MOLTOX®

Molecular Toxicology, Inc.

Salmonella Mutagenicity Test Kit Instruction Manual

31-100.2

INTRODUCTION

The materials contained in this Salmonella Mutagenicity Assay Kit include virtually all of the supplies necessary for the conduct of the "Ames Assay" as described by Maron & Ames (Maron, D. M. and B. N. Ames, Revised methods for the Salmonella mutagenicity test, Mutation Research, 113: 173 - 215, 1983) and Mortelmans & Zeiger (Mortelmans, K. and E. Zeiger, The Ames Salmonella/microsome mutagenicity assay, Mutation Research, 455: 29 - 60, 2000). This manual is a brief summary of the important aspects of testing - **We strongly recommend that you carefully read one of these papers and OECD guideline 471 before you attempt to perform the assay.**

All elements of the MOLTOX® kit are formulated and manufactured using the highest quality components and are consistent with the recommendations of Maron & Ames (ibid.). Certain materials supplied (e.g., STDiscs™ and lyophilized S9) have been specifically developed for inclusion in the assay kit by our laboratory. Most of the materials contained in the kit are accompanied by GLP level Quality Control and Formulation Statements - you may be assured that each element of each kit has been thoroughly tested for performance in the assay.

The MOLTOX® Salmonella Mutagenicity Assay Kit is intended for use by individuals skilled in the science and art of microbiology; the use of strict aseptic technique is essential for the successful application of the materials included in the kit. While the bacterial strains included in the kit (*S. typhimurium* strains TA1535, TA1537, TA98, and TA100) are attenuated, they are potentially pathogenic and must be handled accordingly. **If you have any doubts about the safe handling of the strains included in this kit do not proceed until you have consulted with us at (828) 264-9099 or have obtained the advice of a skilled biochemist or microbiologist.**

The performance of the "Ames Assay" includes several distinct experimental steps; e.g., test design; S9 mix formulation; dosing and plating; phenotype confirmation; target cell titer determination; reading (counting) and analysis. The materials contained in the kit were selected so as to provide the user with considerable flexibility as regards test design. This manual was developed to assist in the utilization of the kit contents; the information provided in the manual is intended to supplement that contained in the Maron & Ames and Mortlemans & Zeiger papers. While presented in a step-by-step manner, the instructions contained in this manual are amenable to modification - if you desire assistance in any phase of the assay, please contact our Customer Service department at (828) 264-9099. We will be happy to work with you to help solve any problems that may arise.

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Salmonella Mutagenicity Assay Kit Components

31-100.2 Salmonella Mutagenicity Assay Kit		
11-05L.2	Lyophilized PB/BNF S9	2 each
21-100	Nutrient Agar Plates	1 each
21-200	ST Quad PC™ Plates	1 each
21-400.2	MGA Plates	8 each
26-505.1	Nutrient Broth, 100 mL	1 each
26-503.1	His/Bio Top Agar, 100 mL	4 each
60-101	ICR 191, 10 µg/vial	1each
60-102	Daunomycin, 60 µg/vial	1 each
60-103	Sodium Azide, 15 µg/vial	1 each
60-107	2-Aminoanthracene, 100 µg/vial	1 each
60-200.4	Regensys™ "A", 40 mL	1 each
60-201.4L	Lyophilized Regensys™ "B", 123 mg	1 each
71-098L	TA98, 10/vial	1 each
71-100L	TA100, 10/vial	1 each
71-1535L	TA1535, 10/vial	1 each
71-1537L	TA1537, 10/vial	1 each

<u>Additional items you will need to complete the assay:</u>

4 - Sterile Erlenmeyer flasks

Sharpie or wax pencil

200 µL and 1000µL sterile pipette tips

1 mL and 5 mL sterile pipets

Pipet-aid or rubber bulb

Test tube rack

13 x 100 mm sterile disposable test tubes

Dimethyl Sulfoxide (DMSO)

Saline, 0.9% NaCl, sterile

Microwave or boiling water bath

45˚C water bath or heating block

Automatic colony counter or magnifying counter

Micropipettes (e.g. Pipette-man, Eppendorf)

Sterile inoculating loops

Sterile swabs (optional)

Ice

Ice bucket

Nitrile gloves

Sterile water

Vortex mixer

Sterile forceps, small (optional)

37˚C incubator with shaker

Water, deionized or distilled, sterile, ice-cold

THE ASSAY

The MOLTOX® kit contains four tester strains: TA1535, TA1537, TA98, and TA100 Each strain was constructed with a different lesion in the histidine operon (see Table 1, Mortelmans & Zeiger). This mutation renders them incapable of synthesizing histidine, i.e. they are histidine auxotrophs requiring exogenous histidine. In addition, TA1535, TA1537, TA98, and TA100 have altered cell walls (*rfa*) that increase the cell's permeability to certain high molecular weight materials. These strains also share a lesion in a DNA repair-coding gene (*uvrB*) which results in an increase in sensitivity to a variety of mutagens. Since this lesion extends through the gene for biotin synthesis (*bio*), biotin is also required for growth]. Tester strains TA98 and TA100 carry a plasmid (pKM101) which acts to increase the activity of an error-prone DNA repair system and to confer resistance to the antibiotic ampicillin, tester strains TA1535 and TA1537 contain no plasmids.

Because of the characteristics of the tester strains, the "Ames Assay" is uniquely suited for the detection of mutagenic activities. The several tester strains differ in their response to DNA-damaging chemical mutagens and therefore are generally employed in combination. Each strain tends to be responsive to specific classes of mutagenic chemicals due to the specific lesion in their histidine operon. However, exposure to mutagens may result in genetic reversions in the histidine operon resulting in restoration of the wild type phenotype; mutants have their histidine operons functionally "restored" and can synthesize histidine. The assay depends on the ability to distinguish between histidine auxotrophs (the tester strains) and histidine prototrophs (the mutants). Accordingly, the target cells are plated on media containing trace quantities of histidine that allows for a few rounds of cell division necessary to "fix" a mutation event; the histidine is rapidly exhausted resulting in cessation of the growth of nonmutated cells. If a mutagenic chemical is present, (comparatively) rare reversions may occur in the altered histidine operon resulting in the continuation of growth after trace histidine exhaustion. Revertant bacterial colonies that appear on histidine-limiting media plates represent prototrophs that arose either spontaneously or due to the action of a mutagen.

The sections that follow describe the procedures for the conduct of the basic four strain assay. For the most part, the methods described are taken directly from Maron & Ames (ibid.) and, subsequently, Mortelmans & Zeiger. If you have no prior experience with the assay, we suggest that you follow these instructions closely; those experienced in the method will find useful information about the use of STDiscs™, QUAD PC™ plates, and lyophilized S9.

A. Getting Started

1. Before setting up the assay, you should gather as much information as possible concerning your test material:

   a. Using the available references, structural analyses or activity data, assemble as much information as is possible concerning your test material or its analogues or closely related congeners. Of particular importance are questions of bacteriostatic or bactericidal activities, hazardous qualities and stability.

   b. Determine the solubility of your test chemical in the appropriate solvent (water and DMSO are preferred solvents - see p. 200, Maron & Ames for listing or check the Merck Index). In many cases, you may find it necessary to dose with suspensions rather that true solutions. If an organic solvent is used (e.g.DMSO, acetone), the test material may precipitate upon addition to the aqueous top agar; e.g. 2-aminofluorene is essentially insoluble in water and is solubilized in DMSO for dosing.

   c. Decide on the doses that you wish to test. The conventional test solution dose volumes is 100 µL per plate, but this can be increased for aqueous formulations or reduced, e.g. if a somewhat toxic organic solvent is used. In general, the upper dose should not exceed 5 mg/plate (50 mg/mL assuming a 100 µL/plate dosing volume). Select 5 to 7 doses separated by factors of 2, 3 (or half logs) or 5. There are 160 minimal glucose agar plates in your kit. This is sufficient for a 5 dose triplicate plate assay (including controls) conducted with and without S9.

2. To avoid difficulties on the day of the assay, design your experiment carefully and well in advance. Examples of questions you will need to have resolved are as follows:

   a. Dosimetry: Top dose? Dose intervals? Number of doses? Solvent? Volume of each dosing solution dilution required for the complete assay?

   b. Replicates: Are you going to dose duplicate plates per condition? Triplicates? In many cases, duplicate assays may be sufficient.

   c. Metabolic activation: Are you going to perform the assay in the presence as well as the absence of S9 mix?

   d. Strains: Which strains are you going to use? Depending on your objective, you may wish to use only TA98 and TA100; e.g. if your test material is a complex mixture, a two strain assay may be desirable.

3. Assemble the supplies and equipment needed to perform the test on the day before.

a. Remove the QUAD PC™ plates from the refrigerator, cut off the plastic sleeve and allow to dry upright at room temperature overnight.

b. Label the Minimal Glucose Agar plates appropriately: Strain number, test material identification and dose, S9 (+/-) and date of test should be included. Writing using a wax pencil or "Sharpie" should be restricted to the dish top or side of the plate - never write on the bottom as such will interfere with scoring. Be sure to include the diagnostic positive and negative controls.

c. Adjust the temperature of your water bath or dry block heater to approximately 45°C. Make sure that your incubator is adjusted to 37°C. You will need a microwave oven or a boiling water bath to melt the top agar. Set your shaker to approximately 100-150 rpm. Note: Some shakers generate excessive heat and cannot be used in standard above ambient bacteriological incubators. It is best if you can connect your incubator shaker to a timing device - depending on the wattage of your equipment, a simple inexpensive residential lighting timer may suffice.

B. Cell Culture - Use of STDiscs™

Your kit is supplied with lyophilized strains in disc format. Each disc contains sufficient viable cells to serve as the inoculum for a 20 - 25 mL culture. STDiscs™ are accompanied by a QC sheet that describes their phenotype. The kit includes materials to confirm the phenotypes of your cultures and you may find it useful to compare your results with those described on the aforementioned QC sheets and with the strain descriptions in the Maron & Ames and Mortelmans & Zeiger papers. To prepare strain cultures for use in the assay:

DAY BEFORE THE ASSAY

***USE ASEPTIC TECHNIQUE ***

1. Label sterile Erlenmeyer flasks with the strain number (e.g., TA1535, TA1537, TA98, and TA100) in accordance with your experimental design.

2. Using aseptic technique, carefully pipet approximately 20 - 25 mL of Oxoid #2 nutrient broth into Erlenmeyer flasks.

3. Remove the STDisc™ vials from the refrigerator and **warm to room temperature** before opening to avoid the formation of condensation on the inner surfaces.

4. Unscrew the vial closures and remove the slotted gray butyl rubber stopper from one vial (this is best accomplished by use of forceps). Do not contaminate the inner surfaces of the stopper (e.g., place the stopper in its correct position in the screw cap closure).

5. Using a sterile loop/needle, pick up one or more discs and drop into the appropriately labeled flask containing nutrient broth. Carefully close the STDisc™ vial.

6. After the flasks are inoculated, transfer to a 37°C incubator and hold stationary overnight. Early the next morning incubate with shaking (125 - 150 rpm, avoid foaming) at 37°C until a density of 1 - 2 X 10^9 bacteria/mL is achieved (approximately 1.0 - 1.4, OD_{650nm}). Correct density is usually achieved within 2 hours.

7. After incubation remove the flask cultures and place them in the refrigerator until you begin the assay.

DAY OF THE ASSAY

*USE ASEPTIC TECHNIQUE *

C. Treatments and Plating

1. Melt the histidine/biotin supplemented top agar in a boiling water bath or microwave oven. **Be sure that you have loosened the container caps - failure to do so may result in a violent explosion due to pressure build-up.** Examine the melted agar carefully - if any opalescence persists, continue heating until a perfectly clear solution is obtained. After melting, place the top agar bottles into a 45°C water bath - allow at least 45 minutes for temperature equilibration.
2. As with any enzyme assay, all materials should be placed on ice prior to use and kept on ice throughout the assay. If you are using the S9 activation system, remove the tear-off seal from one or both lyophilized S9 vials. Rehydrate each vial with 2.1 mL ice cold sterile water and mix to homogeneity. Your Regensys™ system can be used at 5% or 10% S9 concentration. For 5%, add 2.0 mL rehydrated S9 and 2.0 mL sterile water to the Regensys™ "A" bottle. For 10% S9, add a total of 4 mL rehydrated S9. Keep on ice. Just before use, add the contents of the Regensys™ "B" tube (NADP), mix thoroughly, and hold on ice.
3. **Open the CONTROLCHEM™ packages**:

   Nitrile gloves must be worn when handling these chemicals. Latex gloves do not provide adequate protection for chemicals dissolved in organic solvents. Remove gloves carefully to avoid skin contamination. Wash hands after use.

   Add 1.0 mL of the appropriate solvent to each of the CONTROLCHEM™ tubes.

Mutagen	Amount (µg)	Strain	Solvent
Sodium Azide	15	TA1535, TA100	Water
ICR 191 Acridine	10	TA1537	Water
Daunomycin	60	TA98	Water
2-Aminoanthracene (S9 activation control)	100	All	DMSO

4. Perform the dilutions of your test material. Remember that you will be dosing using 100 µl volumes. Therefore, your dosing solutions should be made up at 10x the desired dose. Arrange the test material dilutions so that they follow a logical sequence - e.g., solvent control, low dose to high dose.

5. Load a test tube rack with sterile 13 x 100 mm tubes with closures equal to the number of minimal glucose agar plates labeled in step A 3b. Place rack in 45°C water bath or heating block and pipet 2 mL of molten, 45°C, top agar into each tube. Remove and replace closures carefully so as to avoid contamination.

6. Arrange your previously labeled Minimal Glucose Agar plates by strain and condition (e.g., controls, +/- S9, etc.).

7. Decide which strain you are going to begin with. In the example below it is assumed that TA98 will be used first in a duplicate plate, + and - S9 assay.

8. Assay your Test material:

- WITHOUT S9

a. Add the test material doses to the tubes containing top agar. Begin with the solvent control; add 100 µL of water or DMSO (or other solvent used to solubilize your test material) to the first two tubes. Then, in ascending sequence, add 100 µL of each test material dilution to each additional pair of top agar-containing tubes.

b. Add 100 µL of the TA98 culture to the first two tubes (solvent control tubes).

c. Without delay, gently mix the tube contents using a vortex mixer and decant the mixture onto the surface of the appropriately labeled Minimal Glucose Agar plate. Do one tube at a time. Immediately upon decantation, gently tilt the plate and rotate so as to obtain an even distribution of the plating mixture over the surface of the bottom agar. Place onto a **perfectly level surface,** re-cover plate and allow to harden.

d. Repeat steps 8b. and 8c. for each dose of the test material.

- WITH S9

e. Repeat step 8a. using an additional set of top agar-containing tubes.

f. Add 500 µL of the previously prepared S9 mix to the first two tubes (solvent control tubes).

g. Without delay, add 100 µL of the TA98 culture as described in step 8b (above).

h. Immediately mix the tube contents as before, decant onto the Minimal Glucose Agar plate, re-cover and set aside to harden. Repeat steps 8f. and 8g. for each of the test material doses.

REPEAT THE ABOVE PROCEDURES FOR EACH STRAIN

9. Treat the Positive Control Cultures:

a. Set up 4 tubes for each strain. 2 tubes will be used for the - S9 diagnostic control and 2 will be used for the + S9 positive control.

b. Add 2 mL of molten agar to each tube as before. Add 100 µL of the CONTROLCHEM™ solutions according to the following scheme:

Chemical	Strain	Dose/Plate
Sodium Azide	TA1535, TA100	1.5 µg
Daunomycin	TA98	6.0 µg
ICR 191 Acridine	TA1537	1.0 µg
2-Aminoanthracene	All (+S9)	10.0 µg

c. Following the methods described in Section 8, add 100 µl of the appropriate strain and decant, spread and set aside. For 2-AA, add 500 µl S9 mix and strains as was previously described.

10. Inoculate the QUAD PC™ Plates:

a. Using a sterile loop or swab, wet with the appropriate culture and inoculate each of the four sectors of a QUAD PC™ plate using a "Z" inoculation pattern.

b. Repeat for each strain. After all plates are inoculated, open the vial containing the crystal violet discs and using forceps or an inoculating loop, place single disc on the agar surface in Sector II of each of the Quad PC™ plates.

11. Determine the Titer of the Strain Cultures

a. Arrange sets of 3 sterile tubes with closures for each strain. Pipet 4.95 mL sterile water into each tube.

b. Using your positive displacement pipette, inoculate the first tube with 50 µL of the appropriate strain culture. Mix thoroughly using a vortex mixer at low speed or by use of a 5 mL pipette. This tube contains 1:100 dilution of the sampled culture. Add 50 µL of the 1:100 dilution to the second tube containing 4.95 mL sterile water and mix as before. The second dilution is 1:10,000. Complete the dilutions by adding 50 µL of the 1:10,000 dilution to the third 4.95 mL tube and mix. The final dilution is 1:1,000,000.

c. Arrange sets of 2 sterile tubes with closures for each strain and place in 45°C water bath. Add 2.5 or 3 mL of molten top agar to each tube.

d. Using the positive displacement pipette, inoculate the top agar-containing tubes with 50 µL of the 1:10,000 and 1:1,000,000 dilutions. Mix and pour onto the appropriately labeled Nutrient Agar plates (provided in the kit). The plated volumes result in final dilutions of 5 x 10^{-6} and 5 x 10^{-8} for the 1:10,000 and 1:1,000,000 dilutions in water, respectively.

12. Incubate the Assay

a. Invert the plates and arrange in stacks corresponding to each experimental condition.

b. Place in a 37°C incubator and incubate for approximately 48 h.

13. Read the Assay

a. After the incubation period, remove the inverted plates and allow to come to room temperature. Turn the stacks of plates over so that the tops are up. If excessive condensate has formed on the lids while incubated in the inverted position, remove the condensate by removing the lids one at a time (keeping the plate upside down; agar should face the floor), sharply shaking the lid and replacing before turning the plates over.

b. Colony counting can be performed manually with the aid of a magnifying counter (e.g., "Quebec" counter) or with an automatic colony counter (e.g., Biotran, Synbiosis). Depending on the activity of your test material, large numbers of colonies may develop in certain dose groups. In some cases, it may be desirable to utilize sector-counting techniques rather than full plate counts. However, sector counting is not appropriate if the distribution of colonies is nonuniform across the surface of the agar. Be sure to examine the background lawn using a microscope (40x) or similar instrument. A normal background consists of densely packed microcolonies forming a thin, somewhat granular , film. If a plate contains many very small, just macroscopic "pinpoint" colonies and reveals an absence or "thinning" of the background lawn, the test material dose was toxic. Mutant colony counts from plates exhibiting toxicity should not be considered in activity determinations.

c. After counting and recording the results for the test material treatments, the diagnostic positive control plates should be counted. The colony counts for the positive control treatments should be compared to the values described in the STDisc™ QC sheets. Values of the diagnostic positive control chemicals shown in the Maron & Ames paper (see p. 194) were derived from "dose response curves" and, in our experience may not be representative of the frequencies expected for the doses utilized here.

d. Examine the cell titer (nutrient agar) plates. The 5×10^{-6} plates should be too numerous to count. In contrast, the 5×10^{-8} plates should contain approximately 50-100 colonies. Such a result indicates that the initial population (the stock culture) was in the range of $1 - 2 \times 10^{9}$ “viable” cells per ml. Very much lower or higher initial titers may result in reduced frequencies and background or increased backgrounds, respectively.

e. The QUAD PC™ plates (phenotypic confirmation media) should be examined and the results compared to those described in the relevant strain QC sheets and the Maron & Ames paper.

D. The Results - Evaluation and Interpretation

1. Negative (solvent) Control Counts

The colonies that grew on the Minimal Glucose Agar plates developed from single cells that had regained their ability to grow in the absence of added histidine. The genetic reversion, from histidine auxotrophy to prototrophy, that enabled those cells to grow in the absence of exogenous histidine might have arisen spontaneously or as the result of a mutation induced by the treatments (see Maron & Ames, p. 181). It is important to realize that some of the colonies that arose in the positive control plates would have grown in the absence of treatment; they arose spontaneously. Accordingly, the negative (solvent) control colony counts constitute an important baseline in your evaluation of the test results.

Unfortunately, the spontaneous reversion frequencies for the various tester strains can be quite variable. Nevertheless, large deviations form the “normal” range of spontaneous reversion values may signal systematic problems with the assay. Taking into account the fact that there are significant lab-to-lab differentials, the following ranges for spontaneous reversion values for the several strains may be representative : TA1535, 5 - 20; TA1537, 5 - 20; TA98, 20 - 50, TA100, 75 -200.

2. Diagnostic Positive Control Counts

If you used CONTROLCHEM™ chemicals as suggested, the numbers of revertants (mutants) should fall within the following ranges:

Strain	Chemical	Number of Colonies/Plate
TA1535	Sodium Azide	≥ 200 - 650
TA1537	ICR 191	≥ 40 - 200
TA98	Daunomycin	≥ 450 - 1,850
TA100	Sodium Azide	≥ 300 - 650

In general, the positive control frequencies (number of colonies per plate) should be at least 2.5 times the negative control counts (spontaneous frequency). Large deviations usually indicate problems with cell husbandry; e.g., high spontaneous frequencies (due, perhaps to culture overgrowth) often are paralleled by low induced frequencies. Such eventualities reduce the resolving power of the assay and raise questions regarding the interpretation of the results of the test material treatments.

3. Phenotypic Confirmation

The QUAD PC™ plates are prepared with four different media that provide basic information concerning the genotypes of the strains provided in the kit (see the QC sheets for the specific strains). By sector, the results should be:

Sector	Observation	Genotype
I	No growth (all strains)	*his*
II	Growth. Zonal inhibition around CV disc (all strains)	*his, rfa*
III	Growth of TA98 and TA100 only	pKM101
IV	No growth (all strains)	pAQ1 (present in TA102 only)

4. Test Material Results

Various investigators have applied different criteria for the analysis of the assay results. In general, the 2 or 2.5 times over the background (spontaneous frequency) "rule-of-thumb" may serve as a useful way of distinguishing active mutagens from non-mutagenic materials. The presence of a dose response (not necessarily linear) is often used as an adjunct criterion for and interpretation of positive activity in the assay. We highly recommend that you read pages 195-197 in Maron & Ames (1983) or pages 49 – 50 in Mortelmans & Zeiger for additional information concerning the interpretation of the assay results.

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