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Interim Procedures for Conducting the *Salmonella*/ Microsomal Mutagenicity Assay (Ames Test)

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Foreword

The Ames *Salmonella*/microsomal mutagenicity assay has been developed and used successfully with supporting chemical data by the EPA's National Enforcement Investigations Center in Denver, the Health Effects Research Laboratory, Research Triangle Park, and several EPA Regional Laboratories for air and wastewater characterization and health effects research. However, the method has not been employed in a uniform manner by all Agency Laboratories, and there is some question of comparability of data among these laboratories.

Other EPA Regional and research laboratories and Program Offices with responsibility for toxic and hazardous substances have expressed an immediate need to apply the Ames test in their activities. The test protocols and guidance provided here were prepared — in a joint effort between the Environmental Monitoring Systems Laboratory-Las Vegas, Nevada and the National Enforcement Investigations Center, Denver, Colorado — to meet that need. The standardization of Ames testing methods for EPA use is intended to assure, to the extent possible, that mutagenicity assay data produced by the Agency is valid, defensible, and comparable with assay data produced by other laboratories.

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Acknowledgments

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Section 1
Introduction

The *Salmonella*/mammalian microsome mutagenicity assay commonly known as the Ames test; Ames et al. (1975) has proven to be reliable for, identification of a large number of mutagenic and potentially carcinogenic substances. This test offers a means of obtaining dose-responsive data with a wide variety of environmental samples.

The general applicability of the Ames test has already been demonstrated as a prescreen for potential genetic hazards of complex environmental effluents or products, e.g., tobacco smoke condensates, natural products, hair dyes, soot from city air, fly ash, synthetic fuel oils, aqueous wastes and diesel particulates (Epler et al. 1978, Claxton et al. 1981). Recent applications (e.g., Donnelly and Brown, 1981) of the procedures to solid wastes and waste leachates are further evidence of the broad-spectrum utility of the Ames test for screening and environmental monitoring.

The purpose of this document is to provide an interim standardized Ames test procedure to be used by Agency, State, and contract laboratories conducting mutagenicity testing under EPA's hazardous waste monitoring program. A supplement to this document — that will address sample preparation or chemical fractionation procedures for use with the mutagenicity assay — is planned. A program is currently underway to provide collaborative testing and evaluation of the test protocol contained in this document.

Section 2
Summary of the Method

Plate Assay Method

The test system developed by Ames and his coworkers has been widely used as a rapid-screening procedure for the determination of mutagenic and potential carcinogenic hazards of pure chemicals, complex environmental mixtures, and commercial products. Detailed experimental procedures have been provided in the "methods paper" by Ames et al. (1975)[1] and supplements. An excellent review of the requirements for applying the Ames test is presented by de Serres and Shelby (1979).

These papers are "must reading" as the minimum introduction necessary to carry out the procedures described in this document.

The Ames test involves the use of five standard tester strains of *Salmonella typhimurium* containing a specific mutation in the histidine operon. These genetically altered strains cannot grow in the absence of histidine; when they are placed in a histidine-free medium, only those cells that revert spontaneously to histidine-independence are able to form colonies. The range of spontaneous reverse mutation values for each strain is relatively constant. However, if a chemical mutagen is added to the medium the mutation value is increased significantly.

The sensitivity of the *Salmonella* tester strains has been enhanced by the introduction of two additional mutations, namely *uvrB* and *rfa*. The deletion mutation covering the *uvrB* gene results in elimination of the accurate DNA repair system. Because this deletion also includes the biotin gene, the cells require the addition of biotin to grow. The *rfa* (deep rough) character allows increased cell permeability and greater penetration of chemical mutagens or large chemical molecules such as crystal violet into the bacterial cell, due to partial loss of the lipopolysaccharide (LPS) barrier of the cell surface.

Certain mutagens are directly active in the system while others require activation by mammalian microsomes (e.g., rat-liver enzymes) added to the test system. Generally, these are obtained from Aroclor 1254-induced rats, using the 9000-g supernatant (S-9) of the homogenized rat liver. These microsomes contain enzymes which perform metabolic conversions mimicking those of mammalian organs *in vivo*. Metabolic activation of test materials with these enzymes improves the correlation between mutagenesis in this *in vitro* bacterial test system and carcinogenesis in mammals.

In conducting the test, a tester strain is added to soft agar containing a low level of histidine and an excess of biotin along with varying amounts of the test substance. This mixture is overlaid on mimimal agar plates, and the plates are incubated for at least 48 hours at 37°C. The bacteria undergo several divisions before the trace amounts of histidine are used up and thus form a light film of background growth (lawn) on the plate. In many cases, this growth is necessary for mutagensis to occur. Revertants to histidine independence (*his*$^+$), induced by the mutagenic chemical(s), continue to grow in the absence of histidine to form visible colonies on the plate. These colonies are examined after 48 and 72 hours of incubation. Although visible colonies can usually be obtained with 48-hour incubation at 37°C, the toxicity of some chemicals may delay the appearance of revertants. When this is suspected, plates should be incubated for 72 hours (de Serres and Shelby, 1979). Counts of revertant colonies on these test plates are compared to counts (spontaneous revertants) of the same strain on control plates containing all components but the test substance (solvent controls). The assay is quantitated with respect to doses (amount of chemical added per plate) of mutagen.

The plate assay method described here is basically as used by Ames, and incorporates recommendations of de Serres and Shelby (1979) and Belser et al., (1981), designed to make the test more quantitative.

The desired result of a mutagenicity test is a definitive "positive" or a "negative" with respect to the test system employed. Since a positive dose-response is a primary criterion for our identification of a chemical or mixture as mutagenic, the terminal tests selected are all dose-response assays and the screening tests have dose-response potential under the proper conditions. As a result, it is possible to demonstrate *and confirm* mutagenicity with only two consecutive tests whenever a dose-response can be demonstrated in the preliminary test (see Figure 1). Were we to adopt a rangefinding preliminary test solely for *toxicity*, a minimum of three consecutive tests would be required to obtain confirmation of any test result (positive or negative) noted.

Selection of Specific Tests, Test Modifications

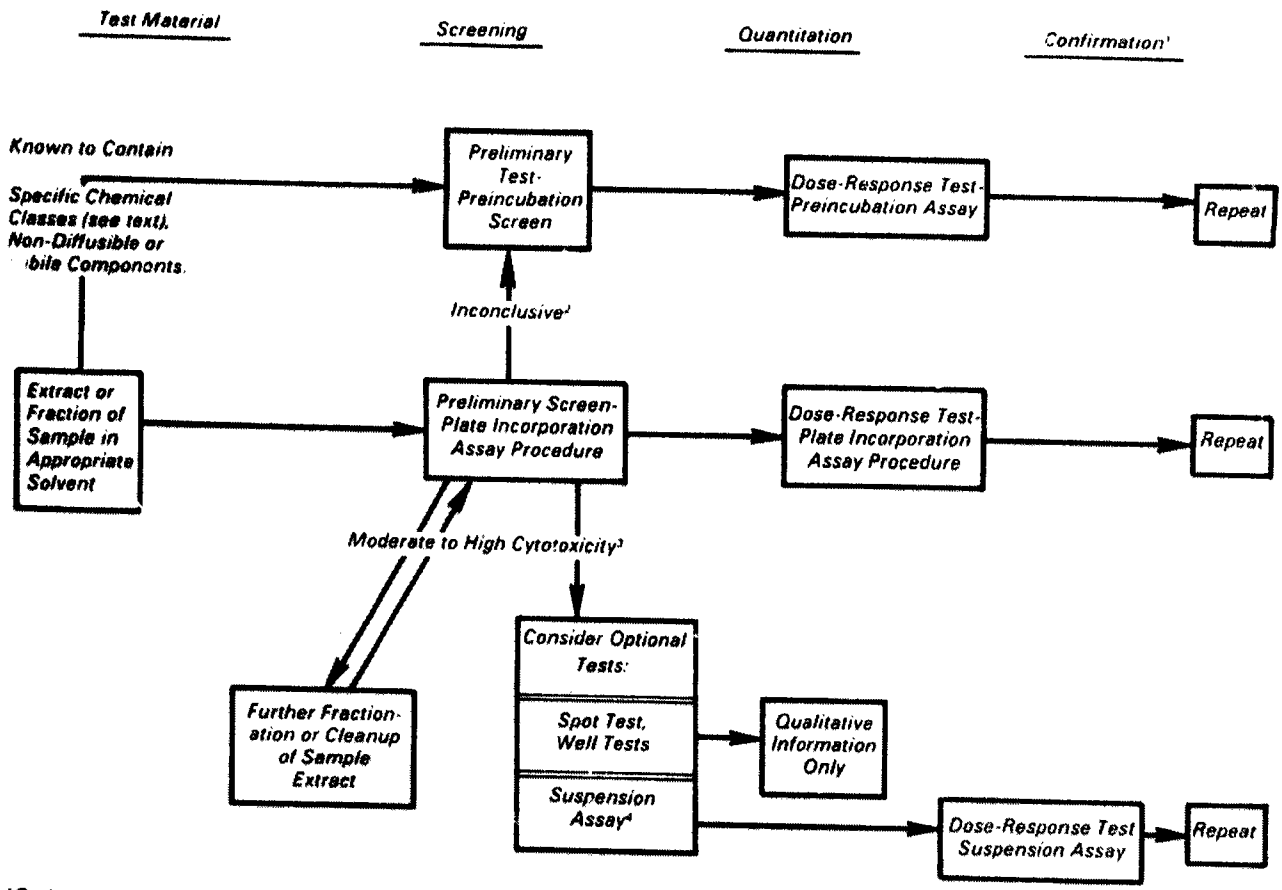
The plate-incorporation procedure described by Ames et al. (1975) is well tested and widely used for testing pure compounds and complex mixtures.

Perhaps the most widely used and successful modification of the plate-incorporation assay is the preincubation method described by Yahagi et al. (1977). Preincubation techniques have been shown to enhance the mutagenic potentials of many chemicals (Sugimura and Nagao, 1980). These techniques may be the method of choice for groups of compounds such as the nitrosamines (Yahagi et al. 1977), pyrrolizidine alkaloids (Yamanaka et al. 1979), and quinoline derivatives (Nagao et al. 1977). Indeed, the preincubation method is now used routinely in some laboratories and is recommended for use in cases where results from the standard plate assay are inconclusive (de Serres and Shelby, 1979).

Modifications of the liquid suspension assay described by Malling (1971) are receiving some attention as possible alternatives for testing samples which have proven too cytotoxic to successfully test with standard plate-incorporation procedures.

Among qualitative screening procedures suggested for rapidly determining the most appropriate tester strain(s), cytotoxicity of the test material, and/or effects of metabolic activation are the spot test (Ames et al. 1975), the well test (Pellizari 1978), and micro-well technique (Loveday, unpublished protocol) and the toxicity screening procedure reported by Waleh et al. All represent efforts to simplify and reduce the testing requirements for obtaining basic screening information.

[1] and as revised (Marson and Ames, *Mutation Research*, in press)



[1] Optional, if positive preliminary test result was confirmed (repeated) by the first "dose-response test."
[2] Results of preliminary test suggest mutagenic activity but do not meet all criteria for a positive test.
[3] Mutagenic activity cannot be resolved from cytotoxicity by dilution alone.
[4] Not as well defined as plate incorporation and preincubation assays; often difficult to perform and interpret satisfactorily.

Figure 1. Flow diagram - recommended mutagenicity testing sequence for complex mixtures or environmental samples.

Section 3
Mutagenesis Assay Procedures

Preliminary Test — Plate-Incorporation Procedure

a. Prepare Master Plates as described in Section 4. On the day prior to performance of the assay, select single isolated well-grown colonies from the Master Plate for each strain and inoculate into nutrient broth. Use tubes or erlenmeyer flasks about five times the volume of the nutrient broth.

b. Incubate cultures with continuous gentle (e.g., 120 rpm) agitation overnight for 14-18 hours at 37°C.

c. Prepare top agar as outlined in Appendix I. Melt prepared top agar in an autoclave for 3 minutes at 121°C, or melt the agar in a microwave oven. Maintain the molten top agar in a 45°C waterbath or warming oven.

d. Prepare a histidine/biotin solution as outlined in Appendix I. Place the histidine/biotin solution in a waterbath and warm to 45°C. Add 20 ml of this solution to 200 ml of top agar.

e. Prewarm minimal agar plates at 37°C for 24 hours before inoculation. This tests for sterility of the medium. The warm plates also aid in uniformly distributing the top-agar overlay.

f. Insert the required number of sterile 13 x 100 mm culture tubes into heating block or waterbath preheated to 45°C. Pipet 2 ml molten top agar supplemented with histidine/biotin solution into each tube.

The next steps in the plate incorporation assay procedure are depicted in Figure 2.

Perform the following steps under an appropriate biological cabinet or a laminar-flow hood; wear fully fastened laboratory coat with solid front and surgical gloves.

g. Add 0.1 ml of fresh nutrient broth culture (from Step 'b') of the desired test strain (approximately 10^8 cells) to each tube (positive and negative/solvent controls and each test dose level in appropriate replication) to be tested. Fresh broth cultures should be kept in an ice bath while preparing the plates. Remember that upon inoculation of the top agar the organisms must not remain at 45°C for more than about 10 minutes.

h. Add 50 µl of a solution of the test material in DMSO[1] to each tube of top agar inoculated with culture. A general guide is to span up to a 3-log dose range, with 1/3 to 1/2-log intervals between doses, for screening. Suggested concentrations of test material include 100, 30, 10, 3, 1 and 0.3 mg/ml for each test strain to yield effective doses of approximately 5, 1.5, 0.5, 0.15, 0.05 and 0.015 mg test material per plate, respectively. Prepare a set of six tubes for each combination of strain and dose.

i. To two of the tubes from each set prepared in 'h', add 0.5 ml of "Low S-9 Mix" (see Appendix I for preparation of S-9 mixes). To the next two tubes from each set add 0.5 ml of "High S-9 Mix" in the same manner. Remove tubes in each case prior to adding S-9 mix and do not return to heating block or 45°C waterbath. The S-9 mix should not be exposed to the 45°C temperature for longer than a few seconds. To the final pair of tubes, substitute 0.5 ml of 0.2 M sodium phosphate buffer solution (see Appendix I) so that volume conditions for the "activated" and non-activated tests will be comparable.

j. Use a mechanical Vortex mixer to thoroughly mix the materials; mix gently for not more than three seconds, taking care to prevent bubble formation in the top agar.

k. Pour the contents of each tube onto the center of a prewarmed minimal agar plate. Gently tilt and rotate each plate to spread the top agar uniformly over the surface of the minimal agar. Uniform distribution can be facilitated by placing the plate on a level table and gently vibrating the table surface, e.g., with a mechanical vibrating device (Belser et al. 1981), but this is not mandatory.

l. Cover each plate and place on a flat surface until the agar sets (several minutes). Then incubate the plates in the dark at 37°C for 48 to 72 hours in an inverted position.[2] Observe the number of revertant colonies shortly after removal from the incubator at 48 and 72 hours.[3] If unable to perform plate counts immediately, storage for up to 2 days at 4°C is acceptable. If satisfactory colony development has occurred by 48 hours, further incubation (i.e., to 72 hours) may be unnecessary. However, until the investigator is confident that he/she can consistently distinguish those tests requiring extended incubation, it is recommended that counts be made at both 48 and 72 hours.

[1] Or other appropriate solvent; if 50 µl of solvent will not solubilize the test material, increase the solvent level uniformly for all dose levels. Do not exceed 100 µl of DMSO (Belser et al., 1981). See Maron, et al. (1981) for information on compatibility of solvents with the Ames test.

[2] If sample is known or suspected to contain significant quantities of volatile chemicals, seal plates in individual plastic bags or place into a dessicator prior to incubating.

Confirmatory Test — Plate-Incorporation Procedure

If the preliminary screen shows increased numbers of revertants over solvent controls or a positive dose-response relationship, repeat all steps of the initial test at least in duplicate using additional doses of test material, *with the most active strains and conditions.* For example, if the 1 mg dose shows mutagenic activity, doses of choice might include 0.25, 0.5, 0.75, 1, 1.5, 2.5, and 4.0 mg to establish a dose-response relationship. Proper test doses must be selected independently for each sample tested.

Metabolic activation with S-9 mix, at the appropriate level, should be used if those were the conditions under which the initial "positives" were detected.

If the preliminary screen does not suggest any mutagenic activity, repeat the test conditions of the preliminary screen but increase the maximum dose level, if necessary, to 5 to 10 mg per plate or that which is clearly cytotoxic. With mixed test materials, one or more components may precipitate on the plate at relatively low concentrations. If the precipitate does not interfere with the scoring of plates, this kind of precipitate should not restrict the upper limit of dose concentration.

Examining Background Lawn

Use a dissecting microscope to examine the background lawn.

The background growth or lawn is a result of the trace amount of histidine present in the top agar. If the test

[3] Toxicity of some chemicals may delay the appearance of revertant colonies beyond 48 hours, in which case incubation should be extended to 72 hours (de Serres and Shelby, 1979). For the same reason, Belser (1981) recommends a 63-hour incubation period.

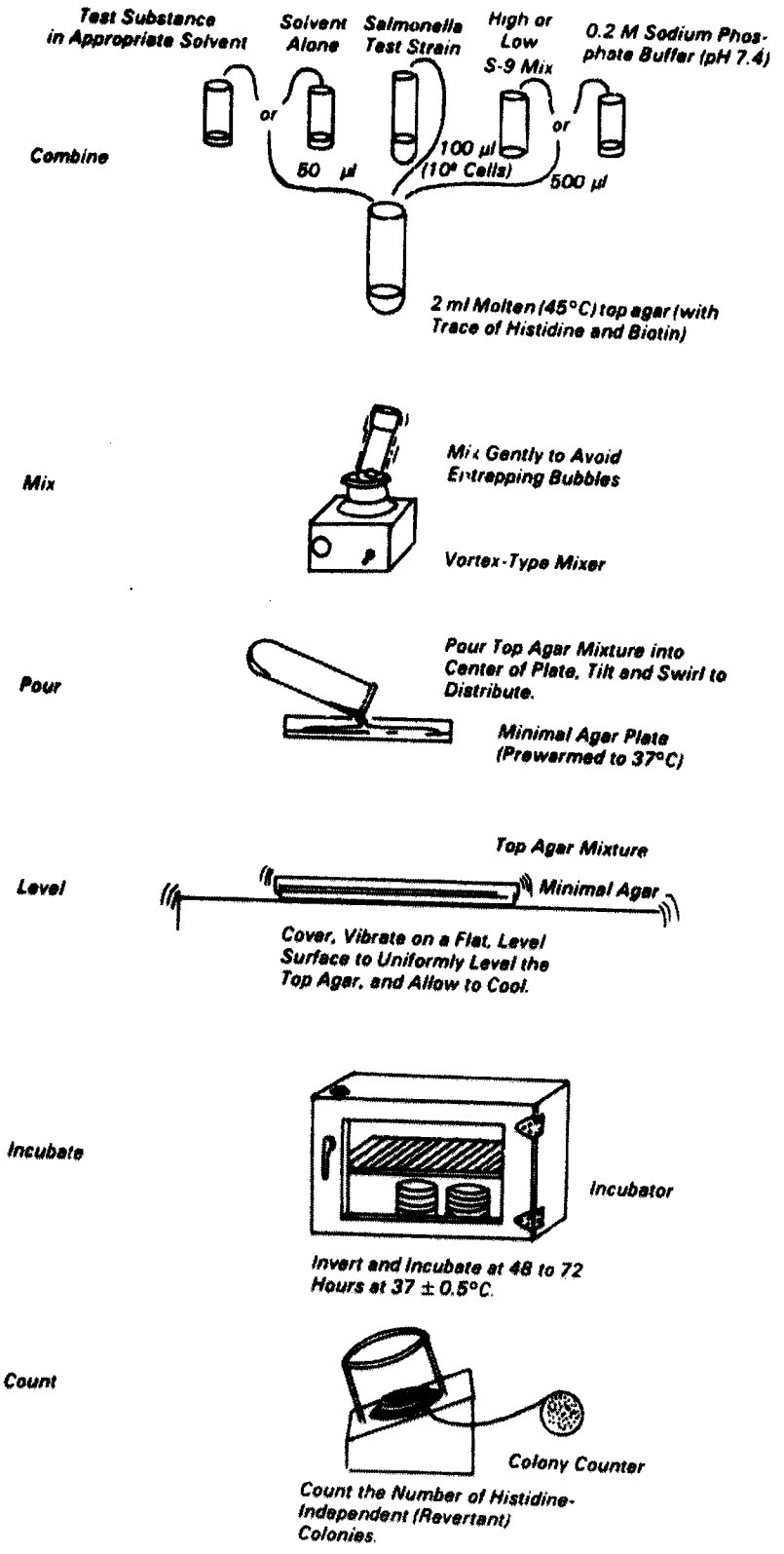


Figure 2. *Steps in the Ames plate-incorporation assay procedure.*

substance is toxic, massive cell death will occur and the background lawn will be sparse or absent compared to control plates. Because more histidine is available to the survivors, they will undergo more cell divisions and may give rise to visible colonies. These colonies can be mistaken for revertants; consequently, the presence or absence of a normal lawn must be noted and recorded.

To verify the histidine independence of resulting colonies, selected control and test plates should be replica plated onto plates containing minimal agar with biotin. Revertant colonies will grow on the minimal agar; phenocopy colonies will not. Replica plating satisfies the question as to whether a statistically adequate number of colonies (especially where the plate count is high) has been checked. Replica plating of the two plates showing highest plate counts (highest revertant levels) should be adequate to evaluate the histidine independence of the colonies.

All positive mutagenic responses should be verified by a repeatable assay.

Preincubation Assay

A most widely used modification to the standard Ames test is the preincubation assay initially described by Yahagi et al. (1977). This procedure is useful for certain types of chemicals (e.g., nitrosamines), or in cases where results of the standard plate assay are inconclusive. Its use as part of the screening assay has been recommended (de Serres and Shelby, 1979). Preincubation tests are performed at least in duplicate. The following preincubation conditions are those recommended by Sugimura and Nagao (1980):

a. Prepare Master Plates as described in Appendix I. On the day prior to performance of the assay, select single isolated well-grown colonies from the Master Plate for each strain and inoculate into nutrient broth. Use tubes or erlenmeyer flasks about five times the volume of the nutrient broth.
b. Incubate cultures with continuous gentle (e.g., 120 rpm) agitation overnight for 14-18 hours at 37°C.
c. Prepare top agar as outlined in Appendix I. Melt prepared top agar in an autoclave for 3 minutes at 121°C, or melt the agar in a microwave oven. Maintain the molten top agar in a 45°C waterbath or warming oven.
d. Prepare a histidine/biotin solution as outlined in Appendix I. Place the histidine/biotin solution in a waterbath and warm to 45°C. Add 20 ml of this solution to 200 ml of top agar.

Perform the following steps under a biological cabinet or laminar-flow hood;

wear fully fastened laboratory coat with solid front and surgical gloves.

e. Add appropriate concentrations of test material to duplicate sterile 13 x 100 ml test tubes which have been placed in an ice bath (see *Preliminary Test — Plate Incorporation Procedure*, Step 'h').
f. Inoculate each tube with 0.1 ml overnight broth culture of the desired test strain.
g. Add 0.5 ml of either High S-9 Mix, Low S-9 Mix, or 0.2 M phosphate buffer solution to each tube containing sample extract and culture.
h. Quickly mix the contents of each tube with a Vortex mixer and incubate with continuous moderate agitation at 37°C for 15 minutes.[1]
i. Add 2 ml of molten top agar with trace amounts of histidine and excess biotin (from Step 'd') to each tube, and vortex each tube for not more than 3 seconds to thoroughly mix the materials, taking care to avoid entrainment of bubbles in the agar.
j. Pour the contents of each tube onto the center of a prewarmed Minimal Agar plate. Gently tilt and rotate each plate to spread the top agar uniformly over the surface of the Minimal Agar. Uniform distribution can be facilitated by placing the plate on a level table and gently vibrating the table surface, e.g., with a mechanical vibrating device (Belser et al. 1981), although this is not mandatory.
k. Cover each plate and place on a flat surface until the agar sets (several minutes). Then incubate the plates in the dark at 37°C for 48 to 72 hours in an inverted position (see footnotes 2 and 3, page 22). Count the number of revertant colonies shortly after removal from the incubator.
l. If increased numbers of revertant colonies are noted, repeat the above steps at least in duplicate (preferably in triplicate) *with the most active strains* using additional dosage levels of test materials (see *Confirmatory Test — Plate Incorporation Procedure*).
m. If the preliminary test does not suggest any mutagenic activity, repeat the test conditions but increase the maximum dose level, if necessary, to 5 to 10 mg per plate or that which is clearly cytotoxic. With mixed test materials, one or more components may precipitate on the plate at relatively low concentrations. If precipitate does not interfere with the scoring of plates, this kind of precipitate should not restrict the upper limit of dose concentration.

[1]Although 15- or 20-minute incubation periods are widely used, optimal conditions for a given sample may require up to 1 hour or more.

General Recommendations

Table 1 provides recommended conditions for conducting the preliminary and confirmatory (dose-response) tests using conventional plate procedure and preincubation assays.

Table 1. *Recommended Experimental Conditions for Conducting* Salmonella *Mutagenicity Assays*

Test Designation	*Assay Type*	*Test Strains*	*S-9 Activation*	*Dose Regimen*	*Plates per Test Condition Recommended Minimum*
Preliminary (PR) Test	*Plate incorporation*	*TA1535 TA1537 TA1538 TA98 TA100*	*High, Low and no S-9 for each strain*	*Minimum of 5 doses over 2- to 3-log range*	*2*
Confirmatory (Dose-Response) Test[1]	*Plate incorporation*	*Strains most active in PR Test*	*Conditions active in PR Test*	*6-8 doses; bracket dose level showing highest activity in PR Screen; space closely (e.g., 8 doses over 1/2-log range)*	*2*
Preincubation (PI) Screen	*Preincubation and plate incorporation*	*TA98 TA100 TA1535 TA1537 TA1538*	*High, Low and no S-9 for each strain*	*Minimum of 5 doses over 2- to 3-log range*	*2*
Confirmatory Preincubation (Dose-Response) Assay	*Preincubation and plate incorporation*	*Strains most active in PI Screen*	*Conditions active in PI Screen*	*6-8 doses; bracket dose level showing highest activity in PI Screen; space closely*	*2*

[1]*For negative results, repeat conditions of preliminary tests or retest material using Preincubation Screen.*

Section 4
Salmonella Test Strains

The five standard *Salmonella* tester strains used in the assay are described in the table below. TA1535 has a missense mutation at the *hisG46* locus and is used to detect mutagens that cause base-pair substitutions (e.g., replacement of one nucleotide base pair in DNA by another). Strains TA1537 and TA1538 carry a frameshift mutation, *hisC3076* and *hisD3052*, respectively, and are used to detect chemicals that cause frameshift mutations.

Strains TA100 and TA98 are derived from TA1535 and TA1538, respectively, and contain the resistance transfer factor plasmid (R-factor pKM101). The R-factor increases sensitivity to certain mutagens, possibly through error-prone repair, confers resistance to the antibiotic ampicillin, and makes strain TA100 sensitive to some frameshift mutagens (Table 2). For example, TA1535 will not detect aflatoxin B, or benzo-a-pyrene, while TA100 will; TA1538 is not sensitive to sterigmatocystin or benzyl chloride, while strain TA98 is.

The five *Salmonella* strains used in the Ames test may be obtained from Dr. Bruce N. Ames, Biochemistry Department, University of California, Berkeley, California 94720. The bacteria are impregnated onto small absorbent discs and are mailed upon written request (together with the latest "Supplement to the Methods Paper").

Processing, Regeneration and Storage

a. Upon receipt of new tester strains, streak each disc onto an individual nutrient agar plate and drop each disc into individual tubes or flasks (approximately 50 ml volume) containing 10 ml of sterile nutrient broth. Close tubes or flasks with closure that allows gaseous exchange, and shake gently at about 120 rpm. As soon as possible, return to the plates you streaked and cross-streak them with a sterile loop to distribute the cells and facilitate obtaining individual colonies. These plates are designated for convenience "Reserve Plates." Incubate plates at 37°C for 24 hours. Place in refrigerator at 4°C. If strains check out (Part C, below) satisfactorily, discard "Reserve Plates." If a tester strain does not check out (i.e., for spontaneous revertant value, genetic markers, and mutagenic activity using a standard mutagen), pick 4 or 5 isolated colonies from the "Reserve Plate" to prepare overnight nutrient-broth cultures and repeat the check-out procedures. Use the isolated culture which best demonstrates the overall desirable characteristics of the strain to prepare frozen permanent stocks.

b. Incubate nutrient-broth cultures of each tester strain with gentle shaking at 37°C for 14 to 18 hours.

c. Pipette 0.8 ml of nutrient-broth culture into the desired number of 2-ml, sterile, glass vials with teflon-lined screwcaps. Add 70 µl of dimethyl sulfoxide (DMSO). Consider one set of frozen permanents as "Routine Use" and the other, "Master Copy" frozen permanents.

   Quick-freeze vials in dry ice and store in freezer at -80°C or lower until needed.

Master Plates

To avoid frequently opening frozen permanents, "Master Plates" can be used as the source of inoculum for overnight cultures used in assays.

To prepare Master Plates, use Minimal Agar plates to which histidine and biotin (and ampicillin for TA98 and TA100) have been added 1 to 2 days prior to inoculation. Spread 0.1 ml of sterile 0.1 M L-histidine and 0.1 ml of 0.5 mM biotin on the surface of each Minimal Agar plate and label properly with a waterproof marker pen. For those Master Plates that will be used to maintain TA98 and TA100 cultures, add 0.1 ml of 7 mg/ml ampicillin to each plate to help retain the R factor. Allow the histidine, biotin and ampicillin solutions to be absorbed into the agar for 24 to 48 hours before inoculation. Streak each prepared Minimal Agar plate with overnight nutrient-broth culture of each tester strain. Incubate for 2 days at 37°C and store in a refrigerator at 4°C. Plates may be used for up to 4 weeks.

Optional Method of Processing Tester Strains

Based on the modifications to the Ames test described by Belser et al. (1981) the following changes may be adopted.

Preparation of Initial Fresh Nutrient Broth Cultures:

- Use 30 ml of sterile nutrient broth in a 150 ml erlenmeyer flask to prepare initial cultures.
- Adjust the bacterial count of the fresh cultures to 1 to 2 x 10^8 cells/ml using a spectrophotometer at 550 nm or a Coulter counter (Salmeen and Durisin, 1981) and standard curves developed within the laboratory to relate instrumental measurements to plate counts of serial culture dilutions. Add DMSO to a final concentration of 8 percent.
- Prepare frozen permanents, each consisting of 0.5 ml of overnight nutrient-broth culture. Quick-freeze, e.g., in a tray of fragmented dry ice and store at -80°C.
- To conduct a mutagenicity assay use entire 0.5 ml of frozen culture to prepare a fresh nutrient-broth culture that is then used as inoculum for the assay (i.e. to add to top agar, etc.).

Checking Characteristics of Tester Strains

Spontaneous Reversion Count

Spontaneous reversion (SR) count tests should be performed upon receipt of the tester strains and in triplicate whenever a mutagenicity test is performed. Results should be maintained as part of the permanent quality control record. These values serve as an indicator of strain activity and as a

Table 2. Genotype of Five Salmonella *Tester Strains Used for General Screening in Mutagen Testing*

Strain	*Mutation*	*Repair*	*LPS*	*R-Factor*
TA1535	*Missense Mutation* hisG46	uvrB	rfa	-
TA100	*Missense Mutation* hisG46	uvrB	rfa	*pKM101*
TA1537	*Frameshift Mutation* hisC3076	uvrB	rfa	-
TA1538	*Frameshift Mutation* hisD3052	uvrB	rfa	-
TA98	*Frameshift Mutation* hisD3052	uvrB	rfa	*pKM101*

negative control for solvent controls used in the mutagenicity test. If the SR values for the negative and solvent controls are significantly different, the test data should be suspect. SR tests are conducted as follows:

a. Prepare top agar (Appendix I). Melt prepared top agar in autoclave for three minutes at 121°C, or melt the agar in a microwave oven. Maintain the molten top agar at 45°C in a waterbath or warming oven.

b. Prepare histidine/biotin solution (Appendix I). Warm histidine/biotin solution in 45°C waterbath. Add 20 ml histidine/biotin solution per 200 ml top agar.

c. Warm the Minimal Agar plates in the incubator at 37°C for 24 hours. This facilitates the formation of a uniform layer of top agar after pouring, and it verifies the sterility of the Minimal Agar plates.

d. Place the required number of sterile, capped, 13 x 100 mm culture tubes into a heating block or waterbath preheated to 45°C. Pipet 2 ml molten top agar (with trace of biotin and histidine) into each tube.

The following steps should be performed in an appropriate hood or glove box.

e. For each strain, add 0.1 ml of fresh nutrient-broth culture containing 1 x 10^8 cells to each of three pairs of tubes. The organisms should remain at 45°C no longer than about 2 minutes to avoid excessive cell death.

f. When performed as part of a mutagenicity assay, add 0.5 ml of the *High S-9 Mix* to each of the first tubes; add 0.5 ml of the *Low S-9 Mix* to each of the second pair of tubes; and add 0.5 ml of 0.2M phosphate buffer (pH 7.4) to each of the third pair of tubes.

g. Mix the material in each tube with a vortex mixer (gently, to prevent bubble formation in the top agar). The addition and mixing in of the S-9 Mix should be carried out in the shortest practicable time (seconds) to avoid inactivation of the enzymes.

h. Pour the contents of each tube onto the surface of a prewarmed Minimal Agar plate. Gently tilt and rotate each plate to spread the top agar over the surface of the minimal agar. Place on a level dry surface, vibrate gently to uniformly distribute the top agar (a mechanical vibrating device, e.g., a vibrating table [Belser et al. 1981] may assist in obtaining uniform distribution), and allow to harden.

i. Incubate the plates at 37°C for 48 hours in an inverted position.

j. Examine the background lawn for proper growth, with and without a dissecting microscope.

k. Count the number of spontaneous revertant colonies and record. Determine whether or not the number of revertant colonies per plate is within an acceptable range.

Expected Spontaneous Revertants for Each Strain

Strain	*No. of Colonies*
TA1535	10-35
TA1537	3-15
TA1538	15-35
TA98	30-50
TA100	120-200

From: Supplement to the Methods Paper (Methods Paper is Ames et al. 1975), revised February 1981.

Note: Revertant values may be slightly higher or lower on plates containing S-9 mixes. Each laboratory should establish an acceptable range of spontaneous revertants per plate to determine if the strains are responding properly. R-factor loss is usually indicated by one or more of the following: ampicillin sensitivity; reduced SR values; and increased sensitivity to uv radiation.

Histidine Requirement (Check as part of each assay)

a. Prepare two minimal agar plates by coating *each plate* with 0.1 ml of sterile 0.5 mM biotin. Spread 0.1 ml of sterile 0.1 M histidine onto *one* of the plates.

b. Apply a single cross-streak of broth culture to each plate with each test strain. Incubate plates at 37°C for 24 hours.

Each strain should show growth on the plate containing histidine; no growth should occur on the plate containing only biotin.

Crystal-violet (rfa character)/ Ampicillin Sensitivity (R-factor) (Check as part of each assay)

a. Pipet 0.1 ml fresh nutrient-broth culture into 2 ml top agar (with trace of histidine and biotin). Pour mixture onto a Nutrient Agar plate and allow to solidify. Using sterile tweezers, place a filter-paper disc (with 10 μg of crystal-violet) off-center on the plate.

b. Place a 10 μg ampicillin-impregnated disc (Difco 6363 "Dispens-o-Disc") off-center, opposite the crystal-violet disc. Use a separate plate for each test strain. Incubate 24 hours at 37°C.

All test strains should have a zone of growth inhibition (approximately 14 mm diameter) around the crystal-violet, indicating the presence of the *rfa* mutation. Strains TA1535, TA1537 and TA1538 should show a zone of growth inhibition around the ampicillin disc. Strains TA98 and TA100 (containing the R-factor) should not be inhibited by the ampicillin.

uv Sensitivity (uvrB-deletion) (Check each strain at least monthly)

The following procedure is suggested as an option to that described in Ames et al. (1975). Cross-streak each test strain on a separate nutrient agar plate. Divide each plate into four approximately equal zones by marking three parallel lines on the bottom of the plates with a waterproof marker. Remove lid, mask all but one of the zones, and irradiate the exposed zone under a 15-watt germicidal lamp at a distance of 33 cm (13 in) for 3 seconds. Move the mask to expose both the previously irradiated zone and the next (adjacent) zone. Repeat the 3-second irradiation. Move mask again so that only the final zone is shielded and repeat 3-second irradiation. At this point zones 1-4 will have been exposed to 9, 6, 3 and 0 sec irradiation, respectively. (Note: The output of a uv light tube diminishes with use. This may require compensatory exposure adjustment). Use caution to avoid looking into the uv lamp or exposing skin surfaces to any unnecessary radiation. Immediately cover and place plate in the dark to prevent photoreactivation.

Incubate all plates at 37°C for 18-24 hours. All strains should retain the *uvrB* deletion: No growth should appear in any of the zones exposed to 9-second irradiation. Plates containing TA1535, TA1537, and TA1538 should show no growth at 6-second exposures either, although slight growth may be visible with TA98 and TA100 strains. Slight to moderate growth is likely in zones exposed to only 3-second irradiation. All unexposed zones should show active growth. The intermediate growth at 3 seconds and marginal growth at 6 seconds provide a rough baseline — for the relative resistance of the tester strains — that should be compared on a regular basis to detect changes in test-strain resistance.

Standard Mutagens

Standard mutagens (positive controls) shall be included *with each assay* to confirm proper tester-strain mutagenic activity and specificity. Standard mutagens may be purchased in diluted quantities from a commercial laboratory. Some mutagenic materials (e.g., 2-Anthramine) are unstable while others

(e g , sodium azide) may be useable for 6 months or more When in doubt, make up standard mutagens fresh each time they are needed if adequate high-hazard facilities are available Dose-response curves should be established for each standard mutagen This curve is used to monitor and possibly adjust the mutagen concentration over time Each laboratory shall use positive controls at concentrations yielding reproducible counts to confirm the reversion properties or mutagenic activity of each strain The standard mutagens are also used to evaluate the metabolic activity of newly prepared S-9 mixtures It is recommended that dose levels of standard mutagens be selected (from the dose-response curves) that generally yield reversion values more than five times the SR value for TA1537 and more than three times the SR value for TA1535, TA1538, TA98 and TA100

Table 3 contains a listing of mutagens found to be useful in plate-incorporation assays for confirming activity of the strains indicated opposite them Often the choice of controls may be influenced by the suspected chemical characteristics of the sample

S-9 Activity

Since the protein content, and hence the enzymatic activity, of S-9 batches can vary widely, it is recommended that each batch of S-9 be tested with standard mutagens and tester strains of known characteristics The following procedure may be used to determine, and adjust if necessary, the activity of a specific batch of S-9

1 Prepare S-9 mix with four different levels (20%, 10%, 5% and 2%) of S-9 preparation
2 Conduct plate-incorporation assays with strain TA1538 using a single dose level of 25 μg of 2-aminofluorene (as the standard mutagen) and each of the four S-9 mixes
3 Concurrently repeat assays using a single dose level of 5 μg benzo-a-pyrene (as a second standard mutagen)
4 Construct dose response curves for each set of data, plotting S-9 percentage against resulting plate counts
5 Compare response levels to those made with previous batches (or preparations) of S-9
6 Adjust, if required, the percentage of S-9 added to the High S-9 and Low S-9 mixes to yield desired revertant levels based upon the response curves obtained with the standard mutagens

Table 3. *Standard Mutagens for Confirming Tester Strain Function*

Mutagen[1]	*Amount/ Plate*[2]	*RLE (S-9)*	*Test Strains Positive*				
			TA1535	*TA1537*	*TA1538*	*TA98*	*TA100*
Sodium azide	*1 μg*[3]	—	X	—	—	—	X
N-Methyl-N'-nitro-N-nitrosoguanidine	*5 μg*	—	X	—	—	—	X
Methylmethanesulfonate (1:50 dilution)	*25 μl*	—	—	—	—	—	X
2-nitrofluorene	*10 μg*	—	—	—	X	X	—
9-aminoacridine	*150 μg*[3]	—	—	X	—	—	—
Daunomycin HCl	*50 μg*	—	—	—	—	X	—
Hycanthone	*2.5 μg*[3]	—	—	—	—	X	—
2-anthramine	*2 μg*[3]	X	X	X	X	X	X
Benzo(a)pyrene	*5 μg*[3]	X	—	X	X	X	X
2-aminofluorene	*25 μg*	X	—	—	X	X	X
Dimethylbenzanthracene	*10 μg*	X	—	—	—	—	X

[1] *All chemical solids are dissolved (1 mg/ml) in DMSO except for Daunomycin and sodium azide, which are dissolved (1 mg/ml) in distilled water. Many of the standard mutagens are available in diluted form from Nanogens International, P O. Box 1025, Watsonville, CA 95076. Additional standard mutagens and sources for obtaining them are provided in Ames (1981).*
[2] *Recommended starting levels; modify concentration as necessary to determine acceptable levels (that reproducibly yield expected results with each strain).*
[3] *Values obtained from McCann, et al (1975).*

Section 5
Quality Control/Quality Assurance

General Requirements

Quality control refers to those procedures that are implemented by the toxicologist, microbiologist, staff members, and supervisor to reduce the variability and bias associated with data generated by their bioanalytical testing programs, and to increase the reliability of the test methods used. The end result of a carefully administered quality control program will, hopefully, be testing data of known accuracy and precision. Necessary steps in conducting a successful testing program include:

- Selection of an appropriate experimental design;
- Rigid adherence to appropriate test protocols and standard operating procedures;
- Assurance of test and data security;
- Careful interpretation and review of test data; and
- Accurate reporting of test results.

The first requirement of good science, that results can be verified by other investigators at other times and places, is a sufficient imperative to adopt and routinely follow a standardized procedure. At the same time, it is vital to have a mechanism to enable significant improvements in testing methodology to be incorporated into the standardized protocols without unnecessary delay so that the user community can benefit from these advances. The principal element of the mechanism is a substantial data base to support the recommended changes (improvements).

Security of the testing operation and of the resulting data must be provided to minimize the loss of irreplaceable testing data. Adherence to standard "good laboratory practices" will help a great deal. Restriction of test-area access to authorized personnel only must be rigorously enforced. This is important from a safety as well as a security standpoint. Responsibilities for control of data records must be clearly defined. Where sample volumes permit, "library samples" (aliquots of the sample material) should be maintained under proper storage conditions, for the duration of the experiment, to allow reruns of samples yielding ambiguous or questionable test data.

Maintenance of the integrity of test data depends upon control over the performance of the experiment, adherence to details of the measurement process, and careful handling of the data. Errors can arise during handling of the data due to transcription, clerical, or typing mistakes; as a result of the use of different statistical methods at different times; computer mistakes or omissions; inclusion of the wrong data; omission of parts of the original data; differences in observational results (as between two microbiologists); and changes in interpretation of the data.

At no point in the testing process is the skill and experience of the analyst/researcher more important than in the interpretation of the test data. Until such time as the analyst has acquired extensive experience in evaluating test data and drawing appropriate inferences therefrom, he should make maximum use of external laboratories for review and confirmation of his findings. A formalized program of data exchange for independent analysis is of great mutual value to the collaborating parties. Participation in interlaboratory studies with known and unknown sample materials (see Quality Assurance) is also of great benefit in establishing a laboratory's competence and in supplementing intralaboratory performance evaluation procedures.

Reports of test results, whether in the public literature or as proprietary submissions to a user group, should either include sufficient data (i.e., all tests, negative control, and positive control data) that the interpretations made by the investigator can be independently evaluated or such data should be readily available upon request. The use of a statistician or statistical staff experienced in the analysis and interpretation of biotesting data is highly recommended. Results of tests or studies should routinely be subjected to a review system prior to the preparation and/or publication of reports.

Quality Control Testing

Table 4 presents tests that are considered vital in a mutagenicity testing program to confirm the condition, genetic integrity, and responsiveness of the test organisms; the sterility of media and test additives; and the characteristics of the colonies resulting from the plate assay. Also included in Table 4 are indications as to when the tests should be performed and how test results may be used to indicate suitable conditions for going forward with the mutagenicity assay. The tests indicated should be performed *in addition to* the strain function confirmation tests presented earlier (i.e., uv radiation, ampicillin, and crystal-violet sensitivities, and the histidine requirement test).

Sterility of minimal or nutrient agar plates (Test SP) is determined by incubating all plates at 37°C for the 24 hours prior to running a QC test series or assay. Any plates showing growth (contamination) after the incubation period should be discarded and the source of contamination identified if possible. To determine the sterility of solvents, reagents, standard mutagens or of the activation mixture (S-9), spread 0.1 ml aliquots of the component in question in 2 ml of top agar onto nutrient agar plates by gently tilting and rotating the plates. Incubate the plates at 37°C for 24 hours, inspect the plates for microbial growth with and without the aid of a dissecting microscope. Discard contaminated materials or, if appropriate, resterilize.

Solvent and positive control tests (CS, PN and PA) are all performed concurrently with the mutagenicity assay, and colony counts are performed after the 48- or, if indicated, 72-hour incubation period. In solvent control tests a 50 μl addition of pure DMSO (or alternate solvent) substitutes for the 50 μl doses of test chemical or sample solutions (in the corresponding solvent) in the experiment. The data yielded by these "zero-dose" tests provide the spontaneous reversion value against which other dose-level data are compared in determining whether or not the sample material is considered positive for mutagenicity by this assay.

It is a useful practice — and one that should be mandatory in laboratories that have not yet developed an adequate data base of their own on spontaneous revertant values for each strain — to run a negative control (spontaneous reversion test; see Section 6) concurrently with each mutagenicity test. The negative control has neither test solution *nor* the equivalent volume of corresponding solvent added, whereas the solvent control incorporates a volume of solvent, equal to the total volume of test solution, into the top agar before pouring the overlay. Comparison of concurrent negative and solvent controls may detect either mutagenicity

Table 4. Quality Control Tests for Ames Plate Assay

Test Designation	Type	When Performed	Expected Results
SP	Sterility Check (minimal or nutrient agar plates).	All plates are incubated at 37°C overnight prior to the assay.	No growth (Discard any plates showing growth).
SH	Sterility Check (Histidine/biotin solution). Plate on nutrient agar.	Perform in duplicate each time histidine/biotin stock solution is prepared.	No growth (If growth, prepare and filter sterilize or autoclave fresh stock solution; recheck sterility).
SS	Sterility Check (Sample material). Plate on nutrient agar at highest test dose level prepared in appropriate solvent.	Perform in duplicate on day before each experiment.	No growth (If growth, filter sample material through 0.8 µ glass-fiber filter; use DMSO as solvent
SA	Sterility Check (S-9 mix, w/and w/out S-9). Plate on nutrient agar plates.	Perform in duplicate when new S-9 batch is received and for cofactor solutions as they are prepared.	No growth (If growth with mix w/out S-9, resterilize stock solutions and retest; if growth with S-9, filter sterilize with 0.45 µm filter).
SM	Sterility Check (Standard mutagens). Plate test levels (in appropriate solvent) on nutrient agar plates.	Perform in duplicate when new solutions of mutagens are received or prepared, or when tester strains are checked out.	No growth (If growth, for solutions - filter through 0.8 µm glass-fiber filter; Suspensions-leave in DMSO overnight).
CN	Negative Control (Spontaneous Reversion Test) Zero dose level, non-activated test conditions.	Perform in triplicate for each bacterial strain, when received and with each assay.	Background growth and spontaneous revertant colonies only.
CS	Solvent Controls Zero dose level, activated and non-activated test conditions.	Perform at least in duplicate for each bacterial strain and condition with each assay.	Background growth and spontaneous revertant colonies only.
PN	Positive Control (Function Check) Non-activated test conditions, direct-acting standard mutagens.	Perform for each test strain, when received and with each assay.	Background growth and revertant growth.

Table 4. (Continued)

Test Designation	Type	When Performed	Expected Results
PA	Positive Control (Function Check) Activated test conditions: standard mutagens requiring S-9 activation	Perform for each test strain, when received and with each assay.	Background growth and revertant growth
GC	Colony Genotype Check-Replica plate colonies onto Minimal Agar with biotin	When mutagenicity is indicated, check plates w/highest mutagenic activity ratio.	Growth of histidine revertant colonies; no growth of phenocopy colonies.
VC	Viability Check-Serial dilutions of overnight culture plated on nutrient agar.	When new tester strains are received, master plates are generated, or overnight cultures to be used in the assay are prepared.	Growth - compare with instrumental density measurements of same cultures to determine cells/ml

or toxicity resulting from the solvent. New solvents, solvent batches, or changes in distilled water supplies or treatment warrant such a comparison, even in laboratories where an adequate historical data base for spontaneous reversion of each tester strain has been developed.

Water or DMSO solutions of standard mutagens (see Table 3) known to yield positive test results (induced revertants ≥2 X spontaneous revertant rate, positive dose-response relationship and background growth within normal range[1]) with specific strains under known activation conditions are used in tests PN and PA, as the test chemical in the assay. These positive controls provide a means of confirming that the test strains are responding predictably and reproducibly. Over time, a large base of positive control data is developed within a testing laboratory. These data are useful in determining whether subsequent tester strains have acceptable mutagenic activity. In order to more quickly build such an adequate data base for each standard mutagen it may be advantageous to limit the number of such mutagens used to the minimum required to show normal mutagenic activity of the strains under the conditions of the testing program. Afterward, it may be useful to add selected standard mutagens (e.g., known chemicals of the same chemical class and with physical/chemical characteristics which approximate those of suspected components in test samples) to optimize the test conditions for a specific assay. A data base for these additional selected standard mutagens should be acquired as soon as feasible.

General criteria for determining the adequacy of *Salmonella* mutagenicity testing data are detailed in Section 6.

To determine if colonies counted are, in fact, true histidine-independent revertant colonies rather than anomalous growth of the histidine-dependent background, colonies should be replated on Minimal Agar medium supplemented with biotin. Histidine-independent cells will form new colonies on the Minimal Agar while the histidine-dependent cells will not grow. Perhaps the most effective way to accomplish this—especially if the number of colonies on the test plate is large—is to "replica plate" the colonies from the test plate to Minimal Agar. By this procedure surface colonies are transferred on a piece of clean, sterile, cotton velvet much as ink is transferred from stamp pad to paper on a rubber stamp. The pattern of surface colonies is reproduced on the minimal agar plate, and direct comparison of "donor" and "recipient" plates confirms the histidine independence of the colonies. Additional confirmation of the histidine independence of "background" colonies can be obtained by replica plating onto biotin-supplemented, Minimal Agar plates *with and without added histidine* and comparing the recipient plates. For successful transfer, the surface of the donor and recipient plates should be dry, and the velvet must be of good quality and "wettable," i.e., free of sizings and other additives (Larimer, pers comm.).[1]

Serial dilutions of overnight cultures of test strains should be plated on nutrient agar and incubated at 37°C overnight to determine the viable cell titer from each culture. Spectrophotometric, colorimetric, turbidimetric, or particle-counter measurements used to adjust culture densities do not estimate *viable* cell count, but rather reflect the density of bacterial material present. It is necessary to develop and periodically reconfirm standard curves within an individual laboratory to relate the viable cell count to instrumental measurements. Changes in a laboratory's standard curve over time may reflect changes in incubation conditions for the broth cultures (viable to total bacterial count) changes in sensitivity or linearity of the measurement procedure, or other problems which must be addressed. Salmeen and Durisin (1981) suggest that order-of-magnitude differences in initial viable cell count can modify plate counts, and the resulting slopes of dose-response relationships. Figure 3 is an example of a form for recording periodic measurements of instrument values relative to plate counts of culture dilutions.

Results of all quality control tests should be properly documented on QC data forms (see Figures 4, 5 and 6) and maintained as part of the permanent data records for the testing program. In addition, consistent with standard

[1]That is, the "background lawn" or growth is not absent or severely inhibited.

[1]Meeting in May 1981 with Dr. Frank Larimer, Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tenn.

Date	Strain/ Culture ID	Initials	Date	Strain/ Culture ID	Initials
Dilution[1]	**Instrument Reading**	**Plate Count**	**Dilution**[1]	**Instrument Reading**	**Plate Count**
10^{-6}			10^{-6}		
10^{-7}			10^{-7}		
10^{-8}			10^{-8}		
10^{-9}			10^{-9}		

Date	Strain/ Culture ID	Initials	Date	Strain/ Culture ID	Initials
Dilution[1]	**Instrument Reading**	**Plate Count**	**Dilution**[1]	**Instrument Reading**	**Plate Count**
10^{-6}			10^{-6}		
10^{-7}			10^{-7}		
10^{-8}			10^{-8}		
10^{-9}			10^{-9}		

Date	Strain/ Culture ID	Initials	Date	Strain/ Culture ID	Initials
Dilution[1]	**Instrument Reading**	**Plate Count**	**Dilution**[1]	**Instrument Reading**	**Plate Count**
10^{-6}			10^{-6}		
10^{-7}			10^{-7}		
10^{-8}			10^{-8}		
10^{-9}			10^{-9}		

1 *Dilute overnight cultures (approximately 10^{9} cells/ml) geometrically with sterile 0.9 percent NaCl.*

Figure 3. *Quality Control Data Form - cell titer and viability measurements.*

"good laboratory practices," the quality of water sources and the performance of laboratory equipment used in the testing program (i.e. waterbaths, autoclaves, incubators, refrigerators, freezers, and cell- and colony-counting equipment) should be periodically confirmed and given regular preventive maintenance in accordance with manufacturers' recommendations. Equipment and water quality QC data should be recorded (Figure 7) and maintained as part of the program's permanent data record.

STRAIN FUNCTION TESTS

Test		Date / Init.	TA1535	TA1537	TA1538	TA98	TA100
1. Solvent / Negative Control[1] (specify)	1						
	2						
	3						
	Average						
2. Ampicillin Sensitivity[2]							
3. Crystal Violet Sensitivity[2]							
4. U.V. Sensitivity[3]	3 sec						
5. Positive Controls[1]	6 sec						
Mutagen (Amt / plate)	9 sec						
a.	( )						
b.	( )						
c.	( )						
d	( )						
e	( )						

Test		Date / Init.	TA1535	TA1537	TA1538	TA98	TA100
1. Solvent / Negative Control[1] (specify)	1						
	2						
	3						
	Average						
2. Ampicillin Sensitivity[2]							
3. Crystal Violet Sensitivity[2]							
4. U.V. Sensitivity[3]	3 sec						
5. Positive Controls[1]	6 sec						
Mutagen (Amt / plate)	9 sec						
a	( )						
b.	( )						
c.	( )						
d.	( )						
e.	( )						

[1] Plate counts
[2] + = growth, - = no growth
[3] ++ = 10^{0} to 10^{-1} x control (not irradiated);
+ = 10^{-1} to 10^{-2} x control;
+ = 10^{-2} to <0 x control; - = no growth

Figure 4. Quality Control Data Form - strain function tests

CULTURE MEDIA RECORD

Media and Lot #	Receive	Open	AMT	pH	Data	Int	Media and Lot #	Receive	Open	AMT	pH	Data	Int

Figure 5. Quality Control Data Form - culture media record

REAGENTS, STANDARDS AND TEST MATERIALS
(Sterility, pH, etc.)

Material	Test	Result	Date	Init		Material	Test	Result	Date	Init

Figure 6. Quality Control Data Form - reagents, standards, and test materials

EQUIPMENT RECORD

Year: 19____
Month ____

Day:												
STILL												
Conductance												
pH												
Drained												
WATERBATHS												
1)												
2)												
3)												
4)												
5)												
AUTOCLAVE												
Pressure												
Temp. (max)												
OVEN												
Temp. (max)												
INCUBATORS												
1)												
2)												
3)												
4)												
5)												
REFRIGERATORS												
1)												
2)												
FREEZERS												
1)												
2)												
3)												
COLONY COUNTERS												
1)												
2)												
OTHER (Specify)												

Figure 7. Quality Control Data Form - equipment record.

Section 6
Data Analysis, Interpretation and Reports

Data Analysis

Steps in Evaluation of Data

Chu et al. (1981) present a series of steps that they have employed in evaluating large volumes of Ames test data from collaborating laboratories:

1. Identification and removal of spurious plate counts;
2. Determination of the adequacy of the remaining data for making decisions on the mutagenicity of the test chemical;
3. Performance of statistical tests; and
4. Interpretation of the results.

This scheme should be followed in evaluating Ames test data

Adequacy of Test Data

Five basic conditions outlined by Dunkel and Chu (1980) for defining the adequacy of test data and removing spurious data have been adopted. These criteria for data acceptance are:

1. Bacterial strain checks must be satisfactory, i.e. the crystal violet and ampicillin checks for strain characteristics should show all strains to be sensitive to crystal violet, and strains TA1535, TA1537 and TA1538 should be sensitive to ampicillin. Strains TA98 and TA100 should be ampicillin resistant.
2. Negative and solvent controls (spontaneous reversion values) must be acceptable, i.e. 2 of 2 or at least 2 of 3 (if in triplicate) negative and solvent control plate counts must fall within empirical 95 percent laboratory-control confidence limits (determined within each laboratory for each tester strain).
3. Positive controls must be acceptable, i.e. 2 of 2 or at least 2 of 3 (if in triplicate) standard mutagen plate counts should exceed the 97.5th percentile of the historical laboratory negative/solvent control single plate count (historical average spontaneous revertant rate for that strain).
4. Four acceptable dose levels must be demonstrated in addition to the solvent control. An acceptable dose has to have at least two acceptable plate counts and not exhibit toxicity. Toxic dose level was defined as any dose level which was greater than that dose eliciting the highest average response (HAR) and in which every plate count was less than the lowest count in the HAR dose level. Outlier plate counts were identified by a Studentized range procedure and eliminated. Unless at least two plate counts were "within range" the dose level was considered unacceptable and eliminated.
5. The test could have no more than one unacceptable dose level lower than that dose giving the highest average response.

In addition to the above conditions (acceptance criteria), replica plating should confirm absence of "non-revertant" (phenocopy) colonies, and all sterility checks must be negative. In addition, all test data obtained from plates with atypically sparse background lawn should be considered questionable. Unfortunately, hard and fast rules with respect to background lawn have not been developed. A recent study (Salmeen and Durisin, 1981) was conducted to quantify background lawn using photomicrographs of plates inoculated over a range of cell concentrations. Tests which directly measure toxicity (Waleh et al., in press) show promise for being successfully coupled to the Ames assay. Additional studies of this nature should be conducted so that unambiguous guidance can be provided as to what constitutes an "acceptable" background growth (both in nature and extent).

Graphical Approaches

A great deal of information about the dose-response nature of an Ames test is obtained by plotting the average plate counts (Y axis) against the corresponding dose level in milligrams (X axis). It may be convenient to plot the data points on log-log paper so that the negative control (spontaneous revertant rate, zero-dose level) count is clearly resolved from the zero line. The spontaneous reversion value of the appropriate strain should be superimposed as a horizontal straight line (see example, Figure 8) as an aid in visually assessing the test-dose responses. The graph may include plots of several "tests" (e.g., for all five strains under one activation condition or a single strain under multiple activation conditions). Appropriate spontaneous reversion values should be included for each strain/condition plotted.

Graphs should be developed for both screening and confirmation level testing. Inflections of dose-response curves in screening tests are used as an aid in selecting the dosing regimen for the confirmatory tests. The shape of resulting plots can also be used to select an appropriate probability model for application to the data (Sexton et al. 1981). All reports associated with graphical representations should include the raw data from which the average plate counts for negative/solvent controls and each test dose are calculated.

Information on automated procedures for graphical and statistical presentation/evaluation of mutagenicity data using the In Vitro Information System (IVIS) is presented in Linhart, et al. (1980).

Statistical Approaches

A generally accepted statistical test for examining the results of the *Salmonella* plate test has not been published (de Serres and Shelby, 1979; Dunkel and Chu, 1980). However, a number of statistical techniques can be applied to the evaluation of Ames Test data. Some of these techniques are useful for determining the adequacy of data for further analysis, e.g., to detect changes in spontaneous revertant values over time, determine the homogeneity of variances among control and test data, determine control limits for standard mutagen response, and identify outlier data points. Other statistical tests are useful to detect the presence and nature of dose effects, e.g., tests of the homogeneity of treatment means and tests for linear trends (dose-response relationships). The statistical tests generally assume that the data are Gaussian distributions; therefore, count and dose data usually must be log-transformed before testing. Dose data should be "coded" (i.e. multiplied by a constant or added to 1 so all values are greater than unity) before transformation to avoid negative logarithms. Table 5 presents a number of useful statistical tests which should be employed to determine (1) the adequacy of the data and (2) the "positiveness" of the test results.

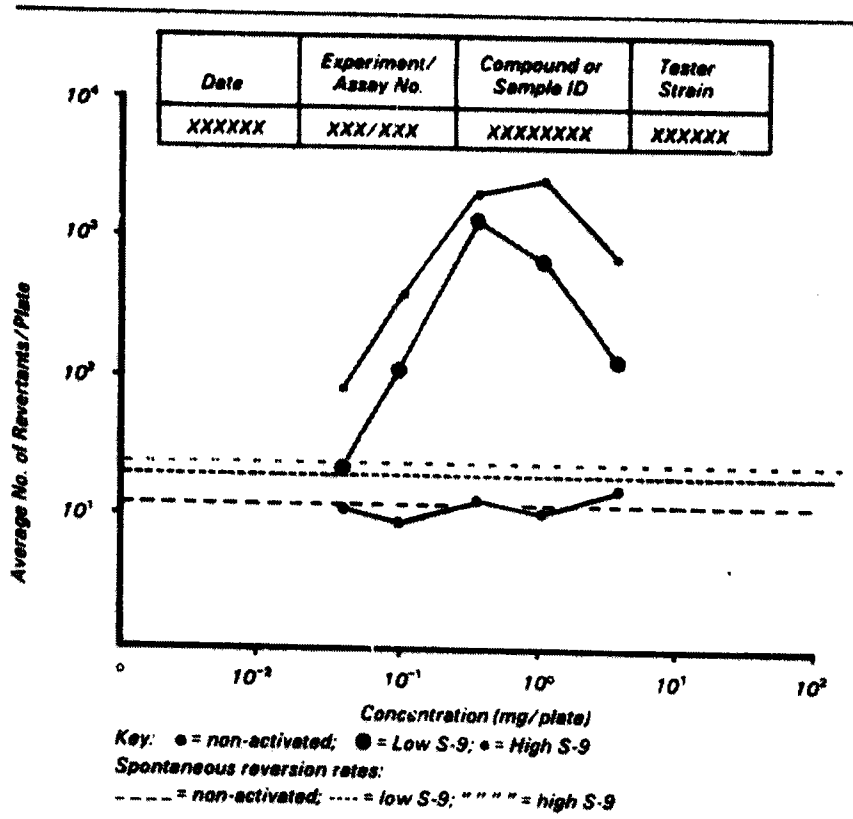


Figure 8. *Example of graphical presentation of* Salmonella *mutagenicity test data (simulated).*

Table 5. *Statistical Techniques Useful for Evaluating Mutagenicity Testing Data*

Test	Reference	Used to Determine
One-Way Analysis of Variance	*Myers, 1979*	*Differences among means in control and test data. Changes in average control counts over time.*
Linear Regression Analysis	*Graybill, 1976 Draper and Smith, 1966*	*Slope and significance of linear data trends.*
Bartlett's Test for the Homogeneity of Variances	*Sokal and Rohlf, 1969*	*Homogeneity of variances among control and test data.*
Confidence Interval Threshold	*Dunkel and Chu, 1980*	*Responses greater than a threshold value (a function of concurrent and historical negative control statistics).*
95% Confidence Interval	*Freund, 1979*	*Acceptability of positive control response data; acceptability of concurrent negative control data (spontaneous revertant value).*
Studentized Range Procedure	*Myers, 1979*	*Outlier values in test and control data.*

Interpretation

Positive and Negative Results

Seven methods were tested with Ames Test data for 17 pure compounds in a major interlaboratory comparison study (Dunkel and Chu, 1980) to determine their effectiveness in making mutagenicity decisions. Rates of disagreement with the consensus determinations of the four laboratories were calculated for each of the following "decision rules":

Decision Rule	False Positive Rate	False Negative Rate
Two-fold [increase] rule	7.1%	1.6%
Modified two-fold [increase] rule	4.1%	1.8%
Positive linear trend	20.0%	0.4%
Positive homogeneity	18.0%	0.7%
Combined statistical tests (linear trend and homogeneity)	11.6%	0.2%
97.5 th percentile	1.8%	3.8%
Confidence interval	1.4%	4.1%

(Dunkel and Chu, 1980)

The two-fold increase rule (Ames et al., 1975) is widely applied to Ames test data as an indicator of positive mutagenic test response. With the two-fold increase rule a test on a single strain of bacteria was considered positive if there was a dose level with an average response that was twice that of the concurrent negative/solvent control. With the *modified two-fold increase rule*, a test was considered positive if two consecutive dose levels (or the highest non-toxic dose level) produced average responses at least twice that of the negative/solvent control *and* at least two of these consecutive doses showed a dose-response relationship.

Tests for linear trends are based upon regression analysis of log-log transformed data (log counts and log (dose + 1.0)) in which the null hypothesis is that the slope is equal to zero. The test is considered positive if the linear trend statistic is significant, i.e., the probability that the departure of the slope from zero is a result of chance alone is less than 5 percent ($P < 0.05$). The test for homogeneity compares the (log-transformed)

responses for each dose using the one-way analysis of variance. The null hypothesis is that the means of the responses for all dose levels are equal. A test result is considered positive if the homogeneity statistic is significant, i.e. the probability that the differences between average responses is a result of chance alone is less than 5 percent ($P < 0.05$).

When the tests for linear trend and homogeneity were combined, each had to be significant ($P < 0.05$ in each test) for the results to be considered positive. Note the very low (0.2%) rate—with the combination of statistical tests—at which tests indicated no mutagenicity when laboratory consensus indicated the compound to be mutagenic.

The 97.5 th Percentile Rule compares the responses for each dose to the empirical 97.5 th percentile of the laboratory negative/solvent control for single plate counts to identify any dose levels having two or more responses (from triplicate plates) greater than the 97.5 th percentile of the laboratory historical controls. A test was considered positive if there were at least two out of three consecutive dose levels above the 97.5 th percentile of the laboratory historical control, and the consecutive doses showed a dose-response relationship. In addition, a test was also considered positive if the highest non-toxic dose was above the 97.5 th percentile.

The Confidence Interval Rule identifies average dose level responses that exceed a threshold level $Y_o + K \cdot SD(h)$, where Y_o equals the average concurrent control value, K is a constant which takes into account the significance level and the number of plates used, and SD(h) is the standard deviation of the log-transformed laboratory historical negative/solvent controls for the strain being used. If the threshold was exceeded by two consecutive dose levels or the last non-toxic dose, and at least two consecutive dose levels showed a dose-response relationship, the test was considered positive.

Several models based upon Poisson (Stead et al. 1981) and negative binomial distributions (Sexton et al. 1981; Margolin et al. 1981) have recently been developed and tested and show promise for increasing the objectivity of Ames test data interpretation.

The selection of test(s) to be applied to Ames test data should be based, in part, on the purpose of the study and the implications of the study findings. For example, it is vital that the tests selected to evaluate data upon which the safety of a consumer product (public health concern) is determined yield the lowest practicable rate of false negatives. That is, we cannot afford to err in a direction that would jeopardize public health by incorrectly ascribing a negative finding to a positive mutagen. Among the tests compared by Dunkel and coworkers, the linear trend, homogeneity, and combined tests yielded the lowest false-negative rate.

On the other hand results of tests which yield an unacceptably high false-positive rate can cause public alarm, adverse economic impacts, and loss or delayed development of useful chemicals or products. The 97.5 th percentile and confidence interval tests yielded the lowest rates of false positives, that is, branding as mutagenic those chemicals that, by laboratory consensus, were not in fact mutagenic by the Ames Test.

As a single test, the modified two-fold rule gave relatively low false-negative *and* false-positive rates. The authors caution, however, that some modification of the two-fold rule was probably used in the decision-making process at each collaborating laboratory and, as a result, one would *expect* good agreement between the two-fold tests and the consensus determinations.

It is recommended that *at a minimum* all plate-incorporation assay data should be tested with the modified two-fold rule. Other tests should also be applied as appropriate to the purpose of the Ames testing activity so that decisions can be made with a high degree of confidence and objectivity.

Ames test results must be reproducible (i.e. from screening test to confirmatory test or among repeated confirmatory tests) before a final decision is made as to whether a sample is mutagenic or not by the *Salmonella* reverse-mutation test system used. Negative statements should not be the natural offspring of inconclusive data, but rather the result of repeated testing which confirms the hypothesis of no difference between negative-control and test-dose counts. Even then, a negative result does not necessarily mean the sample is non-mutagenic, but rather that no mutagenic effect was detectable under the conditions of the test system used. The Ames test measures mutations at specific base sequences in bacterial DNA; it does *not* indicate overall mutagenic potential. Sample materials should show repeatable negative responses when tested at levels of up to 5 - 10 mg/plate (toxicity and solubility permitting) before discontinuing testing. If the confirmatory test that follows a "positive" screening test is negative, recheck the suitability of the test conditions relative to those of the screening test, adjust as appropriate and rerun. Discontinue Ames testing if this retest fails to meet criteria for a positive test and is not confounded by toxic effects.

The tests presented here, and others used by investigators around the world, are simply tools to aid the researcher in making a correct decision based upon the available data. They are not a substitute for the judgement and expertise in Ames test data interpretation developed with years of experience. Data exchanges and independent confirmation of data interpretation are strongly recommended to minimize the chances of making incorrect decisions.

Inconclusive Results

Occasionally a test will yield data that suggest mutagenic activity but do not meet one of the criteria for acceptance as positive. Although these cases can often be resolved by modifying the test conditions (e.g., increasing the number of closely-spaced test doses or optimizing the amount or type of S-9 activation for the sample material), some samples have such a limited range between induction of mutagenic response and cytotoxicity that they cannot be readily characterized with conventional plate-assay procedures. Several options are available to the researcher in such cases:

- Retest with preincubation or suspension assays.
- Employ chemical fractionation schemes to separate, if possible, the mutagenic fraction from other cytotoxic components, and rerun.
- Recommend testing with an alternate mutagenicity test system, e.g., *Saccharomyces* forward/reverse mutation tests.
- Discontinue testing, and report results as inconclusive.

Once again, the option(s) selected may be dictated, in part, by the specific purposes of the testing program being undertaken.

A number of sample preparation/chemical fractionation schemes have been proposed and used by EPA's National Enforcement Investigations Center, Denver, Colorado; EPA's Health Effects Laboratory, Research Triangle Park, North Carolina; Oak Ridge National Laboratory, Oak Ridge, Tennessee; and others to identify those fractions of complex industrial and environmental samples that show mutagenicity. Sample preparative procedures and recommendations for their use with Ames testing will be reported in the near future.

Reporting Test Results

Data Records

Test data should be entered directly onto forms from which the data may be keypunched and input to a database system such as the Interim In Vitro System developed by EPA. A description of the system, reporting forms, and general instructions for their use have been published (Sexton et al. 1981).

The reporting forms developed for the HERL IN VITRO system (Figures 9, 10 and 11) are quite complete and are an excellent record for the laboratory's permanent data files. The system is undergoing some revision at this time (Claxton, pers. comm.)[1] to expand the listings of coded elements and clarify user instructions. The updated listings and instructions for the coded reporting forms can be obtained from Mr. Andrew Stead, MD-57, EPA, Research Triangle Park, NC 27711. Use of the reporting forms is strongly recommended, whether or not the investigator chooses to make use of the data base service at this time or in the future.

Data Presentation

While there are some distinct advantages to standardizing the output format for presenting Ames test data (e.g., to facilitate comparison of findings), it is unlikely that such standardization will be accomplished in the foreseeable future. However, for Ames test data to be acceptable and to make independent evaluation possible, minimum requirements must be met. We agree with the consensus findings reported by de Serres and Shelby (1979) that the following should be essential elements in any Ames testing report:

1. Means and indications of variability (e.g., standard deviation) of the plate counts for the negative control, the positive controls, and each dose of the test compound;
2. The number of replicate plates in each mean; and
3. When possible, individual plate counts.

If the volume of data is prohibitive to report, complete data should be readily available from the investigator upon request.

In addition, the criteria used within the reporting laboratory to determine a positive test should be clearly stated.

[1]Telephone conversation, July 1981 with Dr. Larry Claxton, Genetic Toxicology Division, Health Effects Research Laboratory (EPA), Research Triangle Park, N.C.

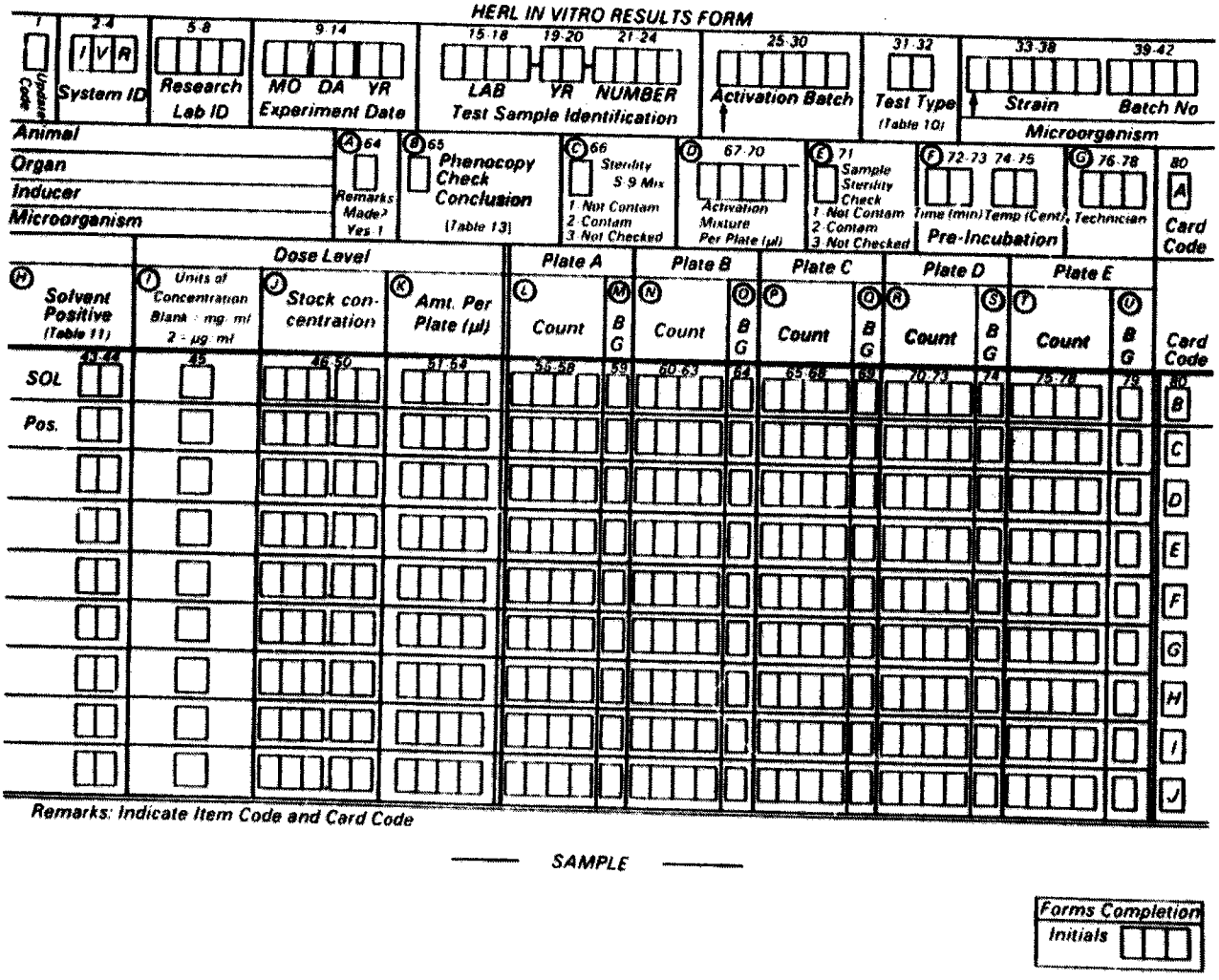


Figure 9. HERL IN VITRO System - results form.

HERL IN VITRO RESULTS CONTINUATION FORM

1 Update Code	2-4 System ID	5-8 Research Lab ID	9-14 MO DA YR Experiment Date	15-18 Lab	19-20 YR	21-24 Number Test Sample Identification	25-30 Activation Batch	31-32 Test Type (Table 10)	33-36 Strain	39-42 Batch No. Microorganism

		Dose Level		Plate A		Plate B		Plate C		Plate D		Plate E		
(B) Solvent Positive (Table 11)	(J) Units of Concentration Blank = mg/ml 2 = µg/ml	(K) Stock Concentration	(L) Amt. Per Plate (µl)	(M) Count	(N) BG	(O) Count	(P) BG	(Q) Count	(R) BG	(S) Count	(T) BG	(U) Count	(V) BG	Card Code
43-44	45	46-50	51-54	55-58	59	60-63	64	65-68	69	70-73	74	75-78	79	80
														K
														L
														M
														N
														O
														P
														Q
														R
														S
														T
														U
														V
														W
				SAMPLE										X
														Y

Figure 10.HERL IN VITRO System - results continuation form.

HERL IN VITRO SYSTEM
SAMPLE IDENTIFICATION FORM (INTERIM)

SAMPLE

System ID
I V S
2-4

Lab	Sample ID Yr	No	Sample Description
5-8	9-10	11-14	15-56
5-8	9-10	11-14	15-56
5-8	9-10	11-14	15-56
5-8	9-10	11-14	15-56
5-8	9-10	11-14	15-56
5-8	9-10	11-14	15-56
5-8	9-10	11-14	15-56
5-8	9-10	11-14	15-56

Forms Completion
Initials

Figure 11. HERL IN VITRO System - sample identification form (interim).

References

American Public Health Association. 1975. Standard Methods for the Examination of Water and Wastewater. 14th Edition. M.C. Rand et al., eds. APHA, Washington, D.C.

Ames, B.N. 1981. Supplement to the Methods Paper [Ames et al. 1975]. Revised February 1981. (Available upon written request from Dorothy Maron, Ames Lab, Department of Biochemistry, University of California, Berkeley, CA 94720).

Ames, B.N., J. McCann and E. Yamasaki. 1975. Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. Mutat. Res. 31:347-364. (Also, as revised April 1981).

Ashwood-Smith, M.J. 1979. Stability of frozen microsome preperations for use in the Ames' *Salmonella* mutagenicity assay. Mutat. Res. 69:199-200.

Belser, W.L., S.D. Shaffer, R.D. Bliss, P.M. Hynds, L. Yamamoto, J.N. Pitts and J.A. Winer. 1981. A standardized procedure for quantification of the Ames *Salmonella*/mammalian-microsome mutagenicity test. Env. Mutag. 3:123-139.

Brusick, D.J., D.W. Matheson, D.R. Jaganath. 1980. Commercial screening of environmental chemicals. *In* Chemical Mutagens, Vol. 6. Eds. de Serres and Hollander, pp. 81-108.

Chu, K.C., K.M. Patel, A.H. Lin, R.E. Tarone, M.S. Linhart and V.C. Dunkel. 1981. Evaluation of statistical analyses and reproducibility of microbial mutagenicity assay. Mutat. Res. 85:119-132.

Claxton, L.D. and H.M. Barnes. 1981. The mutagenicity of diesel-exhaust particle extracts collected under smog-chamber conditions using the *Salmonella typhimurium* test system. Mutat. Res. 88(3):255-272.

de Serres, F.J. 1979. Problems associated with the application of short-term tests for mutagenicity in mass-screening programs. Env. Mutag. 1:203-208.

de Serres, F.J. and M.D. Shelby. 1979. Recommendations on data production and analysis using the *Salmonella*/microsomal mutagenicity assay. Mutat. Res. 64:159-165.

Donnelly, K.C. and K.W. Brown. 1981. The development of laboratory and field studies to determine the fate of mutagenic compounds from land-applied hazardous wastes. *In:* Land Disposal: Hazardous Waste. EPA 600/9-81-002b. pp. 224-239.

Draper, N. and H. Smith. 1966. Applied Regression Analysis. J. Wiley and Sons, Inc., New York, New York.

Dunkel, V.C. and K.C. Chu. 1980. Evaluation of methods for analysis of microbial mutagenicity assays. *In*: The Predictive Value of Short-Term Screening Tests in Carcinogenicity. G.M. Williams, et al. eds. Elsevier/North Holland Biomedical Press. pp. 231-240.

Epler, J.L., B.R. Clark, C.-h. Ho, M.R. Guerin and T.K. Rao. 1978. Short-term bioassay of complex mixtures: Part II, Mutagenicity testing. *In:* Application of Short-Term Bioassays in the Fractionation and Analysis of Complex Environmental Mixtures. EPA-600/9-78-027. Waters, et al. eds. pp. 269-289.

Federal Register. August 22, 1978. FR 37388.

Freund, J.E. 1979. Modern Elementary Statistics, Fifth Edition. Prentice-Hall, Inc., Englewood Cliffs, New Jersey.

Graybill, F.A. 1976 Theory and Application of the Linear Model. Duxbury Press, Wadsworth Publ. Co., Inc., Belmont, California.

King, L.L., M.J. Kohan, A.C. Austin, L.D. Claxton and J. Lewtas. 1981. Evaluation of the release of mutagens from diesel particles in the presence of physiological fluids. Env. Mutag. 3:109-122.

Linhart, M.S., K. Chu, U.N. Evans and V.C. Dunkel. 1980. In Vitro Information System for collection and analysis of experimental data. J. Env. Path. Tox. 4:1-21.

Loveday, K.S. Standard Procedure: *Salmonella* Microtoxicity Assay. Unpublished protocol of Bioassay Systems, Inc., Woburn, Massachusetts 01801.

Margolin, B., N. Kaplan and E. Zeiger. 1981. Statistical analysis of the Ames *Salmonella*/microsome test. Proc. Nat. Acad. Sci. 78:3779-3783. p. 79.

Maron, D., J. Katzenellenbogen and B.N. Ames. 1981. Compatibility of organic solvents with the *Salmonella*/microsome test. Mutat. Res. 88:343-350.

Myers, J.L. 1979. Fundamentals of Experimental Design. 3rd Edition. Allyn and Bacon, Inc., Boston, Massachusetts.

Nagao, M., T. Yahagi, Y. Seino, T. Sugimura and N. Ito. 1977. Mutagenicities of quinoline and its derivatives. Mutat. Res. 42:335-342.

Pellizari, E.D., L.W. Little, C. Sparacino, T.J. Hughes, L. Claxton and M.D. Waters. 1978. Integrating microbiological and chemical testing into the screening of air samples for potential mutagenicity. *In*: Application of Short-Term Bioassays in the Fractionation and Analysis of Complex Environmental Mixtures. EPA-600/9-78-027. Waters, et al., eds. pp. 331-351.

Rosenkranz, H.S., E.C. McCoy, M. Anders, W.T. Speck and D. Bickers. 1978. The use of microbial assay systems in the detection of environmental mutagens in complex mixtures. *In*: Application of Short-Term Bioassays in the Fractionation and Analysis of Complex Environmental Mixtures. EPA-600/9-78-027. Waters, et al., eds. pp. 3-42.

Salmeen, I. and A.M. Durisin. 1981. Some effects of bacterial population on quantitation of Ames *Salmonella*-histidine reversion mutagenesis assays. Mutat. Res. 85:109-118.

Sexton, N., L. Myers and T. Hughes. 1981. A plan to develop and implement a quality assurance program for the Ames/*Salmonella* test. EPA-600/2-81-054. pp. 1-27 and Appendices A-D. Available through National Technical Information Service, Springfield, VA. 22161.

Sokal, R.R. and F.J. Rohlf. 1969. Biometry. Freeman and Co., San Francisco, California. pp. 370-775.

Stead, A., V. Hasselblad, J. Creason and L. Claxton. 1981. Modelling the Ames test. Mutat. Res. 85:13-27.

Sugimura, T. and M. Nagao. 1980. Modification of mutagenic activity. *In*:Chemical Mutagens, Vol. 6. F.J. de Serres and A. Hollaender, eds. Plenum Publ. Corp., New York, New York. pp. 41-59.

U.S. Environmental Protection Agency. 1978. Microbiological Methods for Monitoring the Environment - Water and Wastes. EPA-600/8-78-017. R. Bordner and J. Winter, eds. EPA/EMSL, Cincinnati, Ohio.

Waleh, N.S., S.J. Rapport and K. Mortelmans. Development of a toxicity test to be coupled to the Ames *Salmonella* assay and the method of construction of the required strains. Mutat. Res. (In press).

Yahagi, T., M. Nagao, Y. Seino, T. Matsushima, T. Sugimura and M. Okada. 1977. Mutagenicities of N-nitrosamines on *Salmonella*. Mutat. Res. 48:121-130.

Yamanaka, H., M. Nagao, T. Sugimura, T. Furuya, S. Atsuko and T. Matsushima. 1979. Mutagenicity of pyrrolizidine alkaloids in the *Salmonella*/mammalian microsome test. Mutat. Res. 68:211-216.

Y. Inokawa, and M. Ishidate. 1980. Stability of S-9 during frozen storage in *Salmonella*/S-9 assays. Mutat. Res. 74:389-391.

Zeiger, E., D.A. Pagano and I.G.C. Robertson. 1981. A rapid and simple scheme for confirmation of *Salmonella* tester strain phenotype. Envir. Mutag. 3:205-209.

Appendix I
Preparation of Media and Reagents

Stock Solutions

Vogel-Bonner Medium "E" (50X)

(For Minimal Agar)

Ingredient	Per Liter of Solution
Magnesium Sulfate ($MgSO_4 \cdot 7H_2O$)	10.0 g
Citric acid (monohydrate)	100.0 g
Dipotassium hydrogen phosphate (K_2HPO_4, anhydrous)	500.0 g
Sodium ammonium phosphate ($NaNH_4HPO_4 \cdot 4H_2O$)	175.0 g
Distilled water	To final volume of 1,000 ml

Preparation. Slowly dissolve, in order, the first four ingredients in 600 ml of warm (45°C) distilled water on a magnetic stirring hot plate. The K_2HPO_4 dissolves slowly, therefore add no more than 50 g at a time until completely dissolved. Bring to 1,000 ml volume by adding the distilled water. Pour 500 ml of solution into each of two clean 1-liter glass bottles labelled "50 X VBME Solution" and label with the date prepared. Autoclave the two bottles, loosely capped, for 15 minutes at 121°C. When bottles have cooled, tighten caps and store in a cabinet at room temperature. Storage should not exceed 2 months before use.

Calcium Chloride Solution (10 mM)

(For Minimal Agar)

Ingredient	Per Liter of Solution
Calcium chloride (anhydrous)	0.115 g
Distilled water	To final volume of 1,000 ml

Preparation. Dissolve 0.115 g $CaCl_2$ in 1,000 ml of distilled water. Transfer 500 ml to each of two clean 1-liter glass bottles labelled "$CaCl_2$ Solution, 10mM". Autoclave, cool and store at room temperature. Stable as long as solution is sterile.

Glucose Solution (20%)

(For Minimal Agar)

Ingredient	Per Liter of Solution
D-glucose	200 g
Distilled water	To final volume of 1,000 ml

Preparation. Dissolve 200 g D-glucose in about 600 ml of distilled water in a 1-liter volumetric flask. Add distilled water to make up the total volume to 1 liter. Sterilize by autoclaving or filtering through a 0.22 μm membrane filter. Store in sterile glass bottle(s) at room temperature. Discard if, on visual inspection, solution appears turbid or a surface film has formed. Solution is stable on storage, as long as sterility is maintained.

L-Histidine HCl (0.1 M)

(For Master Plates)

Ingredient	Per 100 ml of Solution
L-Histidine·HCl (M.W. = 191.56)	1.916 g
Distilled water	To final volume of 100 ml

Preparation. Prepare in a volumetric flask; shake vigorously to dissolve. Sterilize by autoclaving or filtering through a 0.22 μm membrane filter. Store in properly labelled sterile glass bottle, at 4°C for up to 1 month; wrap bottle in aluminum foil to protect from light during storage. Discard solution if it has yellowed.

D-Biotin (0.5 mM)

(For Master Plates)

Ingredient	Per 100 ml of Solution
D-Biotin	0.012 g
Distilled water	To final volume of 100 ml

Preparation. Prepare in a volumetric flask; shake vigorously, and warm if necessary, to dissolve. Sterilize by autoclaving or filtering through a 0.22 μm membrane filter. Store up to 3 months at room temperature in properly labelled glass bottle(s). Heat gently before use if the solution has precipitated on storage.

L-Histidine HCl (0.5 mM)/ D-Biotin (0.5 mM) Solution

(For addition to top agar before use)

Ingredient	Per 250 ml of Solution
L-Histidine·HCl (M.W. = 191.56)	0.024 g
D-Biotin (M.W. = 244.31)	0.030 g
Distilled water	To final volume of 250 ml

Preparation. Sterilize by filtration through a 0.22 μm membrane filter. Store at 4°C in properly labelled glass bottle(s) for up to 1 month. Wrap bottle(s) in aluminum foil to protect from light. Discard solution if it has yellowed.

Salt Solution

(For S-9 Mix)

Ingredient	Per 500 ml of Solution
1.65 M Potassium chloride (KCl)	61.42 g
0.4 Magnesium chloride ($MgCl_2 \cdot 6H_2O$)	40.66 g
Distilled water	To final volume of 500 ml

Preparation. Autoclave to sterilize. Store at room temperature in properly labelled glass bottle(s). Stable for long periods if sterility is maintained.

Sodium Phosphate Buffer (0.2 M, pH 7.4)

(For S-9 Mix)

Ingredient	Per 500 ml of Solution
Sodium dihydrogen phosphate ($NaH_2PO_4 \cdot H_2O$	3.77 g
Disodium hydrogen phosphate ($Na_2HPO_4 \cdot 7H_2O$)	19.51 g
Distilled water	To final volume of 500 ml

Preparation. Dissolve the dibasic salt in 300 ml H_2O, and the monobasic salt in

150 ml H_2O. Add dibasic solution to monobasic solution and adjust volume to 500 ml. Adjust pH to 7.4 with HCl; autoclave to sterilize. Store at room temperature in properly labelled glass bottle(s). Stable for long periods if sterility is maintained.

Nicotinamide Adenine Dinucleotide Phosphate (NADP) (0.1 M)

(For S-9 Mix)

Ingredient	Per 100 ml of Solution
NADP (MW = 765.4)	7.65 g
Distilled water	To final volume of 100 ml

Preparation. Sterilize by filtration through a 0.22 μm membrane filter. Store in labelled, 13 x 100 mm, stoppered glass test tubes in 2 ml volumes in freezer at -20°C. Note: Check the molecular weight of each lot, as it varies according to impurities present.

Glucose-6-Phosphate (G-6-P) (1 M)

(For S-9 Mix)

Ingredient	Per 10 ml of Solution
G-6-P (MW = 282.2)	2.82 g
Distilled water	To final volume of 10 ml

Preparation. Sterilize by filtration through a 0.22 μm membrane filter. Store in labelled, 13 x 100 mm, stoppered glass test tubes in 0.3 ml volumes in freezer at -20°C.

Nutrient Broth

Composition:

Ingredient	Per Liter of Medium
Oxoid Media #2 Nutrient Broth Powder	25 g
Distilled Water	To final volume of 1,000 ml

Preparation. Dissolve nutrient broth powder completely and dispense 10 ml aliquots of the broth into sterile 50 ml screw-capped culture or centrifuge tubes. Label appropriately (broth, volume, date of preparation). Autoclave for 15 minutes at 121°C (15 lb pressure). Store in dark up to 2 months at room temperature.

Nutrient Agar

Composition:

Ingredient	Per Liter of Medium
Purified agar (Oxoid # L28 or equivalent)	10 g
Nutrient broth (prepared as above)	To final volume of 1,000 ml

Preparation. Heat in a boiling water bath to dissolve completely. Autoclave 15 minutes at 121°C (15 lb pressure). Remove from autoclave and cool to 45°C in water bath. Dispense 25 ml/plate into 100 x15 mm petri dishes using an automatic dish filler which has been adjusted to level. Place plates in a sealed container and store up to 1 month at room temperature. In general, do not prepare more plates than will be used during a 2-week period.

Minimal Agar

Composition:

Ingredient	Per Liter of Medium
Oxoid #L28 agar (or equivalent)	15 g
50 X VBME stock solution	20 ml
20% glucose stock solution	100 ml
(10 mM $CaCl_2$ stock solution[1]	1 ml)
Distilled water	To final volume of 1,000 ml

Preparation of Minimal Agar Medium (For 1 liter - approximately 40 plates). Add 15 g of Oxoid #L28 agar to 880 ml of distilled water in a 2-liter flask. Adjust to 1 liter volume with distilled water, if necessary. Autoclave for 35 minutes using slow exhaust. When solution has cooled slightly, add 20 ml of "VBME 50 X" salt solution, 100 ml of 20 percent glucose, and 1 ml 10 mM $CaCl_2$ solution. Mix and place in 45°C waterbath.

[1]Optional; use if needed to control filamentous growth of *Salmonella* cells.

Top Agar

Composition:

Ingredient	Per Liter of Medium
Purified agar (Oxoid #L28 or equivalent)	6.0 g
Sodium chloride (NaCl)	5.0 g
Distilled water	To final volume of 1 liter

Histidine/biotin solution (20 ml added to each 200 ml portion of medium before use).

Preparation. Heat in an autoclave with flowing steam or in a boiling water bath to completely dissolve the agar. Dispense into screw-capped glass bottles, 200 ml/bottle. Autoclave 15 minutes at 121°C. Store in refrigerator. Before use, melt top agar in autoclave or microwave oven, cool and maintain at 45°C in a waterbath. Add 20 ml of sterile histidine/biotin solution (pre-warmed to 45°C) to each 200 ml portion of top agar, and swirl contents thoroughly to obtain a uniform mixture. Dispense the top agar into 100 mm disposable, sterile test tubes (2 ml/tube); cap the tubes and place in a 45°C heating block to equilibrate (about 5 - 10 minutes). Top agar should only be melted once, as repeated meltings may cause crystals to form when top agar is poured on plates and make colony counting more difficult.

Strain Function Test Materials

Ampicillin Solution (7 mg/ml)

Ingredient	Per 100 ml of Solution
Ampicillin trihydrate	0.7 g
Sodium hydroxide (0.02 N)	To final volume of 100 ml

Preparation. Sterilize by filtration through a 0.22 μm membrane filter. Store up to 1 month in glass bottle at 4°C or up to 6 months frozen.

Crystal Violet Solution (0.1 %)

Ingredient	Per 100 ml of Solution
Crystal violet (Gentian violet, methyl-rosaniline chloride)	0.1 g
Ethanol solution (70%)	100 ml

Preparation. Bring up to 100 ml in a volumetric flask. Store in glass bottle at room temperature. Stable for prolonged periods if stored out of direct light.

Preparation of Crystal Violet discs (10 µl of 0.1% Crystal Violet per disc). Prepare 100 ml of crystal violet in 70% alcohol. Using a sharp (new), clean paper punch and Whatman filter paper #1 or #2, punch out individual discs (or multiple discs and separate the layered discs). Arrange discs on a large petri dish (single discs, no overlapping). Add 10 µl of the crystal violet solution to each disc, allow to dry and autoclave at 121°C for 15 minutes.

Rat Liver Enzyme Mix (S-9 Mix)[1]

Composition:

Ingredient	Per 50 ml of Mix	
	Low S-9 Mix	High S-9 Mix
Rat Liver S-9 Arochlor-1254-induced)	2.0 ml	10.0 ml
Salt Solution	1.0 ml	1.0 ml
Glucose-6-Phosphate	0.25 ml	0.25 ml
NADP	2.0 ml	2.0 ml
Sodium Phosphate Buffer	25.0 ml	25.0 ml
Sterile Distilled Water	19.75 ml	11.75 ml

Preparation of S-9 Mix. On the day of the test, combine the ingredients indicated above (under *Composition*), using aseptic technique, in a sterile graduated cylinder that has been placed in an icewater bath. Freshly prepared S-9 Mix can be kept on ice several hours before running the test. Alternatively, it is convenient, and acceptable, to mix all ingredients of the S-9 Mix (*except* the rat liver homogenate) in large batches, dispense into convenient aliquots, and store at -20°C so that only the microsomal preparation (S-9) need be added on the day of the test.

It is recommended that laboratories purchase the microsomal preparation (S-9) from a commercial biological supply company or private laboratory performing mutagen research. This material is available from Litton Bionetics, 5516 Nicholson Lane, Kensington, MD 20795; or from the Meloy Laboratories, c/o Dr. Carol Richardson, 6715 Electronic Drive, Springfield, VA 22151. Quick-freeze with dry ice immediately after preparation, and store at -80°C in 2-ml plastic vials. The frozen S-9 has been shown to retain full activity for at least 1 month at -80°C (Yoshikawa et al., 1980) and up to several years for selected enzymes, if maintained at temperatures below -130°C (Ashwood-Smith, 1979).

S-9 preparations vary in protein content. It is recommended that new S-9 batches be tested with standard mutagens and strains of known activity.

[1]Other rat tissues and tissues of other mammals may be used as a source of S-9 preparation. Also other chemicals (e.g., phenobarbital) may be used to induce the mammal.

Appendix II
Facilities, Equipment and Supplies

General Requirements

Microbial assays for mutagenicity should be performed in a stationary laboratory. Generally, support equipment used in a typical water microbiology laboratory is suitable for use in the Ames test. This equipment includes good quality autoclaves, colony counters, sterilization ovens, incubators, waterbaths, water distillation systems, dishwashers, refrigerators, freezers, balances (analytical and top loading) and the usual pipettes and glassware for preperation of media and reagents. These items are described in the EPA's microbiological methods manual (EPA 1978) and *Standard Methods* (APHA 1975). Facility requirements are also detailed in Brusick et al., (1980).

Autoclaves

Unit(s) selected must maintain standard autoclave conditions and be capable of accepting large volume reagent bottles and flasks. It is recommended that an exhaust hood be installed over the autoclave to evacuate volatile chemicals, heat, and moisture from the laboratory area.

Incubators

Unit(s) should employ forced-air (or a mechanism of equivalent effectiveness) to minimize temperature differences throughout the chamber and must be able to maintain 37° ± 0.5°C. Incubator(s) should be directly or indirectly (e.g., through an exhaust hood with appropriate filtration) exhaustible; the use of a microswitch on doors to activate an exhaust fan is recommended. It is recommended that a temperature recorder be attached to each incubator to verify temperature stability over prolonged periods of operation. Units which provide positive humidity control are useful to minimize dessication of, or condensation on the plates. Incubators should not be overloaded in use; overloading can result in the establishment of temperature gradients (Belser, et al. 1981) which affect growth rates.

Refrigerators and Freezers

Units should be lockable. If used for solvent or organic chemical storage, they must be explosion proof. Temperature recorders should be provided to monitor the performance of the refrigerator and freezer units. A visual or audible alarm should be provided to indicate power outages and significant temperature deviations. An auxiliary power supply for freezer units can protect deep-frozen culture materials from the damaging effects of power interruption, and is strongly recommended.

Laminar-Flow Safety Cabinets

Must be an OSHA-approved type, designed to protect both the personnel and work area. Intake air must be filtered with a high efficiency particulate air (HEPA) filter with not greater than 30 percent recirculation and 100 percent of intake air must be exhausted. Exhaust air should flow through a HEPA filter and an appropriate trap (e.g., activated charcoal) for organic chemicals.

A general list of equipment, supplies, media and reagents (requirements for 20-30 samples) necessary for environmental mutagenesis testing is presented below. For many items, equivalent products are available from other sources. Listing does not constitute a specific endorsement.

Major Equipment

Essential Items

Item	No. Needed	Suggested Source
● Membrane filtration system, for sterilization of heat-labile materials	2	Nuclepore Corp.; Millipore Corp.
● Bacterial colony counter, darkfield, with electronic register	1	Scientific Products, Inc.
● Automated Colony Counter	1	New Brunswick Scientific Co., Inc.
● Laminar-flow safety cabinet	1	Contamination Controls, Inc.
● Dri-block heater, w/accessories, to hold 13x100 mm test tubes	3	Scientific Products, Inc.
● Incubator shaker (e.g., Controlled Environment Incubator Shaker)	1	New Brunswick Scientific Co., Inc.
or		
Shaker waterbath, for culturing test strains (37°C)	1	New Brunswick Scientific Co., Inc.
● Waterbath, for tempering media	1	Scientific Products, Inc.

Item	No. Needed	Suggested Source
● Ultra-freezer (-80°C) (e.g., So-Low PR120E, 5 cu. ft. capacity)	1	So-Low Environmental Equipment Co.
● Incubator (e.g., Forma Scientific model 3028 CO_2 incubator)	1	Forma Scientific
● Refrigerator-compact, explosion-proof, lockable, for storage of standard mutagens.	1	Scientific Products, Inc.
● Spectrophotometer, Turbidimeter, *or* Particle Counter (Coulter-type). Determination/adjustment for bacterial culture density.*	1 (Choice)	Beckman Co.; Coulter Electronics; Perkin-Elmer Co.
● Micro-Volume Pipettes (1 μl - 1000 μl volumes)	6	Cole-Parmer Instrument Co.
Optional Items (recommended)		
● Petri dish filler/stacker	1	New Brunswick Scientific Co., Inc.
● Mechanical Pipetting Device	2-3	Bellco Glass, Inc.
● Bag Sealing Device (for sealing petri dishes in plastic bags)	1	Sears-Roebuck and Co.

Expendable Equipment and Supplies

Item	No. Needed	Suggested Source
● Sterile Disposable Tips for Micro-Volume Pipettes	1 Box of 1,000 for each Volume Pipette	Cole-Parmer Instrument Co.
● Sealable Plastic Bags, for sealing petri dishes	4-5 cases of 500	Sears-Roebuck and Co.
● Surgical Gloves, latex, disposable	1-2 cases of 500	Pharma Seal Laboratories
● Petri Dishes 15x100 mm, gamma-irradiation sterilized, disposable; or #1028 Muta-assay® cold-sterilized plates.	1-5 cases of 500	Falcon Plastics, Inc.
● Test Tubes, 13x100 mm, disposable	4-5 cases of 1,000	Bellco Glass, Inc.
● Volumetric Flasks, 10, 25, 50, 100, 500 and 1,000 ml	6 each	Cole-Parmer Instrument Co.
● Membrane Filters (pore size $\leq$0.22 μm)		Nuclepore Corp.; Millipore Corp.
● Glass-fiber Filters (pore size $\leq$0.8 μm)		Whatman Corp.
● Laboratory Tape, white, and heavy-base dispenser	Dispenser and 1 doz. rolls	
● Caps, for 13x100 mm test tubes, color coded	1 case of 1,000 ea.	Bellco Glass, Inc.

*NOTE: Whichever method is selected, standard curves must be prepared (and periodically reconfirmed) with viable cell counts determined by the dilution and plate method.

Item	No. Needed	Suggested Source
● Test Tubes, 20x125 mm, screw-capped	1 case of 500	Corning Glass, Inc.
● Reagent Bottles, screwcapped for storage of media and reagents, 100, 200, 300, 400, 500 ml volume	1 doz. ea	Bellco Glass, Inc.
● Pipettes, TD, disposable, sterile, glass, 1 ml, 2 ml, 5 ml and 10 ml volumes	1 case of each volume	Scientific Products Co.
● Erlenmeyer Flasks, 50, 125, 250, 500, 1,000, 2,000 ml volumes with Morten culture tube closures or equivalent	1 doz. ea	Kimball Glass Co.

Reagents (Reagent grade unless otherwise indicated)

Item	Amount	Suggested Source
● Ampicillin, diagnostic reagent (special preparation, high purity)	5 g	Bristol Laboratories
● D-Biotin (M.W. 244.31)	5 g	Eastman Kodak Co. (# 14635)
● Calcium Chloride ($CaCl_2$)	1 lb.	J.T. Baker Chemical Co.
● Citric Acid	500 g	J.T. Baker Chemical Co.
● Crystal-violet	10 g	Difco Laboratories, Inc.
● Glucose	500 g	Difco Laboratories, Inc.
● Methylene Chloride (Dichloromethane) distilled in glass	5 gal	Burdick and Jackson Laboratories
● Dimethyl Sulfoxide (DMSO) spectrophotometric quality	1 gal	Matheson, Coleman and Bell (# MX1454)
● Dipotassium Hydrogen Phosphate (K_2HPO_4)	1 lb.	Mallinckrodt Chemical Works
● Disodium Hydrogen Phosphate ($Na_2HPO_4 \cdot 7H_2O$)	1 lb.	Mallinckrodt Chemical Works
● Glucose-6-Phosphate (M.W. 282.1) anhydrous	10 g	Sigma Chemical Co. (# G7879)
● Hydrochloric Acid (HCl)	9 lb.	J.T. Baker Chemical Co.
● L-Histidine (M.W. 192.7) anhydrous	10 g	Sigma Chemical Co. (# H8125)
● Magnesium Chloride ($MgCl_2 \cdot 6H_2O$)	1 lb.	J.T. Baker Chemical Co.
● Magnesium Sulfate ($MgSO_4 \cdot 7H_2O$)	1 lb.	J.T. Baker Chemical Co.
● Nicotinamide Adenine Dinucleotide Phosphate (M.W. 765.4) anhydrous	10 g	Sigma Chemical Co. (# NO505)
● Potassium Chloride (KCl)	1 lb.	J.T. Baker Chemical Co.
● Sodium Ammonium Phosphate ($NaNH_4 PO_4 \cdot 4H_2O$)	1 lb.	J.T. Baker Chemical Co.
● Sodium Chloride (NaCl)	1 lb.	J.T. Baker Chemical Co.
● Sodium Dihydrogen Phosphate ($NaH_2PO_4 \cdot H_2O$)	1 lb.	Fisher Scientific Co.

Item	Amount	Suggested Source
● Sodium Hydroxide (NaOH)	1 lb.	J.T. Baker Chemical Co.
● Sodium Sulfate (Na_2SO_4) anhydrous	5 lb.	J.T. Baker Chemical Co.

Prepared Media

Item	Amount	Suggested Source
● Ampicillin, "Dispens-o-Discs," 10 μg	250	Difco Laboratories (# 6363)
● Purified Agar (Oxoid # L28 or equivalent)	5 lb.	K.C. Biological Inc.
● Nutrient Broth Powder (Oxoid #2 or equivalent)	5 lb.	K.C. Biological Inc. (# CM67)
● Rat Liver Enzymes (Induced with Aroclor 1254) (S-9 preparation)		Litton Bionetics; AMC Cancer Research Center; Meloy Laboratories, Inc.

Additional sources of equipment and materials for *Salmonella*/microsomal mutagenicity tests are suggested in Ames (1981).

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