates plus appropriate sterility controls except when the same solvent/vehicle is used, in which case it would be $48 \times 5 = 240$ plates for each additional compound plus sterility plate(s).

In the example, test article dose number 0 corresponds to the vehicle control and A, B, C indicate the order of additions. Where more than one test article is being assessed, there is no need to duplicate vehicle or positive controls. If this occurs in GLP studies at a Contract Research Organization (CRO), then the test article name should be replaced by a code name to ensure anonymity in case of client review of raw data.

Table 4.7: Study design, example

Plate No.	A Treatment	Dose No.	Dose Vol. μL	Dose μg/Plate	C Strain	B S9	Preinc Time, From to	Count	Observations (If Any)
1	Untreated	–	–	–	–	0			
2	Untreated	–	–	–	–	+			
3	Test article	0	100	0	–	0			
4	Test article	8	100	5000	–	0			
5	NaAz	1	100	0.5	–	0			
6	9AC	1	100	50		0			
7	2NF	1	100	1		0			
8	NQO	1	100	0.5		0			
9	2AA	2	100	20		0			
10	BaP	1	100	5		0			
11	NaAz	1	100	0.5	TA1535	0			
12	NaAz			0.5	TA1535	0			
13	NaAz			0.5	TA1535	0			
14	2AA	1	100	2	TA1535	+			
15	2AA			5	TA1535	+			
16	2AA			5	TA1535	+			
17	9AC	1	100	50	TA1537	0			
18	9AC			50	TA1537	0			
19	9AC			50	TA1537	0			
20	BaP	1	100	5	TA1537	+			
21	BaP			5	TA1537	+			
22	BaP			5	TA1537	+			
23	2NF	1	100	1	TA98	0			
24	2NF			1	TA98	0			
25	2NF			1	TA98	0			
26	BaP	1	100	5	TA98	+			
27	BaP			5	TA98	+			
28	BaP			5	TA98	+			
29	NaAz	1	100	0.5	TA100	0			
30	NaAz			0.5	TA100	0			
31	NaAz			0.5	TA100	0			
32	BaP	1	100	5	TA100	+			
33	BaP			5	TA100	+			
34	BaP			5	TA100	+			
35	NQO	1	100	0.5	WP2uvrA	0			
36	NQO			0.5	WP2uvrA	0			
37	NQO			0.5	WP2uvrA	0			
38	2AA	2	100	20	WP2uvrA	+			
39	2AA			20	WP2uvrA	+			
40	2AA			20	WP2uvrA	+			
41	Test article	0	100	0	TA1535	0			
42					TA1535	0			
43					TA1535	0			
44					TA1535	+			

(*Continued*)

Table 4.7: (Continued)

Plate No.	A Treatment	Dose No.	Dose Vol. μL	Dose μg/Plate	C Strain	B S9	Preinc Time, From to	Count	Observations (If Any)
45					TA1535	+			
46					TA1535	+			
47	Test article	1	100	1.58	TA1535	0			
48					TA1535	0			
49					TA1535	0			
50					TA1535	+			
51					TA1535	+			
52					TA1535	+			
53	Test article	2	100	5	TA1535	0			
54					TA1535	0			
55					TA1535	0			
56					TA1535	+			
57					TA1535	+			
58					TA1535	+			
Init./date									

4.10.4 Examination of the Plates

After the incubation period, the plates can be examined immediately or stored refrigerated for a few days prior to examination if more convenient. Subsequently, the plates should be stored refrigerated until the results have been compiled and reviewed in case any additional checks are needed. Plates should be evaluated for the quality of the background lawn, the presence of precipitate, and the number of revertant colonies. A microscope should be available to check the quality of the lawn and for the presence of precipitate in case of any doubt following visual examination. Colony counts may be performed "manually" with the aid of a bench-top tally counter and a marker pen. This type of manual counting can be facilitated using a Quebec colony counter illumination system; however, because of the effort involved in manual counting, laboratories performing tests routinely use an automatic colony counter that relies on image analysis to enumerate colonies. Automatic colony counters are generally adjusted to ignore the edge of the plate (which otherwise could be falsely interpreted as bacterial colonies), so a standard mathematical adjustment may be appropriate to provide the equivalent full plate count to allow direct comparison with manual counts. This calculation can be done automatically by systems specifically designed for bacterial mutation testing as follows:

corrected (reported) count = frame count × plate area ÷ frame area

Precipitate, highly colored test article, or minor microbial contamination can interfere with automatic colony counting, in which case a visual count can usually be made.

Depending on the throughput of the laboratory and the GLP status of the test, results may be entered by pen or by direct keyboard entry into the study design, or by direct data capture if an automatic colony counter is used—the latter two methods are preferred because they avoid transcription errors. If precipitation interferes with observation of the background lawn, then this is recorded. Whatever system of data entry is used, it should allow manual input of comments regarding the plates; usually, this is facilitated by the use of standardized abbreviations such as:

cx	microbial contamination obscuring count
c	microbial contamination not obscuring count
il	incomplete lawn (toxicity)
mc	microcolonies (toxicity)
nl	no lawn (toxicity)
poc	precipitate obscuring count
pol	precipitate obscuring assessment of background lawn
ppt	precipitate
vc	visual count
vr	visual recount

4.10.5 Interpretation of Results

4.10.5.1 Evaluation of toxicity

Toxic effects of the test item are normally indicated by the partial or complete absence of a background lawn (colony counts, if any, should not be reported in this case) or a substantial dose-related reduction in revertant colony counts compared with lower dose levels and concurrent vehicle control taking into account the laboratory historical control range; for example, a fold response of less than 0.6-times the concurrent control can be selected empirically to trigger a comment (either considered to be "indicative of toxicity" or, if no supporting information from associated points, "considered to be due to normal variation"). Where precipitation obscures observations on the condition of the background lawn, the lawn can be considered normal and intact if the revertant colony counts are within the expected range based on results for lower dose levels and historical control counts for that strain.

4.10.5.2 Validity of the study

Normally, the bacteria will have been evaluated for appropriate phenotypic markers and response to diagnostic mutagens in advance of use in an individual study. Where these checks are performed concurrently with the study, results for any strain that do not pass the check should be considered invalid.

The spontaneous mean revertant colony counts for each strain should lie close to or within the current historical control range of the laboratory. Note that historical control ranges for

the test can drift over time so, providing that the laboratory has sufficient background data, the historical control range should cover only the past 2 years. At a minimum, the historical data should be from at least 10 and preferably 20 independent experiments [53]; the laboratory will have accumulated at least this number of results during their own internal validation of the test. The chosen positive controls (with S9 where required) evaluated concurrently as part of the study should produce substantial increases in revertant colony numbers with the appropriate bacterial strain.

In the case where part of the study is invalid based on criteria described in the protocol (e.g., the positive control does not induce an appropriate response with an individual strain or there is generally poor growth of the background lawn with that strain), detailed results for that part of the study do not need to be reported. The affected part of the study would normally be subjected to an automatic repeat, in which case a protocol amendment with supporting justification may be appropriate for GLP studies if this scenario is not fully covered in the original protocol.

4.10.5.3 Criteria for negative/positive/equivocal outcome

The mean number of revertant colonies for all treatment groups is compared with those obtained for the concurrent vehicle control level. The mutagenic activity of the test item is routinely assessed by applying the following criteria:

The results are considered positive (i.e., indicative of mutagenic potential) if:

- The results for the test item show a substantial increase in revertant colony counts, i.e., response two-times or more the concurrent vehicle control level values, with mean value(s) outside the laboratory historical control range (beyond the 98% tolerance limit). Otherwise, results are considered negative. *Note that this two fold rule is conventional but somewhat arbitrary: depending on a laboratory's experience with the strain it may be more appropriate to require a higher fold increase for strains with a low spontaneous revertant colony count (e.g., TA1537) and a lower threshold for strains with a high spontaneous reversion rate* (*e.g., 1.5-fold for strains TA97a, TA100, TA102, and WP2 uvrA pKM101). Apparent isolated responses in only a single replicate plate occur only rarely and should be viewed with suspicion; generally, they would be regarded as outliers and reported only in parentheses with appropriate justification for their exclusion from calculation of mean values*.
- The above increase must be dose-related and/or reproducible (i.e., increases must be obtained at more than one experimental point, more than one dose level, more than one occasion, or with different methodologies).

If the second criterion is not met, then the results may be classified as equivocal, and further testing may be appropriate to clarify such results using an appropriately modified study design, (e.g., a narrower dose interval with the appropriate strain) [52]. Parallel

testing with appropriate modifications to the test, e.g., using the preincubation method if the original test used the plate incorporation method or also testing TA1537 if the original apparent effect was seen with TA97a. In such cases, if no substantial increase (as defined) is obtained in the confirmatory test, then the results will be considered negative.

It may be that a consistent apparently treatment-related increase in revertant colony counts is obtained on more than one occasion, but the increase does not meet the two fold criterion described. In this case it may be appropriate to consider the outcome as borderline or equivocal, which would put more emphasis on follow-up testing using relevant (often *in vivo*) systems.

4.10.5.4 Unexpected and borderline results

Certain classes of chemicals (i.e., those with structural alerts or with specific modes of biological activity) can be expected to give positive results in the bacterial mutation test. In some cases, bacterial mutagenicity may be due to formation of unique *in vitro* metabolites or degradants; alternatively, the effect may be bacterial-specific and therefore not considered relevant in terms of hazard assessment. In cases where an apparent effect is unexpected for that class of material, it is advisable to check the reproducibility of the results to preclude any experimental error. This is particularly true when an increase in revertant colony counts is seen in only part of the experiment (e.g., in one strain only in the absence but not the presence of S9) and no evidence of genotoxicity has been obtained in other test systems. In this case, only the affected part of the experiment needs repeating over an appropriate dose range using a narrower dose interval, where appropriate. When testing materials in accord with OECD guideline 471, the results of the confirmatory test are normally expected to be very similar to those seen in the initial test even though slightly modified methods may have been used, (e.g., plate incorporation in the initial test and preincubation method in the confirmatory phase).

If the unexpected increase is found to be reproducible, then consideration should be given to the possible presence of a mutagenic impurity in the test article. In this case, different batches of the test material and a highly purified lot of the test material can be compared using the appropriate strain(s). If a substantial difference in response is seen with different batches, then chemical analysis and examination of the synthetic route intermediates can be used to identify the responsible impurity, which, in turn and if stable, could be isolated or synthesized before testing in its pure form.

4.10.6 Presentation of Results

The report should, at a minimum, include all the items listed in the OECD guideline 471 and FDA Redbook 2000 [17,39]. Results should be tabulated to show individual, mean, and standard deviations for revertant colony counts. Any individual counts that are reported but

not used for calculation of mean values should be indicated in parentheses and a reason for their exclusion should be footnoted in the tables.

Mean colony counts and their SDs should be rounded and presented to the nearest whole number. Fold values should be reported to a minimum number of significant figures except when clarification is required to define borderline results (e.g., 0.3, 1.8, 11, 1.95). Related values such as concentrations should be decimal-aligned in columns (Tables 4.8 and 4.9).

Results should be presented for all evaluated dose levels for each strain/S9 combination plus at least one toxic level (in cases where toxicity has been observed). At least three of the reported dose levels should not show toxic effects (see next section). Results for all three plates at each reported experimental point should be presented; occasional loss of a plate (e.g., due to microbial contamination) is permissible. Individual plate counts may be considered as potentially erroneous if they are well outside expected levels based on historical control values and results for related plates. In such cases, we suggest reporting the colony count in parentheses and excluding the value from any calculation while presenting justification in a footnote to the table. Although OECD mentions the possibility of using duplicate plates, we advise against it because of the greater potential for invalidating results in the event of loss of an individual plate. Although duplicate plates may be justifiable for the positive controls, the potential very minor savings in workload do not seem to warrant their use.

Negative/spontaneous historical control values for relevant experiments performed within the recent past under similar conditions should be presented in detail to give an idea of how often values at the upper limits occur by chance. If values obtained with different methodologies (e.g., with and without S9, using plate incorporation and preincubation methods) are similar, then historical control values can be presented as combined results for the sake of simplicity. In some laboratories revertant counts for some strains may be somewhat higher in the presence of S9. In that case the laboratory should consider presenting without and with S9 historical control results separately once a sufficiently large database has been accumulated. However, all appropriate details including method, dose volume, and vehicle should be recorded in the historical control database in case there is a reason to consider historical control data for specific conditions separately. The following examples give suggested layouts; footnotes that are not used in a particular report can be deleted. Note that certain authorities also require submission of tabulated summary reports (e.g., eCTD as described by ICH) [54]; these may be supplied separately or in the first (summary) section of the report.

The chart (Figure 4.4) is an example of the historical mean revertant colony counts for triplicate plates obtained in previous QA-audited experiments performed between the first and the last experiment. In this example, the grand mean is 14, SD is 4, and 97.5% of the results fall within the range of 7 to 23 revertants per plate (Table 4.10).

Table 4.8: Compound A: plate incorporation test in the absence of S9 mix.

Strain	Concentration (μg/Plate)	Number of Revertants					Plate Observations[a]			Fold Response[b]	
		x_1	x_2	x_3	Mean	SD	x_1	x_2	x_3		
TA1535	0.9% NaCl	27	23	24	**25**	2				1.0	
	15.8	14	18	15	**16**	2				0.6	
	50	12	21	15	**16**	5				0.6	
	158	19	14	(3)[c]	**17**	(4)				0.7	
	500	31	20	23	**25**	6				1.0	
	1580	19	19	16	**18**	2				0.7	
	5000	7	7	—	**7**	(0)			il	0.3	T
TA1537	0.9% NaCl	13	16	22	**17**	5				1.0	
	15.8	14	17	16	**16**	2				0.9	
	50	22	13	14	**16**	5				1.0	
	158	14	22	14	**17**	5				1.0	
	500	29	23	27	**26**	3				1.5	
	1580	24	18	14	**19**	5				1.1	
	5000	—	—	—	—	—	il	il	il		T
TA98	0.9% NaCl	31	34	27	**31**	4				1.0	
	15.8	34	26	30	**30**	4				1.0	
	50	41	38	39	**39**	2				1.3	
	158	71	65	83	**73**	9				2.4	+
	500	143	152	139	**145**	7				4.7	+
	1580	69	77	103	**83**	18				2.7	+
	5000	—	—	—	—	—	il	il	nl		T
TA100	0.9% NaCl	143	103	122	**123**	20				1.0	
	15.8	117	135	110	**121**	13				1.0	
	50	103	148	111	**121**	24				1.0	
	158	111	128	113	**117**	9				1.0	
	500	103	143	122	**123**	20				1.0	
	1580	95	106	110	**104**	8				0.8	
	5000	—	—	—	—	—	nl	nl	nl		T
WP2 *uvrA*	0.9% NaCl	43	46	41	**43**	3				1.0	
	50	40	36	44	**40**	4				0.9	
	158	53	43	59	**52**	8				1.2	
	500	46	50	69	**55**	12				1.3	
	1580	41	63	46	**50**	12				1.2	
	5000	21	20	23	**21**	2				0.5	T

Notes: SD Sample standard deviation;
NA Not applicable
T Toxic as indicated by low revertant colony counts (fold response <0.6) or incomplete/no background lawn (no meaningful count results for plates with il or nl)
L Low count considered due to normal variation rather than toxicity because not clearly dose-related and not outside normal limits based on historical control values
+ Substantial increase in revertant colony counts
[a]Comments on the plate or background lawn: incomplete lawn (il), no lawn (nl), precipitate (ppt), contamination did not obscure count (c), precipitate obscured assessment of background lawn (pol).
[b]Mean revertant count÷concurrent vehicle control value.
[c]Value excluded from the mean because outside expected range based on results for vehicle control and adjacent plates (possible technical error).

Table 4.9: Positive controls for the plate incorporation assay.

Strain	Treatment	Concentration (μg/Plate)	S9	Number of Revertants					Fold
				x_1	x_2	x_3	Mean	SD	Response[a]
TA1535	NaAz	0.5	0	327	328	334	**330**	11	12
TA1537	9AC	50	0	356	379	532	**422**	97	22
TA98	2NF	1	0	142	143	96	**127**	27	4.8
TA100	NaAz	0.5	0	537	503	500	**513**	13	5.0
WP2 *uvrA*	NQO	0.5	0	1180	1201	1170	**1193**	20	27
TA1535	2AA	5	+	247	252	192	**230**	33	15
TA1537	BaP	5	+	104	121	101	**109**	11	6.1
TA98	BaP	5	+	227	263	289	**260**	31	6.2
TA100	BaP	5	+	721	769	749	**746**	24	5.2
WP2 *uvrA*	2AA	20	+	228	227	206	**220**	12	3.8

Notes: SD Sample standard deviation
0S9 Without S9
+S9 With S9
[a]Fold response in mean revertants compared to concurrent vehicle control.

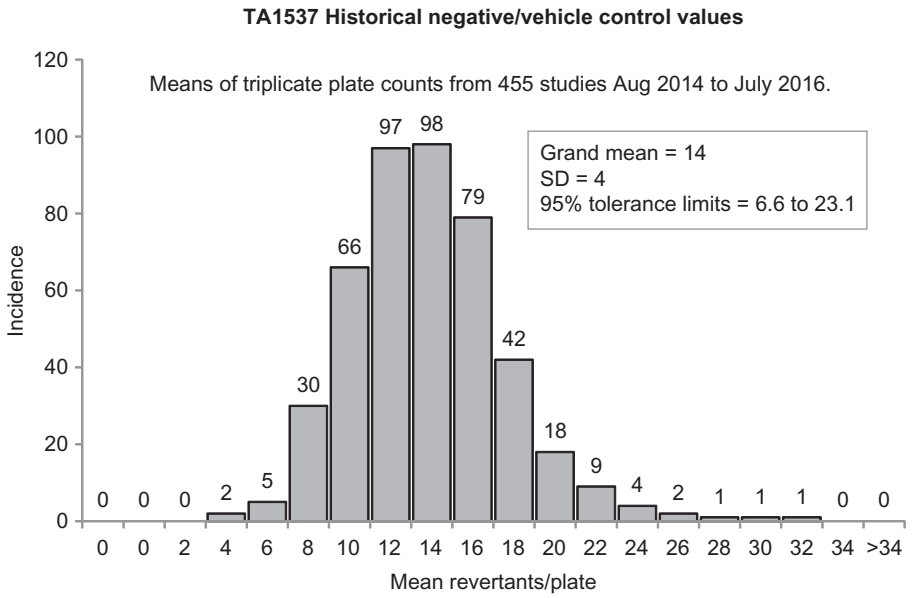

Figure 4.4
Example of historical negative/vehicle control results.

Table 4.10: Historical positive control results

Strain	Treatment	Dose (μg/Plate)	S9	Mean	SD	Range
TA1535	NaAz	0.5	0	320	45	151–655
TA1537	9AC	50	0	337	172	29–2010
TA98	2NF	1	0	166	41	43–330
TA100	NaAz	0.5	0	530	59	250–1003
WP2 *uvrA*	NQO	0.5	0	610	201	82–2220
TA1535	2AA	5	+	380	91	44–699
TA1537	BaP	5	+	110	24	35–189
TA98	BaP	5	+	369	81	179–681
TA100	BaP	5	+	1040	216	408–1669
WP2 *uvrA*	2AA	20	+	300	165	108–2444

Notes: SD Standard deviation
0S9 Without S9
+S9 With S9
Note that in comparison to negative control counts, positive control values can vary widely, so the value of making comparisons of mean values with the values obtained in any specific experiment is limited.

Sometimes it is useful to compare mutagenic potencies of materials tested concurrently (e.g., extracts of soil samples prepared before and after amelioration). In such cases, results are plotted graphically using a spreadsheet program such as Microsoft Excel or OpenOffice. Results obtained at toxic levels should be excluded and then a line of best fit is used to calculate slope expressed as induced revertants (per gram of soil in our example). The dose-response is not necessarily expected to be linear (especially over a wide dose range), so an appropriate transformation may help straighten the curve. Because of this lack of linearity, it is extremely important for equivalent dose levels to be compared; in our example, the doses should be expressed in terms of weight of soil rather than mg of extract.

4.10.7 Testing of Volatile and Gaseous Compounds

The standard plate incorporation and preincubation assays must be specially adapted to allow reliable detection of mutagenic gases and volatiles [55,56]. In both cases bacteria are plated out in the top agar with either buffer or S9 before sealing them in commercially available Tedlar gas sampling bags. Alternatively, they can be exposed in a desiccator jar [57]. As much air as practical is removed before measured amounts of air and the test gas are injected via a septum or valve in the bag using a gas syringe or via a wet gas meter; a flow meter is not suitable for accurate measurement of volume unless it has been calibrated for that particular gas. A range of concentrations of the gas are evaluated; the calculated concentrations should take into account the dead volume of residual air in the plates after the bag has been partially evacuated. Using standard mutagenic gases, the plates can be stacked inverted with lids on as usual. After incubation for the standard period (e.g., 65 h), the bags are vented and removed in a fume cupboard; after the gas has dissipated, plates are

evaluated as usual. Note that many types of plastic are porous to gases (Tedlar tends to be less so), and atmospheres of up to 100% gas are possible if the bag is purged with the gas because the bacteria are facultative anaerobes. The laboratory should validate the system using gaseous mutagenic agents. At least one of these can be included in individual studies as proof of competency; otherwise, standard positive controls are sufficient. In the case of volatile materials, a measured amount of test article is added to a glass Petri dish that is immediately sealed in the bag prior to injection of air.

4.11 Screening Tests

Given the importance placed on the outcome of the bacterial mutation test by regulatory authorities, many companies screen their compounds for microbial genotoxicity at a very early stage of the development. At this point, test article availability and resources are often limited, so it is not necessary or feasible to run a full standard test. Individual laboratories may use "cut-down" versions of the Ames test, such as testing only in the presence of S9, using a reduced top dose level, fewer replicate plates, reducing the number of strains examined (often using only TA98 and TA100), or a combination of these [58]. The advantage of this approach is that results can be directly extrapolated to what might be expected in the subsequent GLP test; the disadvantage is that there will be some difficulty in detection of weak mutagens or, in the case of reducing the number of strains, that some mutagens will be missed. However, a positive result in such a test is sufficient to categorize the chemical as a mutagen. Numerous other approaches to preliminary screening have been proposed; some of the modified standard methods and other approaches to screening in current use in the pharmaceutical industry have been summarized and evaluated by Escobar et al. [59].

4.11.1 Simplified Test Systems

Screening tests based on a single or matched pair of test organisms to detect forward mutations or DNA damage generally have very low test material and resource requirements; their main utility seems to be in very early stage screening. However, their predictive ability (in terms of correlation with the eventual Ames test results) seems to be generally weak, so they are only described briefly here. Examples in current use include:

1. Forward mutation systems involve loss of functionality of a non-vital gene leading to drug resistance. Miller et al. [60] described use of strain TA100 to assess induction of forward mutation to 5-fluorouracil resistance in a system with close parallels to the mouse lymphoma mammalian cell mutation assay. An additional mutation was added to the tester strain to prevent cross-feeding (metabolic cooperation). The test uses 30 mg of compound compared with more than 10-times that much for a standard Ames test.

This particular test seemed to perform well during validation with known mutagens but was much less predictive of the Ames test in routine use in one laboratory [59].

2. Bacterial DNA repair tests assess differential killing of matched pairs of tester strains, one of which is repair-deficient. In theory, the repair-deficient strain should be more sensitive to toxins that primarily target DNA [43,61–64]. Toxicity can be assessed using either liquid or semisolid (diffusion-based) systems.
3. Several systems have been developed that detect the biochemical stress response of the organism to DNA damage. These include the "umu test" in which TA1535 incorporates a pSK1002 plasmid containing the umuC gene fused to a lacZ reporter gene. The umuC gene is activated as part of the bacterial SOS response, which, in turn, promotes the β-galactosidase activity associated with lacZ, which is assessed by a colorimetric reaction. Related tests include SOS chromotest in an *E. coli* strain with a deficient cell wall [65]. Vitotox measures induction of a luciferase bioluminescence gene under transcriptional control of a SOS response gene [66,67].
4. Greenscreen and Bluescreen are related licensed reporter gene assays with the same advantages of high-throughput, miniaturization, and low compound requirements. The technology was originally developed in yeast and subsequently transferred to human transformed cell lines. Because the systems use eukaryotic cells, they are not necessarily predictive of the outcome of the Ames test, although they may be capable of detecting a wider range of relevant genotoxic effects [68].

4.11.2 Screening Tests Using Standard Tester Strains

Although some screening tests use non-standard strains or even mixtures of strains, these modifications are not recommended when trying to predict the outcome of the eventual GLP study because of expected lack of correlation, difficulty in interpretation, and reduced sensitivity. Assays using the standard test strains in pure form are discussed briefly.

1. In the Spot Test [69,70] the bacteria are mixed with S9 and top agar and then plated out on MGA using the same components as in a standard bacterial mutation test. A 100-μL volume of test solution is added to a 6-mm filter disc or to a central well created using an alcohol sterilized cork-borer. The test article can also be added directly to the surface of the plate as a solid or as a liquid in a dose volume of 10 μL. During the subsequent incubation at 37°C for 48–72 h, the test article usually diffuses out from center, creating a concentration gradient. Potent mutagens cause a halo around the center, showing an increased density of revertant colonies; often, this halo will have a central zone with no background lawn or with a decreased colony density due to toxicity. The method has obvious deficiencies with respect to sensitivity, its nonquantitative nature, and the requirement for the mutagen (or its active metabolite) to diffuse into the medium.

2. The sloped (gradient) plate method [71] and the Spiral Plate [72–74] both create concentration gradients using physical methods. Although the spiral plates can be analyzed using image analysis equipment, interpretation of results is not as straightforward as in standard testing and both methods use nonstandard equipment; consequently, neither method seems to have gained widespread acceptance.
3. The microsuspension assay was originally developed to detect mutagenic metabolites in urine extract samples [75] to cope with low sample volumes. Bacteria are concentrated to 10-times the normal density by centrifugation and then preincubated with the test sample and S9 (where appropriate) for 90 min prior to plating out in the normal way. Compared with pour-plate methods, a higher density of bacteria are exposed during this preincubation period to high concentrations of the test material, which should mean that a similar response is obtained with reduced compound requirements. However, the bacteria are not dividing during the preincubation period and exposure to the test compound is greatly reduced after plating, both of which are expected to reduce sensitivity of the test as compared with the standard preincubation method. A 96-well version of the microsuspension test has recently been briefly described that simplifies the dosing procedure [59].
4. The BioLum method involves bioluminescent derivatives of the standard test organisms. The bacteria are plated with top agar, S9 (or buffer), and test article in a 24-well plate format that reduces test article usage by at least 95%. Revertant colonies are easy to detect by their phosphorescence and can be scored using a custom-built system [59,76].
5. The fluctuation test originally formulated by Nobel Prize winners Luria and Delbruck was adapted for mutagenicity screening in the 1970s [43,77]. In a more convenient version of the test with very low test article requirements, bacteria are diluted in medium with S9 (where appropriate) before inoculation into microtiter plates [78,79]; both 96- and 384-well versions of the test are used routinely. After overnight incubation, the medium is replaced by medium without histidine/tryptophan before incubation for an additional 3 days; bromocresol purple or bromothymol blue indicator is added at the replenishment point or after completion of incubation and then the proportion of wells showing bacterial growth (as evidenced by pH (color) change and the presence of microscopic colonies) is recorded. Because the bacteria are directly exposed to the test compound during the initial exposure period, and because the spontaneous frequency of mutation can be accurately measured using this method if survival is assessed, it can be more sensitive than pour-plate methods. To optimize sensitivity, the number of viable bacteria dispensed into each well must be controlled to yield approximately 20% positive wells in the vehicle controls. Cultures of TA100 are usually diluted with nine volumes of culture medium immediately before use; a lower histidine concentration may also be appropriate for this strain to optimize the number of spontaneous positive wells [52]. Results can be interpreted using a one-sided chi-square

look-up table [77]; use of the 1% rather than 5% critical significance will help avoid false-positive claims associated with numerous comparisons with the concurrent control. The method may be more problematic and slightly more work than a pour-plate test but, because of its sensitivity, is suited to testing dilute environmental samples, has lower sample requirement, and may be amenable to automation [80–83]. Several commercial kits are available for performing fluctuation tests [84], but they are not necessarily suited to those unfamiliar with genotoxicity testing.

4.11.3 Reduced Format Tests Using Standard Tester Strains

These use the same methods and principles as the standard test except the volume of all components is reduced proportionately. The six-well Miniscreen involves an 80% reduction in volume and consequently utilizes 20% test compound compared with the standard method with a top dose of 1000 μg/well [85]. The 24-well version of Miniscreen is a minor adaptation of a 25-well version originally described by workers in the United Kingdom [86,87] and is now referred to as the micro-Ames [59], or μAmes, with test article usage 95% lower than the conventional Ames test (Figure 4.5). It is advisable to enter results directly into the study design spreadsheet to reduce workload and the chance of transcription error. Where a large increase in revertants is evident, the number of colonies should be estimated (e.g., entering values of >100 and >200 colonies as 101 and 201 in the spreadsheet). An appropriate statistical method can be used to help evaluate the strength of any response because the somewhat arbitrary "two fold rule" to specify positivity is inappropriate, particularly for strains with low background revertant colony counts (i.e., the nonplasmid-containing strains). Results can be extrapolated and compared directly with those obtained using the conventional method. The tests are best suited for use with strains having higher mutation rates because relative increases are easier to identify. The disadvantage of strains with low spontaneous counts can be overcome to some extent by substitution of strains (e.g., using TA97a in place of TA1537) and, in the case of μAmes, by increasing the number of vehicle control replicates from 3 to 12 wells to more accurately define the background rate of reversion. In the 24-well plate version, the compound is added directly on to the bottom agar before addition of a premix of bacteria + S9 (or buffer) + top agar, allowing it to be performed by a single operative in substantially less time than a standard test. Because image analysis systems have not been developed to score plates in these formats, colonies are necessarily scored by eye, which is facilitated using a benchtop tally counter and light box or a Quebec colony counter.

The standard size (positive control) plate illustrates the normal variation in colony size. The smaller colonies probably grow from late-occurring mutants, whereas the larger colonies often show a denser central spot, giving a "poached egg" appearance. These are colonies that have broken out onto the surface of the agar and spread out. Smaller colonies

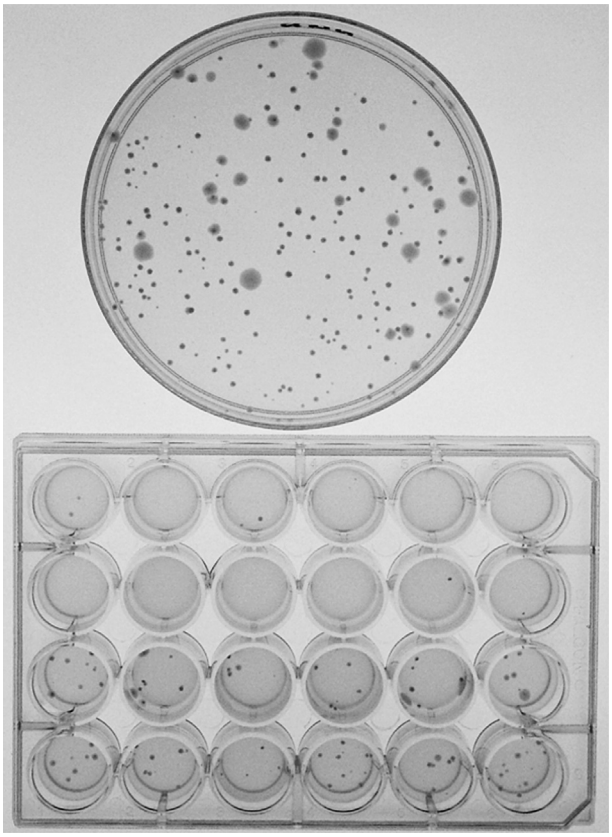

Figure 4.5
Comparison of a standard and a 24-well plate format.

(sometimes torpedo-shaped) are those that remain embedded in the matrix of the medium. The 24-well plate shows a fairly flat dose-response in the presence of S9. The top 12 wells have been treated with the vehicle, whereas the lower wells have been treated with benz[a]pyrene.

4.12 Appendix 1: Growing and Monitoring Suspension Cultures

Technical problems in individual laboratories or experiments such as generally poor background lawns, micro-colonies, and spurious control counts can be due to issues with the top or bottom agar (e.g., top agar too hot or MGA incorrectly formulated). However, problems with individual strains are usually caused by use of unhealthy (often overgrown) suspension cultures or inappropriately maintained stocks. Standardization of culture conditions and appropriate monitoring of growth will help to prevent these issues.

Suspension cultures are usually initiated by addition of freshly thawed bacterial suspension of known density or an isolated colony from a streak into, typically, 25 or 30 mL of

nutrient broth in a flask. If a frozen ampoule of bacterial suspension is used, then the suspension should be thawed rapidly in a 37°C water bath while agitating; as soon as the ampoule is thawed, a measured amount of the culture is added to the nutrient broth to dilute the cryoprotectant. The flask is secured on the platform of a gyratory shaker in an incubator and incubated at 37°C at a speed below that causing foaming (typically approximately 100 rpm depending on the shaker and the flask) until, based on growth curve experiments described in the next section, the targeted OD is achieved and the concentration of bacteria has been confirmed by direct count. The density of the culture can also be confirmed by plating, but results will not be available until the next day.

The culture should then be held at room temperature for use on the same day, either for testing or for preparation of frozen aliquots as suggested here. If the suspension is stored chilled for a few hours, then it should be brought to room temperature before addition of top agar to minimize any lag phase and to avoid the possibility of thermal shock [57].

It is important for an optimal and standardized number of actively growing bacteria to be exposed to the test material and its metabolites during the most critical phase of the test (i.e., soon after dosing). Bacteria should have just reached the late log phase of growth at the time of use (target density is $1-2 \times 10^9$ organisms per mL). A common error is to use an apparently very dense overnight culture in late stationary phase that contains a low titer of viable bacteria. Determination of the growth characteristics of bacteria under standardized conditions in the laboratory as described here will ensure that bacterial cultures prepared later for characterization or routine tests achieve an appropriate density at a convenient time in the working day. Accurate assessment of density is complicated by the fact that bacterial size and morphology change during different phases of growth; in addition, bacteria tend to form chains and may clump.

Bacterial density can be estimated by turbidimetric measurement, plate counts, or direct counts using a counting chamber or a Coulter-type particle counter. Whatever method is used, rough pipetting will help disrupt any chains of bacteria prior to measurement. Most laboratories use a spectrometer to measure optical density (OD) of the bacterial suspension at 650 nm as a surrogate for turbidity; a 1/10 dilution in saline may be used because the correlation between OD and density is better at lower concentrations. OD does not correlate directly with turbidity or cell density and can depend on the conditions used; therefore, each laboratory should generate its own standard curve based on the growth curve methods described here. Once the growth characteristics of the strain have been established and the inoculated number of live bacteria and incubation conditions have been standardized, the OD will give a reasonable idea of when the culture has reached an appropriate growth phase.

A Coulter counter suitable for bacterial density measurement may be available in a laboratory that performs cell counts routinely. If not, then a Petroff-Hausser-type bacterial

Figure 4.6
A Petroff-Hausser bacterial counting chamber.

counting chamber (Hausser Scientific) (Figure 4.6) or a Helber Thoma chamber (Hawksley) should be purchased. This operates on the same principles as a hemocytometer but has finer divisions and is much shallower (depth 0.01 or 0.02 mm). Bacterial counting chambers are comparatively fragile and expensive ($100–$600) and should be handled and cleaned directly over the bench. Some chambers are supplied with thin and fragile coverslips—obtaining a few (thicker) hemocytometer coverslips to act as replacements is recommended.

A phase-contrast microscope is ideal for performing direct counts but, if not available, a standard microscope can be used with the condenser diaphragm closed down. To focus on the gridlines and locate the appropriate zone near the middle of the mirrored area prior to counting, place the unloaded chamber slide on the stage of the microscope and use a medium power objective (e.g., 10×); then, refocus using a high-power air objective (e.g., 40×). Remove the chamber and clean it and the coverslip thoroughly with a moistened medical wipe (alcohol may help to remove any grease). Place the chamber on a smooth, flat surface and then mount the coverslip on the chamber using a small drop of water (approximately 0.5 μL) in the frosted raised areas on each side of the grid. Position and press down gently on the sides of the coverslip while moving it slightly back and forth to secure it. Prepare a 1 in 10 dilution of the bacterial culture in 0.1 mM copper sulfate in 0.9% saline—copper is toxic and will reduce the motility of the live bacteria to facilitate counting. Use a micropipette to transfer 2.5 μL (for a 0.02 mm deep chamber) of mixture to the silvered central region adjacent to the edge of the coverslip so that the suspension is drawn under the coverslip by capillary action. If the chamber does not fill properly, then clean and reload it. The bacteria should be allowed to settle for a short time before counting.

The central square millimeter of the chamber is ruled into 25 groups of 16 small squares, with each group separated by triple lines, the middle one of which is the boundary. Count the bacteria in a group of 16 small squares (shaded area in Figure 4.7). Bacteria touching the two boundary lines indicated by the arrow are counted while those touching the other

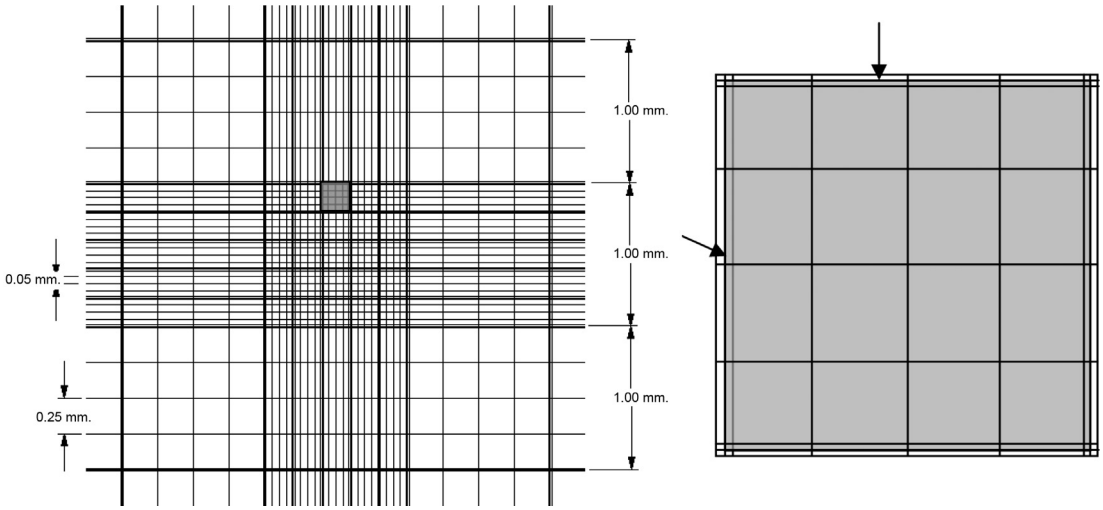

Figure 4.7
Bacterial counting chamber grid.

two lines are ignored. A Denominator-type multichannel bench-top tally counter should be used to facilitate counts.

The volume of a 4×4 square of a 0.02-mm deep chamber is 800×10^{-9} mL; therefore, the bacterial density equal is calculated as:

chamber count ÷ dilution factor ÷ 800×10^9 bacteria/mL.

For example, if the culture was diluted with nine volumes of diluent, a count of 120 equates to 1.5×10^9 bacteria/mL ($120 \div 1/10 \div 800 \times 10^9$ bacteria/mL). Although the direct count does not necessarily equal the viable count, virtually all the bacteria counted in a log phase suspension will be viable. The condition of the bacteria in terms of morphology, motility (TA100 in particular is highly motile), and absence of clumping will help confirm their health.

To clean the chamber, carefully slide the coverslip from the chamber. Wipe the coverslip and chamber with a dry paper tissue. Spray the chamber and coverslip with purified water and then wipe dry with a fresh tissue. Clean again with 70% v/v propanol and discard all tissues as appropriate for contaminated material.

The bacteria can also be plated to confirm their density. In this case, the suspension is serially diluted in 0.9% saline to approximately 1000 bacteria/mL (e.g., by two serial 10 μL + 10 mL dilutions using a fresh micropipette tip for each dilution); then, 0.1 mL is either spread directly across the surface of a nutrient agar plate or mixed with 2.5 mL molten top agar at 45°C before spreading across the plate. After a few minutes to allow

absorption or gelling on a level surface; the plate should be incubated at 37°C for approximately 24 h before counting colonies. Provided that care is taken when performing the dilutions, the calculated number of colony-forming units per mL of original culture should be equal to the density of viable bacteria and the number of bacteria in the actively growing culture because nearly all the bacteria are expected to be viable. However, some variance is expected as a result of experimental error.

When performing growth curve experiments, OD_{650nm}, viable counts, and direct counts should all be plotted against time. Exponentially growing suspension cultures of *Salmonella* strains are expected to have a doubling time of approximately 30 min, whereas *E. coli* strains generally grow slightly faster. Thus, if a 25 or 30 mL culture flask is inoculated with 200 μL of a healthy *Salmonella* suspension (e.g., as obtained from a frozen culture), then the suspension will achieve the density of the original inoculum after approximately 4.5 h taking into account a brief lag period.

For routine use (i.e., standard testing), if the suspension is inoculated from a freshly thawed frozen suspension of known density, the growth curve experiments can be used to specify inoculation volumes and incubation periods for each of the strains so that suspension cultures can be inoculated and are ready for use at convenient times of the day. Suspensions can be inoculated in the evening at room temperature and then placed in the shaking incubator, which is connected to a timer set to switch the incubator on at a specified time in the early morning so that cultures are ready for use when staff come into the laboratory.

References

[1] Zeiger E. History and rationale of genetic toxicity testing: an impersonal, and sometimes personal, view. Environ Mol Mutagen 2004;44(5):363–71.

[2] Demerec M. Studies of the streptomycin-resistance system of mutations in *E. coli*. Genetics 1951;36 (6):585–97.

[3] Witkin EM. Mutations in *Escherichia coli* induced by chemical agents. Nucleic acids and nucleoproteins. Cold Spring Harbor Symp Quant Biol 1947;12:256–69.

[4] Demerec M. Mutations induced by carcinogens. Br J Cancer 1948;2:114–17.

[5] Bertani G. A method for detection of mutations, using streptomycin dependence in *Escherichia coli*. Genetics 1951;36(6):598–611.

[6] Szybalski W. Special microbiological systems: II, observations on chemical mutagenesis in microorganisms. Ann NY Acad Sci 1985;76:475–89.

[7] Berger, et al. Analysis of amino acid replacements resulting from frameshift and missense mutations in the tryptophan synthetase A gene of *Escherichia coli*. J Biochem Mol Biol 1968;34(2):219–38.

[8] Hill RF. Dose-mutation relationships in ultraviolet-induced reversion from auxotrophy in *Escherichia coli*. J Gen Microbiol 1963;30(2):281–7.

[9] MacPhee DG. Development of bacterial mutagenicity tests: a view from afar. Environ Mol Mutagen 1989;14(Suppl. 16):35–8.

[10] Mortelmans K, Riccio ES. The bacterial tryptophan reverse mutation assay with *Escherichia coli* WP2. Mutat Res 2000;455(1–2):61–9.

[11] Hartman PE, Ames BN, Roth JR, Barnes WM, Levin DE. Target sequences for mutagenesis in *Salmonella* histidine-requiring mutants. Environ Mutagen. 1986;8(4):631–41.
[12] Gabridge MG, Legator MS. A host-mediated microbial assay for the detection of mutagenic compounds. Proc Soc Exp Biol Med 1969;130(3):831–4.
[13] Malling HV. Dimethylnitrosamine: formation of mutagenic compounds by interaction with mouse liver microsomes. Mutat Res 1971;13(4):425–9.
[14] Ames BN, Durston WE, Yamasaki E, Lee FD. Carcinogens are mutagens: a simple test system combining liver homogenates for activation and bacteria for detection. Proc Natl Acad Sci USA 1973;70(8):2281–5.
[15] Bartsch H, Malaveille C, Montesano R. *In vitro* metabolism and microsome-mediated mutagenicity of dialkylnitrosamines in rat, hamster, and mouse tissues. Cancer Res 1975;35:644–51.
[16] Yahagi T, Degawa W, Seino Y, Matsushima T, Nagao M, Sugimura T, et al. Mutagenicity of carcinogenic azo dyes and their derivatives. Cancer Lett 1975;1(2):91–6.
[17] OECD Guideline for Testing of Chemicals, Genetic Toxicology No. 471, Organisation for Economic Co-Operation and Development, Paris; 21 July 1997.
[18] ICH Harmonised Tripartite Guideline S2(R1). Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use; June 2012.
[19] Gatehouse D, Haworth S, Cebula T, Gocke E, Kier L, Matsushima T, et al. Recommendations for the performance of bacterial mutation assays. Mutat Res 1994;312(3):217–33.
[20] Zeiger E, Pagano DA, Robertson IGC. A rapid and simple scheme for confirmation of *Salmonella* tester strain phenotype. Environ Mutagen 1981;3(3):205–9.
[21] Wilcox P, Wedd DJ, Gatehouse D. Collaborative study to evaluate the inter/intra laboratory reproducibility and phenotypic stability of *Salmonella typhimurium* TA97a and TA102. Mutagenesis 1993;8(2):93–100.
[22] Pagano DA, Zeiger E. The stability of mutagenic chemicals stored in solution. Environ Mutagen 1985;7 (3):293–302.
[23] Maron DM, Ames BN. Revised methods for the *Salmonella* mutagenicity test. Mutat Res 1983;113 (3–4):173–215.
[24] Ong T-M, Mukhtar H, Wolf CR, Zeiger E. Differential effects of cytochrome P450-inducers on promutagen activation capabilities and enzymatic activities of S-9 from rat liver. J Environ Pathol Toxicol 1980;4(1):55–65.
[25] Eliott B, Coombs R, Elcombe C, Gatehouse D, Gibson G, MacKay J, et al. Report of UKEMS working party: alternatives to Aroclor 1254 induced S9 in *in vitro* genotoxicity assays. Mutagenesis 1992;7 (3):175–7.
[26] Hakura A, Suzuki S, Satoh T. Advantage of the use of human liver S9 in the Ames test. Mutat Res 1999;438(1):29–36.
[27] Hakura A, Suzuki S, Satoh T. Improvement of the Ames test using human liver S9 preparation. Methods Pharmacol Toxicol 2004;:325–36.
[28] Prival MJ, King VD, Sheldon Jr. AT. The mutagenicity of dialkyl nitrosamines in the *Salmonella* plate assay. Environ Mutagen 1979;1(2):95–104.
[29] Prival MJ, Mitchell VD. Analysis of a method for testing azo dyes for mutagenic activity in *Salmonella typhimurium* in the presence of flavin mononucleotide and hamster liver S9. Mutat Res 1982;97(2):103–16.
[30] Prival MJ, Bell SJ, Mitchell VD, Peiperl MD, Vaughan VL. Mutagenicity of benzidine and benzidine-congener dyes and selected monoazo dyes in a modified *Salmonella* assay. Mutat Res 1984;136 (1):33–47.
[31] Levin DE, Hollstein M, Christman MF, Schwiers EA, Ames BN. A new *Salmonella* tester strain (TA102) with A.T base pairs at the site of mutation detects oxidative mutagens. Proc Natl Acad Sci USA 1982;79 (23):7445–9.
[32] Watanabe K, Sakamoto K, Sasaki T. Collaborative study of interlaboratory variability in *Salmonella typhimurium* TA102 and TA2638 and *Escherichia coli* WP2/pKM101 and WP2 uvrA/pKM101. Mutagenesis 1995;10(3):235–41.

[33] Gatehouse D. Bacterial mutagenicity assays: test methods. Methods Mol Biol 2012;817:21–34.
[34] Zeiger E. Bacterial mutagenicity assays. Methods Mol Biol NY 2013;1044:3–26.
[35] Wilcox P, Naidoo A, Wedd DJ, Gatehouse DG. Comparison of *Salmonella typhimurium* TA102 with *Escherichia coli* WP2 Tester strains. Mutagenesis 1990;5(3):285–91.
[36] Herbold A, Arni P, Driesel AG, Engelhardt G, Jäger J, Joosten HFP, et al. Criteria for the standardization of *Salmonella* mutagenicity tests: results of a collaborative study II. Studies to investigate the effect of bacterial liquid culture preparation conditions on *Salmonella* mutagenicity test results. Teratog Carcinog Mutagen 1983;3(2):187–93.
[37] Göggelmann W, Grafe A, Vollmar J, Baumeister M, Kramer PJ, Pool BL. Criteria for the standardization of *Salmonella* mutagenicity tests: results of a collaborative study IV. Relationship between the number of his- bacteria plated and number of his+ revertants scored in the *Salmonella* mutagenicity test. Teratog Carcinog Mutagen 1983;3(2):205–13.
[38] de Raat WK, Willems MI, de Meijere FA. Effects of amount and type of agar on the number of spontaneous revertants. Mutat Res. 1984;137(1):33–7.
[39] US FDA Redbook. Short-Term Tests for Genetic Toxicity; 2000.
[40] EPA Health Effects Test Guideline OPPTS 870.5100: Bacterial Reverse Mutation Test.
[41] Hollstein M, McCann J, Angelosanto FA, Nichols WW. Short-term tests for carcinogens and mutagens. Mutat Res 1979;65(3):133–226.
[42] Pagano DA, Zeiger E. Conditions for detecting the mutagenicity of divalent metals in *Salmonella typhimurium*. Environ Mol Mutagen 1992;19(2):139–46.
[43] Green MH, Muriel WJ. Mutagen testing using TRP^+ reversion in *Escherichia coli*. Mutat Res 1976;38 (1):3–32.
[44] Mitchell I, de G, Dixon PA, Gilbert PJ, White DJ. Mutagenicity of antibiotics in microbial assays: problems of evaluation. Mutat Res 1980;79(2):91–105.
[45] Gatehouse DG, Rowland IR, Wilcox P, Callander RD, Forster R. Bacterial mutation assays. In: Kirkland DJ, editor. UKEMS subcommittee on guidelines for mutagenicity testing, report, part I revised, basic mutagenicity tests: UKEMS recommended procedures. Cambridge: Cambridge University Press; 1990. p. 13–61.
[46] Thompson C, Morley P, Kirkland D, Proudlock R. Modified bacterial mutation test procedures for evaluation of peptides and amino acid-containing material. Mutagenesis 2005;20(5):345–50.
[47] Barber ED, Donish WH, Mueller KR. The relationship between growth and reversion in the Ames *Salmonella* plate incorporation assay. Mutat Res 1983;113(2):89–101.
[48] Benigni R., Bossa C., Jeliazkova N., Netzeva T., Worth A. The Benigni/Bossa rulebase for mutagenicity and carcinogenicity – a module of Toxtree. European Commission report EUR 23241, <http://ihcp.jrc.ec.europa.eu/our_labs/computational_toxicology/doc/EUR_23241_EN.pdf>; 2008.
[49] Maron D, Katzenellenbogen J, Ames BN. Compatibility of organic solvents with the *Salmonella*/microsome test. Mutat Res 1981;88(4):343–50.
[50] Vedmaurthy RB, Padmanabhan S, Vijayan M, Jamal ZA, Kumjumman J, Narayanan ML. Compatibility of different solvents with *Salmonella typhimurium* mutant strains in bacterial reverse mutation assay. Int J Pharm Pharm Sci 2012;4(1):283–4.
[51] Ayrton AD, Neville S, Ioannides C. Cytostolic activation of 2-aminoanthracene; Implications in its use as diagnostic mutagen in the Ames test. Mutat Res 1992;265(1):1–8.
[52] Vennit S, Crofton-Sleigh C, Forster R. Bacterial mutation assays using reverse mutation. Mutagenicity testing: a practical approach. Oxford, UK: IRL Press; 1984.
[53] Hayashi M, Dearfield K, Kasper P, Lovell D, Martis H-J, Thybaud V. Compilation and use of genetic toxicity historical control data. Mutat Res 2011;723(2):87–90.
[54] ICH Harmonized Tripartite Guideline. The Common Technical Document for the Registration of Pharmaceuticals for Human Use: Safety–M4S (R2), Nonclinical Overview and Nonclinical Summaries of Module 2. Organisation of Module 4.
[55] Hughes TJ, Simmons DM, Monteith LG, Claxton LD. Vaporization technique to measure mutagenic activity of volatile organic chemicals in the Ames/*Salmonella* assay. Environ Mutagen 1987;9(4):421–41.

[56] Araki A, Noguchi T, Kato F, Matsushima T. Improved method for mutagenicity testing of gaseous compounds by using a gas sampling bag. Mutat Res 1994;307(1):335–44.
[57] Mortelmans K, Zeiger E. The Ames *Salmonella*/microsome mutagenicity assay. Mutat Res 2000;455 (1–2):29–60.
[58] Zeiger E, Risko KJ, Margolin BH. Strategies to reduce the cost of mutagenicity screening with the *Salmonella* assay. Environ Mutagen 1985;7(6):901–11.
[59] Escobar PA, Kemper RA, Tarca J, Nicolette J, Kenyon M, Glowienke S, et al. Bacterial mutagenicity screening in the pharmaceutical industry. Mutat Res 2013;752(2):99–118.
[60] Miller JE, Vlasakova K, Glaab WE, Skopek TR. A low volume, high-throughput forward mutation assay in *Salmonella typhimurium* based on fluorouracil resistance. Mutat Res 2005;578(1–2):210–24.
[61] Slater EE, Anderson M, Rosenkranz HS. Rapid detection of mutagens and carcinogens. Cancer Res 1971;31:970–3.
[62] Kada T, Tutikawa K, Sadaie Y. *In vitro* and host-mediated "rec-assay" procedures for screening chemical mutagens; and phloxine, a mutagenic red dye detected. Mutat Res 1972;16(2):165–74.
[63] Leifer Z, Kada T, Mandel M, Zeiger E, Stafford R, Rosenkranz HS. An evaluation of tests using DNA repair-deficient bacteria for predicting genotoxicity and carcinogenicity. A report of the U.S. EPA's Gene-TOX Program. Mutat Res 1981;87(3):211–97.
[64] de Flora S, Zanacchi P, Camoirano A, Bennicelli C, Badolati GS. Genotoxic activity and potency of 135 compounds in the Ames reversion test and in a bacterial DNA-repair test. Mutat Res 1984;133(3):161–98.
[65] Quillardet P, de Bellecombe C, Hofnung M. The SOS Chromotest, a colorimetric bacterial assay for genotoxins: validation study with 83 compounds. Mutat Res 1985;147(3):79–95.
[66] Verschaeve L, Van Gompel J, Thilemans L, Regniers L, Vanparys P, Van der Lelie D. VITOTOX bacterial genotoxicity and toxicity test for the rapid screening of chemicals. Environ Mol Mutagen 1999;33(3):240–8.
[67] Westerink WM, Stevenson JC, Lauwers A, Griffioen G, Horbach GJ, Schoonen WG. Evaluation of the Vitotox and RadarScreen assays for the rapid assessment of genotoxicity in the early research phase of drug development. Mutat Res 2009;676(1–2):113–30.
[68] Simpson K, Bevan N, Hastwell P, Eidam P, Shah P, Gogo E, et al. The bluescreen-384 assay as an indicator of genotoxic hazard potential in early-stage drug discovery. J Biomol Screen 2013;18 (4):441–52.
[69] Iyer VN, Szybalski W. Two simple methods for the detection of chemical mutagens. Appl Microbiol 1958;6(1):23–9.
[70] Ames BN, McCann J, Yamasaki E. Methods for detecting carcinogens and mutagens with the *Salmonella*/ mammalian-microsome mutagenicity test. Mutat Res 1975;31(6):347–64.
[71] McMahon RE, Cline JC, Thompson CZ. Assay of 855 test chemicals in ten tester strains using a new modification of the Ames test for bacterial mutagens. Cancer Res 1979;39(3):682–93.
[72] de Flora SA. "Spiral test" applied to bacterial mutagenesis assays. Mutat Res 1981;82(2):213–27.
[73] Diehl M, Fort F. Spiral *Salmonella* assay: validation against the standard pour-plate assay. Environ Mol Mutagen 1996;27(3):227–36.
[74] Claxton LD, Houk VS, Warren S. Methods for the spiral *Salmonella* mutagenicity assay including specialized applications. Mutat Res 2001;488(3):241–57.
[75] Kado NY, Langley D, Eisenstadt E. A simple modification of the *Salmonella* liquid-incubation assay. Increased sensitivity for detecting mutagens in human urine. Mutat Res 1983;121(1):25–32.
[76] Aubrecht J, Osowski JJ, Persaud P, Cheung Ackerman JR, Lopes SH, Ku WW. Bioluminescent *Salmonella* reverse mutation assay: a screen for detecting mutagenicity with high throughput attributes. Mutagenesis 2007;22(5):335–42.
[77] Green MH, Muriel WJ, Bridges BA. Use of a simplified fluctuation test to detect low levels of mutagens. Mutat Res 1976;38(1):33–42.
[78] Gatehouse D. Detection of mutagenic derivatives of cyclophosphamide and a variety of other mutagens in a "microtitre"® fluctuation test, without microsomal activation. Mutat Res 1978;53(3):289–96.

[79] Gatehouse DG, Delow GF. The development of a “microtitre®” fluctuation test for the detection of indirect mutagens, and its use in the evaluation of mixed enzyme induction of the liver. Mutat Res 1979;60(3):239–52.
[80] Bridges BA. The fluctuation test. Arch Toxicol 1980;46(1–2):41–4.
[81] Näslund M, Kolman A. On the sensitivity of the fluctuation test. Mutat Res. 1980;73(2):409–13.
[82] Reifferscheid G, Maes HM, Allner B, Badurova J, Belkin S, Bluhm K, et al. International round-robin study on the ames fluctuation test. Environ Mol Mutagen 2012;53(3):185–97.
[83] Hubbard SA, Green MHL, Gatehouse D, Bridges JW. The fluctuation test in bacteria. In: Kilbey BJ, Legator M, Nichols W, Ramel C, editors. Handbook of mutagenicity test procedures. 2nd ed. Elsevier; 1984. p. 141–60.
[84] Pant K. In: Steinberg P, editor. Ames II™ and Ames liquid format mutagenicity screening assays, in high-throughput screening methods in toxicity testing. Hoboken (NJ, USA): John Wiley & Sons, Inc.; 2013.
[85] Diehl MS, Willaby SL, Snyder RD. Comparison of the results of a modified miniscreen and the standard bacterial reverse mutation assays. Environ Mol Mutagen 2000;35:72–7.
[86] Brooks TM. The use of a streamlined bacterial mutagenicity assay, the MINISCREEN. Mutagenesis 1995;10(5):447–8.
[87] Burke DA, Wedd DJ, Burlinson B. Use of the Miniscreen assay to screen novel compounds for bacterial mutagenicity in the pharmaceutical industry. Mutagenesis 1996;11(2):201–5.
[88] Josephy PD, Gruz P, Nohmi T. Recent advances in the construction of bacterial genotoxicity assays. Mutat Res 1997;386(1):1–23.