# Procedure for Conducting the Host-Mediated Assay Utilizing Bacteria ('Salmonella typhimurium')

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*Abstract*

**Irrespective of the indicator system selected, several concentrations of a compound under investigation should be used and a dose-response curve is essential. A standard mutagen as well as an adequate negative control should always be included in the test. At the present state of the art, it is quite possible that the host-mediated assay will play a significant role in characterizing chemical mutagens and establishing a correlation between carcinogenic and mutagenic substances. Future developments in this field will include attempts to localize the site of action of a specific agent by the use of chambers (FISCOR et al. [2]), the development of a single indicator microbial strain that could detect multiple genetic lesions, and use of experimental animals other than the mouse. In addition to the conventional host-mediated assay, the indicator system described in this report will no doubt be used as one of the few available methods for detecting the presence of chemical mutagens and carcinogens in man, by appropriate analysis of body fluids.**

The recent development of the field of chemical mutagenesis, a new area of toxicology, parallels the development of appropriate methods that allow for the detection of chemical mutagens after activation by a mammalian host. The host-mediated assay, a procedure that detects mutagenic substances after metabolic activation in an intact mammal has played a prominent role in the development of this area of toxicology. In this assay, the mammal, during treatment with a potential chemical mutagen, is injected with an indicator organism in which mutation frequency can be measured. After a sufficient time period, the organisms are withdrawn from the animal and the induction of mutants is determined. The comparison between the mutagenic action of the compound, (1) on the organism directly, and (2) in the host-mediated assay indicates whether (a) the host can detoxify the compound or (b) mutagenic products can be formed as a result of host metabolism. This is an extremely flexible procedure, allowing the use of a wide variety of indicator organisms.

Since the original publication, a variety of indicator organisms have become available and by selecting the proper indicator strains, a variety of genetic alterations can be detected. Although several modifications have been introduced with this assay, the following steps are usually carried out in this procedure:

(a) Animal, usually a rodent is treated by a appropriate route with the chemical under investigation. The compound can be administered by a single injection or multiple injections.

(b) The indicator organism is injected into the animal usually into the pertioneal cavity.

(c) After a suitable period of time, optimally to allow several replications of the indicators organism, the organism is recovered from the host.

(d) The indicator is plated and mutation frequency of the organism recovered from the treated animals and the appropriate control animals are compared.

The comparison of the induction of mutants in the host and the direct effect of the chemical on the organism in the absence of the host renders useful information as to the nature of the active chemical. If the administered drug is not active in the direct test, but active in the host, it is likely that a metabolite of the administered chemical is responsible for the mutagenic activity. This method has become one of the major procedures used in characterizing mutagenic substances for the following reasons: (a) ability to detect point

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mutations, (b) simplicity and (c) short term duration of the test.

This method, like other procedures commonly used in the field of toxicology, has definite limitations. The indirect nature of the test, inability to take into account repair responses of the host, and the inability to conveniently localize the site of action of the mutagenic substances are obvious limitations of the host-mediated assay. This procedure should be incorporated as one of the battery of tests to be used for characterizing mutagenic substances. Table 1 depicts the advantages and disadvantages of this method.

In the last few years, a variety of indicators including bacteria, yeast, molds and mammalian cell cultures have been utilized. The selection of the proper indicator is one of the major factors in successfully conducting this assay.

Although no perfect single indicator system exists, recent studies would indicate that three optimum systems in this assay are (a) various strains selected from this histidine auxotroph of *Salmonella typhimurium*, (b) yeast, where mitotic recombinant can be scored and (c) the Fischer murine leukemic system. Table 2 is a summary depicting the advantages, disadvantages and status of the various indicators that have been used in the host-mediated assay.

**Histidine auxotroph of 'Salmonella typhimurium'**

Although several indicator organisms have been studied in this assay, the histidine auxotrophos of *S. typhimurium* has been the most extensively used. The histidine genes of *S. typhimurium* are among the best characterized operons in microorganisms. The structural genes for the enzymes of this histidine operon (the enzymes that convert the 5-carbon chain of phosphoribosyl pyrophosphate to histidine) are in a cluster on the *Salmonella* chromosome. More than a thousand histidine requiring mutants have been located on a fine structure map of the operon (WHITFIELD, MARTIN and AMES [8]). A series of tester strains that include compounds that test for both base analogue and frameshift mutation in repair deficient as well as repair competent strains have been utilized. The utility of this method in detecting active metabolism in the animal has been amply demonstrated utilizing this auxotroph with well over a hundred compounds including compounds such as cycasin and nitrosamines (LEGATOR and MALLING [5]) have been shown to be active with this system.

In the last few years, as a result of the work primarily by AMES [1], rapid progress has been made in the development of new indicator strains that are extremely sensitive and have greatly expanded our ability to detect mutagenic agents when directly tested in this reverse mutation procedure. The availability of strains that have a defective excision repair system (a deletion through uvrB) and deep rough mutants of *S. typhimurium* that lack a large portion of the lipopolysachride (a component of the bacterial outer membrane),

*Table 1*
Host-mediated assay.

| Advantages | Disadvantages | Present status | Future development |
|---|---|---|---|
| Only feasible screening method that detects point mutation, can detect transient metabolites as well as direct acting chemical. | Indirect indication of point mutations | Practical screening procedure, over 1000 compounds tested. | Indicators, such as the Fischer murine leukemic line that can divide in host and mutate at same rate as in vitro (i.e., mouse lymphoma system). |
| Indicate detoxification as well as potentiation by comparison with direct test on indicators. | localization of active metabolite as to organ or tissue may be difficult. | | Localization of genetic effect in various organs. |
| Simple, economical test. | | | In bacteria detection of multiple genetic events with a single indicator. |
| Moderately skilled investigator. | | | |
| Can be used to correlate carcinogenicity with mutagenicity. | | | |

represent significant progress in the devlopement of tester strains. With the repair deficient strain, three strains (TA 1531, 1532 and 1534) are designed to detect frameshift mutagenesis, and the fourth (TA 1530) is similar to the missense mutant G-46 and will detect base pair substitutions. The deep rough mutant is sensitive to certain chemicals such as the epoxide of carcinogenic polycyclic hydrocarbons where maximum penetration is a prerequisite for induction of mutations. The deep-rough mutant has yet to be utilized in the host-mediated assay. The tester strains, depicted in Table 3, should be used in studies with host-mediated assay to maximize the detection of a chemically induced mutation of varying specificity.

The following is the general protocol followed with this procedure [9].

### (1) Media

The *Salmonella* strains are maintained on tryptone-yeast agar slants and transferred in tryptone-yeast broth (1.0% tryptone, 0.5% yeast extract). For enumeration of his$^+$ revertants, Spizizen's minimal medium is used, supplemented with 0.5 μg biotin/ml to allow for growth of the deletion containing strains.

*Table 3*
Recommended tester strain of *S. typhimurium* to be used either in the direct test or host-mediated assay.

| Mutant | Class |
|---|---|
| G-46 | Missense |
| TA-1530 | Missense and (repair deficient) |
| C-203 | Frameshift |
| C-207 | Frameshift |
| TA-1531 | Frameshift and (repair deficient) |
| TA-1532 | Frameshift (repair deficient) |
| TA-1534 | Frameshift (repair deficient) |
| TA-1537 | Frameshift (deep-rough mutant) |

### (2) Chemical administration

This assay can be used for either acute, subacute or chronic studies. The solvent used to dissolve the drug is extremely important. With non-water soluble compounds, DMSO, corn oil, and ethanol have been employed. It is essential to run a suitable solvent control with each experiment.

In a typical acute study, the compound under investigation can be administered by gavage using a blunt tipped 18 gauge (1 inch) hypodermic needle. A total volume of 0.5 ml will be inserted into the stomach. The indicator organism is injected within a suitable time period (i.e., four hours) after administration of the test compound.

*Table 2*
Representative indicator organisms in the host-mediated assay.

| Indicator | Genetic alterations detected | | | Ease of detecting genetic events | Genetic validity of detected change | Growth division in host as compared to in vitro | Spont. mutation frequency in host as compared to in vitro | Ability to localize genetic effect in host | Utility |
|---|---|---|---|---|---|---|---|---|---|
| | Mutations Forward | Reverse | Miotic gene conversion and recombination | | | | | | |
| *S. typhimurium* (Histidine auxotroph) | No | Yes | No | Excellent | Established | Similar | Similar | With difficulty | Good |
| *N. crassa* (Adenine 3 locus) | Yes | Yes | No | Fair | Established | No growth or division | – | With difficulty | Questionable |
| *S. cerevisiae* | Yes | Yes | Yes | Fair | Established | Slight growth No division | – | With difficulty | Good |
| Chinese hamster | Yes | Yes | No | Good | Questionable | No growth or division | – | With difficulty | Questionable |
| Murine leukemic | Yes | Yes | No | Good | Questionable | Similar | Similar | With difficulty | Good |

**(3) Inoculation techniques**

The bacteria are grown to a density of approximately $3–5 \times 10^8$ cells/ml (log phase). A 23 gauge needle is used to inoculate 2.0 ml of this cell suspension into the peritoneal cavity of male Swiss albino mice (after the area of inoculation has been swabbed with ethanol). The mice weight is 25–30 g and they are maintained on a diet of Purina Mouse Feed or equivalent. The experiment is allowed to run for three hours after inoculation of the bacterial suspension.

**(4) Autopsy and recovery techniques**

The abdominal regions of the dead mice are swabbed with ethanol, and 1–2 ml of sterile saline is injected into the peritoneal cavity of each mouse. The peritoneal cavity is then opened a septically and the exudate withdrawn using a tuberculin syringe without a needle. The peritoneal exudates from each mouse will be run separately.

**(5) Dilutions and plating**

(A) *Dilution:* Dilution blanks, containing 4.5 ml of sterile saline are prepared in advance. Tenfold serial dilutions are made of each peritoneal exudate (0.5 ml + 4.5 ml) yielding a concentration series from 10° (undiluted peritoneal exudate) through $10^{-7}$.

(B) *Plating:* For enumeration of the total bacterial counts, the $10^{-6}$ and $10^{-7}$ dilutions are plated in tryptone-yeast agar: three plates per sample, 0.20 ml of sample per plate. Each sample is spread over the surface of the plate with a bent glass rod immersed in 95% ethanol and flames just prior to use. In plating for the total mutant counts on minimal agar, the 10° dilution is used; the $10^{-1}$ and $10^{-2}$ dilutions are only plated when the sample is known to contain a significant number of revertants. With the 10° dilution is plated, five plates are used (0.20 ml sample/plate); when it is necessary to plate additional dilutions, three plates are used for each dilution. The plating procedure is identical to that followed for the tryptone-yeast agar plates. All plates are incubated at 37°C.

**(6) Scoring**

No. colonies/No. of plates $\times 5$ = colony-forming units (CFU) per ml of sample plated. CFU/ml $\times$ 1/dilution factor ($10°–10^{-7}$) = CFU/ml in diluted exudates. The mutation frequency (MF) is calculated for each sample.

$$MF = \frac{His^+ \text{ revertants/ml}}{\text{CFU/ml in undiluted exudate}}$$

$$MFt/MFc = \frac{\text{MF of experimental sample}}{\text{MF of control sample}}$$

(MFt/MFc = 1.00 for control sample)

**Genetic effects of various chemicals**

In an earlier report, data on standard mutagenic substances as well as selected test compounds was presented (LEGATOR and MALLING [5]). ZEIGER et al. [11], ZEIGER and LEGATOR [10] have evaluated a series of nitrosamines using primarily the G-46 tester strains. Dimethylnitrosamine, diethylnitrosamine, nitrosomorphiline, 1-nitrosopiperazine, 1,4-dinitrosopiperazine, 1-nitroso-4-methylpiperazine and 1-nitropiperidine were positive. Nitrosoiminodiacetic acid, diphenylnitrosamine and nitrosomethylanaline were reported to be negative. Cyclophosphamide, methyl-nitroso-urea and butyl nitroso-urea were reported to be active with G-46 (PROPPING et al. [6]).

**Analysis of body fluids by use of various indicator systems**

The availability of various biological indicators, such as those employed in the host-mediated assay, can be exploited for the detection of mutagenic agents in body fluids in experimental animals and man. In 1969, the mutagenic activity of streptozotocin was detected in body fluids of treated animals [4] and FISCOR and MUTHIANI [3] detected mutagenic activity of streptozotocin in tissue homogenates of treated mice. Recently the mutagenic activity of cyclophosphamide was demonstrated in the urine of treated animals [7]. The animal work can now be extended to man and it should be possible to detect in man mutagenic and potential carcinogenic substances by analyzing blood and/or urine using suitable extraction and concentration procedures after drug administration.

*Conclusion*

Irrespective of the indicator system selected, several concentrations of a compound under investigation should be used and a dose-response curve is essential. A standard mutagen as well as an adequate negative control should always be included in the test. At the present state of the art, it is quite possible that the host-mediated assay will play a significant role in characterizing

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