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Health Effects Research
Laboratory
Research Triangle Park NC 27711

Research and Development

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Project Summary

Comparison of Ames *Salmonella typhimurium* Plate Incorporation Test Protocols

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This document is a companion volume to the Compilation of Ames *Salmonella typhimurium* Plate Incorporation Test Protocols. Its purpose is to tabulate the differences between the submitted protocols and to note the modifications from the 1975 Ames reference. The tables show the differences obtained between laboratories in bacterial strain maintenance, metabolic activation systems, chemical controls, preparation of media and solutions, and of the assay procedure itself. The recommendations of de Serres and Shelby and any revisions by Ames are also included.

No attempt is made to evaluate any laboratory or indicate a preferable method for the test. Some laboratories may have Standard Operating Procedures (SOPs) that are more detailed than the submitted protocols. Changes or revisions that have taken place in a protocol since submission are not noted in this document. In addition, laboratories that use Ames' reference (1975) as their main protocol may have only sent their modifications.

This Project Summary was developed by EPA's Health Effects Research Laboratory, Research Triangle Park, NC, to announce key findings of the research project that is fully documented in a separate report of the same title (see Project Report ordering information at back).

Introduction

In 1975, Ames *et al.*, published a detailed protocol that incorporated the use of a mammalian metabolizing system, thereby establishing the Ames test as a routine screening system for mutagenicity and potential carcinogenicity. Since then, many laboratories have introduced a variety of changes and/or additions.

The U.S. Environmental Protection Agency (EPA) therefore decided to conduct an informal survey of laboratories that utilize the Ames test. A list of laboratories routinely performing the test was obtained from Dr. Mike Shelby at the National Institute of Environmental Health Sciences, Research Triangle Park, NC. An initial letter was sent to the 46 laboratories, 33 of which responded. These included six laboratories that no longer performed the Ames test, one laboratory that had closed and 25 laboratories that submitted protocols. The other responding laboratory elected to restrict usage of their protocol. A list of names and addresses of participating laboratories is found in Table 1. This publication is a tabulated qualitative summary and comparison of the submitted protocols.

Bacterial Strains

Bacterial Strains Routinely Used

The Ames reference (1975) suggested using three standard strains for routine testing (TA1535, TA1537, and TA1538) to be used in combination with the newer derivative strains (TA98,TA100).

The majority of the participating laboratories use all five tester strains

Table 1. *Names and Addresses of Participating Laboratories*

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routinely. Two of the laboratories do nc use TA1538 routinely. One uses onl three strains: TA98, TA100, and TA153 Another does not say what strains uses. One laboratory appears to subst tute TA1575 for TA1535.

Storage of Strains

Ames is now employing master plate for the temporary storage of strains. Hi laboratory stores master plates in a col room, where all culturing is performed The master plates are kept for one to tw months. Their preparation involve making minimal glucose agar an spreading histidine and biotin. Ampicillir is also spread on plates used for strain TA98 and TA100. The plates sit for on or two days to allow the compounds tc diffuse into the media. The six labora tories that list a procedure report usinç the same procedure as Ames. Perma nent storage is in a 2 ml sterile glass via with rubber-lined screw tops. The culture is stored at a concentration of 0.8 ml of a 16-h nutrient broth culture tc 0.07 ml DMSO. It is kept at -80°C in a Revco freezer. The laboratories that list a procedure agree with the Ames permanent test and revised temporary storage procedures.

Histidine Requirement Test

Ames' recommendations for the histidine requirement test specify minimal media petri plates. The *Salmonella* is streaked onto the plate, an aliquot of 0.1 ml of a 0.1 M solution of L-histidine is then spread. Biotin is also used with strains that have the uvrB deletion. An aliquot of 0.1 ml of a 0.5 mM solution is spread onto the plates. Plates without histidine are used as a negative control. Nine laboratories do not mention the methods they employ to monitor this requirement. Of the ones who mentioned a procedure, five laboratories say that they check this requirement but give no description. Nine laboratories repeat the Ames procedure in their protocol. Two others use an overlay rather than streak the bacteria on the plate.

Determination of rfa Character

Ames uses nutrient agar plates and a 16-h incubation of 10^8 concentration *Salmonella* in nutrient broth culture. A total of 0.1 ml of the tester strain is added to an agar overlay and poured on the nutrient agar plate. Ames gives two possibilities for a sensitivity test compound: an aliquot of 10 μl of a 1 mg/ml solution of crystal violet or 2 mg

sodium deoxycholate. The test compound is applied to a sterile filter paper disc which is then placed on the petri plate. The plate is incubated for 12 h at 37°C and then interpreted. If the mutation is still present there will be a zone of inhibition around the disc. The size of the zone is approximately 14 mm for crystal violet and 13 mm for deoxycholate. Six laboratories do not list a method of determination, and four mention that they do the procedure. Of the remaining laboratories, nine repeat Ames' procedure, and two use minimal agar rather than nutrient agar. One laboratory mentions the compound and adds that it checks for this requirement. One laboratory streaks the *Salmonella* rather than using an overlay. Two others use Mueller-Hinton plates in combination with a swab-streak of *Salmonella.*

Ampicillin Resistance

Ames (1975) recommends using nutrient agar plates for the ampicillin resistance test. A total of 10 µl of an 8 mg/ml ampicillin solution in 0.02 N NaOH is streaked on the plate and cross-streaked with *Salmonella.* After 12-24 h of incubation at 37°C, the plates are evaluated. If the mutation is present (TA98, TA100) there should be no zone of inhibition around the streak. De Serres and Shelby recommended that the ampicillin be put on the plate in a disc form and used in combination with a top-agar overlay. Six laboratories do not list a procedure. Six others say they perform this test but give no instructions. Three follow the procedure given in Ames, 1975. Four laboratories follow the recommendation of de Serres and Shelby. Two use a Mueller-Hinton plate with an Ampicillin disc and a swab-streak of *Salmonella.* Three others spread the ampicillin over the entire plate and score the mutation as present with growth on the plate. One laboratory does not check for this mutation.

uvrB Deletion

Ames uses nutrient broth agar with a streak of *Salmonella* for this test. Half the plate is irradiated with a UV light for 6 seconds (8 seconds for TA98, TA100) at a distance of 33 cm (i.e., plate to lamp distance). The plate is then incubated for 12-24 h at 37°C and scored. If the mutation is present, no growth will occur on the exposed side. Eight laboratories perform this test as described. Eight do not list a procedure, and four say they perform the test. One differs from Ames only in the use of Mueller-Hinton plates. Two laboratories use an overlay rather than a streak for the *Salmonella* and also employ different time intervals. One laboratory irradiates sections of the plate from 10 sec to one minute, and incubates for 48 h. One laboratory does not list a procedure but says it uses Ames' identically.

Compound Sensitivity Verification

Ames recommends comparing TA98 and TA100 to its parent strains in a standard assay with Aflatoxin B_1 and methyl methanesulfonate to show increased mutagenesis by TA98 or TA100. Only one laboratory mentions this procedure.

Spontaneous Reversion Rate

Ames suggests that the spontaneous reversion rate be checked in a standard assay; an acceptable interpretation is that plate counts fall within the standard individual labortory's limits. Five laboratories check the reversion rate but give no details. Eleven others follow the Ames suggestion, and nine do not mention the procedure.

Metabolic Activation System

Preparation of S-9 Fraction: I. Induction of Animal

Ames uses the PCB mixture Aroclor 1254 for inducing male rats weighing approximately 200 g, and suggested using the Spraque-Dawley/Biol strain. The method of induction is a single intraperitoneal injection five days before sacrificing the animal. The dosage is 500 mg of Aroclor per kg of body weight, and the concentration is 200 mg/ml suspended in corn oil. Food and water are given *ad libitrium* until 12 h before sacrifice, at which time the food is removed. Three laboratories do not describe their induction method. Five laboratories restrict their discussion to the strain of rat and/or the induction chemical used. Nine others use essentially the same procedure described by Ames. One laboratory differs from Ames only in the strain of rat used. Five laboratories differ from Ames in the time rats are deprived of food — 24 h rather than 12. One laboratory sacrifices the rats by gassing CO_2. One laboratory uses Aroclor 1242 rather 1254 as an alternative to corn oil; sesame oil is mentioned by one laboratory.

Preparation of S-9 Fraction: II. Preparation of Fraction

Ames gives a detailed procedure for the fraction preparation after sacrifice of the rat. All work is done between 0 and 4°C using sterile materials and solutions. The liver is removed and washed twice with cold 0.15 M KCl. It is then minced with sterile scissors and homogenized using a Potter-Elvehjen homogenizer with a Teflon pestle. After homogenizing, a Sorvall RC_2-B centrifuge with a SS-34 head is used for 10 min at 8700 rev/min (9000xg). The decanted supernatant fraction is stored in a 2 ml plastic liquid nitrogen storage tube. It is quick-frozen on dry ice and stored in a Revco freezer at -80°C. Five laboratories make no mention of the procedure. Five others list only the centrifuge speed or organ used or storage temperature. Eight laboratories give essentially the same procedure as the one described by Ames. Three laboratories differ only in the amount of fraction in a storage container. One expands the Ames protocol to include a detailed homogenizing procedure. One laboratory used a Polytron J21C centrifuge instead of a Sorvall. Two use a sucrose wash in combination with two KCl washes. One laboratory that uses a sucrose wash also centrifuges for 25 min rather than 10.

Preparation of S-9 Mix

Ames lists the following ingredients and amounts for the S-9 mix:

S-9	0.04-0.1 ml/ml mix
$MgCl_2$	8 µmole/ml mix
KCl	33 µmole/ml mix
G-6-P	5 µmole/ml mix
NADP	4 µmole/ml mix
sodium phosphate buffer	100 µmole/ml mix

He recommends keeping the mix on ice for the assay procedure and only retaining it for several hours. Most laboratories use the quantities listed above. One laboratory does not list quantities. The only significant difference is that one laboratory listed the use of 100 µmole G-6-P/ml mix, 5 µmule NADP/ml mix, and 4 µmole sodium phosphate buffer/ml mix. One laboratory omits KCl as a component in the mix.

Stock Solutions

Sodium Phosphate Buffer

Ames recommends using a 0.2 M sodium phosphate buffer in the S-9 mix.

The pH of the buffer is 7.4 and the amount used per ml of mix is 100 μmole. One laboratory does not say what buffer it uses, and two list the pH. Five laboratories list the pH and amount of buffer used. Two others list the pH and the molarity as described by Ames. Eight laboratories give essentially the same description as Ames, and four use different buffer molarities (0.125, 0.4, 0.1 or 1 M). The laboratory using 1 M buffer uses 0.1 ml buffer/ml mix. One other uses an NADPH generating system. One laboratory uses a mixture of monobasic and dibasic sodium phosphate.

Microsomal Salt Solution

Ames says that the microsomal salt solution should consist of 0.4 M $MgCl_2$ and 1.65 M KCl. The quantities per ml of mix are 8 μmole $MgCl_2$ and 33 μmole KCl.

Twenty laboratories list the same quantities and/or molarity as Ames. One laboratory does not give any information for the microsomal salt solution. One laboratory gives the molarity as 0.4 M for both $MgCl_2$ and KCl. One laboratory reports 0.65 M concentration for KCl. Another reports standard molarities but gives gram weights 10x higher than expected. One laboratory uses an NADPH generating system.

Glucose Stock Solution

Ames lists no directions for the preparation of a glucose stock solution. Five of the laboratories make no mention of a glucose stock solution. Sixteen laboratories say they use a 2% solution. One laboratory uses a 0.8% solution and two use a 1% solution. One uses a stock solution of 20% but does not give the quantity of this solution that is used in the agar.

Histidine/Biotin Stock Solution

Ames reports a molarity of 0.5 mM for both the histidine and the biotin in the His/Bio stock solution or quantities of 8.7136 mg/l histidine and 11.1045 g/l biotin. He recommended using 10 ml of the solution per 100 ml top agar. Fourteen of the laboratories use these amounts. Two do not give instructions for this solution. One laboratory reports using trace amounts of His/Bio. Four use 0.05 mM solutions. One laboratory uses 39 mg/l of histidine. One laboratory uses 10 μg/ml of histidine and 12 μg/ml of biotin in their stock solution. One laboratory uses 0.0976 g biotin/l and 0.0525 g histidine/l in the solution. Another uses 61.0 mg biotin/500 ml and 48.0 mg histidine/500 ml and 100 ml of the stock solution per liter of top agar.

VBME Salt Solution

Ames does not give instructions for the preparation of the VBME solution.

Vogel's reference gives the following gram amounts per liter of solution:

$MgSO_4 \cdot 7\,H_2O$	- 10 g
Citric Acid $\cdot\, H_2O$	- 100 g
K_2HPO_4 ·Anhydrous	- 500 g
$NaNH_4 \cdot HPO_4 \cdot 4\,H_2O$	- 175 g
H_2O	- 670 ml

The pH is 7.0 and the solution is a 50x concentration. Fourteen of the laboratories use these same instructions and 10 give none. One laboratory gives the same gram amounts but lists the concentration of the solutions at 25x.

Media

Top Agar Overlays

Ames recommends a solution of 0.6% Difco agar and 0.5% NaCl for top agar. Before using, the top agar is melted in a steam bath and 10 ml of a His/Bio solution is added to 100 ml of top agar. The His/Bio solution consists of a 0.5 mM concentration of histidine and a 0.5 mM concentration of biotin. Aliquots of 2 ml are pipetted into culture tubes and kept warm until use. Nine laboratories prepare overlays by this procedure. Nine others give the quantities of the ingredients or the His/Bio concentration but do not elaborate further. One laboratory does not list information on the overlays. Three use media other than Difco, and three use concentrations different from those recommended by Ames.

Petri Plate Agar

Ames specifies Falcon plastic 100 x 15 mm petri plates, and 30 ml of a 1.5% Bacto-Difco agar with 2% glucose and VBME salt solution for the bottom agar. Two laboratories do not discuss their petri plate agar. For most, the amount of agar in a plate ranges from 20 to 30 ml. Three laboratories prefer nonethylene oxide sterilized plates. Two laboratories do not use Difco agar. Thirteen laboratories follow the recommendations of Ames. One laboratory uses 16 g/l of agar. Four laboratories do not indicate the agar concentration.

Nutrient Broth

Ames originally used Difco Nutrien Broth but has since recommende Oxoid #2 broth. Seven laboratories sa they use Oxoid #2. Five laboratories us Difco; one uses Columbia Broth, an one recommends Lab-Lemco powder an peptone. The 11 remaining laboratorie do not indicate the type of broth used i this laboratory.

Chemical Controls

Control Mutagens/ Carcinogens Routinely Used

For activation assays Ames recommends using 2-aminoflourene, aflatoxin B_1, and benzo(a)pyrene. De Serres and Shelby recommend using 2-aminoanthracene solely. Fourteen laboratories use 2-aminoanthracene exclusively. Two laboratories use 2-aminofluorene exclusively. Two others use 2-aminoanthracene and benzo(a)pyrene. One laboratory uses 2-aminoflourene and aflatoxin B_1, and another uses benzo(a)-pyrene, 2-aminoanthracene, and proflavine. Five laboratories do not indicate what chemical they use.

For nonactivation assays using strain TA1535 Ames recommends N-methyl-N-nitrosoguanidine (MNNG) and de Serres and Shelby recommend methyl methanesulfonate (MMS). Six laboratories use MNNG exclusively. Nine laboratories use sodium azide solely. Another uses 1,3-propane sultone. One laboratory uses MNNG, MMS, and sodium azide. Another uses sodium azide and 4-fluoro-3-nitrophenyl azide. Still another uses sodium nitrite, N-hexyl-N-nitro-N-nitrosoguanidine. One laboratory uses MNNG, MMS, and 4-Nitroquinoline-N-oxide, and five do not say what chemical they use.

When using TA1537 Ames and de Serres both recommend using 9-aminoacridine. Eighteen laboratories follow this recommendation. Five laboratories do not say what chemical they use. One laboratory uses 4-fluoro-3-nitrophenyl azide, and another uses MNNG and 9-aminoacridine.

For TA1538 Ames suggests 2-aminofluorene and de Serres recommends 4-nitro-O-phenylene diamine, 2-nitrofluorene, and hycanthone methanesulfonate. Thirteen laboratories use 2-nitrofluorene only. Seven do not list the chemical they use. One laboratory uses Daunomycin exclusively, and another uses 2-aminofluorene exclusively. One laboratory uses 2-nitrofluorene and 4-nitro-O-phenylene diamine while one

uses 2-nitrofluorene and 4-fluoro-3-nitrophenyl azide. One laboratory uses MNNG and 4-nitroquinoline-N-oxide.

When using TA98 Ames recommends 4-nitroquinoline-N-oxide, Daunomycin, 2-aminofluorene, and aflatoxin B_1. De Serres recommends using 4-nitro-O-phenylene diamine, 2-nitrofluorene, and hycanthone methanesulfonate. Fourteen laboratories use 2-ntirofluorene only. Six laboratories do not list a chemical. One laboratory uses Daunomycin only and one uses 4-nitroquinoline-N-oxide only. One laboratory uses 2-nitrofluorene and hycanthone methanesulfonate. Another uses 2-nitrofluorene and 4-fluoro-3-nitrophenyl azide. Still another uses Daunomycin, MMS, and 4-nitroquinoline-N-oxide.

For strain TA100 Ames suggests MMS, MNNG, 2-aminofluorene, aflatoxin B_1, and 4-nitroquinoline-N-oxide. De Serres' recommendations are sodium azide, MMS, and nitrofurantoin. Nine laboratories use sodium azide only, and three use MNNG only. One laboratory uses 1,3-propane sultone, and one uses MMS; another uses benzo(a)pyrene only. Five laboratories do not list the chemical they use. One laboratory uses MMS and MNNG. One laboratory uses MNNG, MMS, sodium azide, and nitrofurantoin. One laboratory uses sodium azide, 4-fluoro-3-nitrophenyl azide. Another uses MMS and 2-nitrofluorene. Still another uses MNNG, MMS, and 4-nitroquinoline-N-oxide.

Preparation of Control Mutagens/Carcinogens

Ames recommends the disposable sterile polystyrene screw cap tube as the container of use for carcinogens and lists four possible solvents: H_2O, DMSO, ethanol, or p-dioxane. Fourteen laboratories do not discuss their method of preparation. Two laboratories follow Ames' procedure. Five others list only the solvent used. Four laboratories list either their storage container or procedure.

Assay Procedure

Ames recommends the following procedure for the assay. An aliquot of 0.1 ml from a 10^8 culture of *Salmonella* is added to a tube containing 2 ml molten top agar at 45°C, followed by the sample to be tested and 0.5 ml of S-9 mix. The tube is rotated between the palms and, within 20 sec, poured onto a petri plate. The plates are left to harden for one hour and then incubated in the dark for two days at 37°C. De Serres and Shelby recommended a culture of 1 or 2 x 10^9 concentration of *Salmonella* and an extension of the incubation time from 48 h to 48-72 h. They suggested duplicate plating as the minimum with triplicate plating preferred, and used aliquots of 0.2 μg to 5 mg of sample. They recommended both that sterility controls include the solvent and S-9 mix, and that spot checked phenotype monitoring be performed.

The most variation among the laboratories comes in the order in which constituents are added. Half the laboratories indicate a preference to the order of addition: *Salmonella*, sample, and S-9. The other laboratories use different permutations or do not list an order. Temperatures for top agar vary from 43 to 56°C. Incubation times range from 36 to 72 h. Duplicate or triplicate plating is recommended. Five laboratories substitute the volume taken up by S-9 by using KCl, saline, or buffer in a nonactivation assay. Three laboratories place their plates in sealed bags for the incubation time.

Ames has revised his mixing technique: he now uses a vortex at low speed. Eight laboratories mention using a vortex in their protocols.

Three laboratories indicate use of only a preincubation technique with the *Salmonella* and sample.

Discussion

As a first step in establishing Quality Assurance/Quality Control (QA/QC) procedures for *in vitro* bioassays, the Ames *Salmonella* plate incorporation bioassay protocols from 25 laboratories were compared. At this stage, no attempt was made to evaluate a protocol or to designate a preferred method.

In general, most laboratories follow most of the procedures as outlined by Ames; however, it appears that each laboratory deviates from this standard protocol in some manner. There was strong concordance with Ames on the selection of tester strains, the preparation of the S-9 mix, and the preparation of the microsomal salt solution.

Most laboratories differ from the Ames, *et al.*, 1975 protocol in the selection of some control compound and in the actual assay exposure procedure. Only one laboratory prescribed the routine verification of compound sensitivity for strains TA98 and TA100. Most procedures and media preparation instructions were left out of one or more protocols; however, a laboratory SOP may have included these instructions.

Comparison of current protocols may improve both individual experimental procedures and the consistency of results from different laboratories. In addition, this study may help identify monitorable parameters within the test procedures so that adequate QA/QC procedures can be established.

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The complete report, entitled "Comparison of Ames Salmonella typhimurium *Plate Incorporation Test Protocols," (Order No. PB 82-234 253; Cost: $10.50, subject to change) will be available only from:*

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The EPA Project Officer can be contacted at:

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