

INTRODUCTION

The materials contained in this *E.coli Mutagenicity Assay Kit* include virtually all of the supplies necessary for the conduct of the assay as described by Green, M.H.L., and Muriel, W.J., (*Mutation Research*, **38**: 3-32, 1976) - **we strongly recommend that you carefully read this paper before you attempt to perform the assay.**

All elements of the MOLTOX™ kit were formulated and manufactured using the highest quality components and are consistent with the recommendations of Green and Muriel (*ibid.*). Certain materials supplied (e.g., ECDiscs™ and LS-9) have been specifically developed for inclusion in the assay kit by our laboratory. GLP level Quality Control and Formulation Statements accompany most of the materials contained in the kit - you may be assured that each element of each kit has been thoroughly tested for performance in the assay.

The MOLTOX™ *E.coli 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 [*E.coli* strains WP2 *trp* (72-187) and WP2 *trp uvrA* (72-188)] are, in a practical sense, attenuated, they are etiologic agents 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 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 Green and Muriel paper. 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 might arise.

THE ASSAY

The MOLTOX™ kit contains two tester strains; WP2 *trp* (72-187) and WP2 *trp uvrA* (72-188). Each strain was constructed with a lesion (tryptophan mutation) in the tryptophan operon (see p. 4, Green and Muriel). WP2 *uvrA* also contains a lesion in a DNA repair-coding gene (*uvrA*) that increases sensitivity to certain mutagenic activities.

The *E. coli trp*⁺ assay is well suited for the detection of mutagenic activities. Due to the specific lesion in their tryptophan operon, both strains are tryptophan requiring mutants (tryptophan auxotrophs). Thus, the tester strains all require exogenous tryptophan for growth. However, exposure to mutagens may result in genetic reversions in the tryptophan operon resulting in restoration of the wild type phenotype; mutants have their tryptophan operons functionally “restored” and can synthesize tryptophan. The assay depends on the ability to distinguish between tryptophan auxotrophs (the tester strains) and tryptophan prototrophs (the mutants). Accordingly, the target cells are plated on media containing trace quantities of tryptophan that allows for a few rounds of cell division necessary to “fix” a mutation event; the tryptophan 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 tryptophan operon resulting in the continuation of growth after trace tryptophan exhaustion. Bacterial colonies that appear on tryptophan-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 assay. For the most part, the methods described are taken directly from Green & Muriel (*ibid.*). 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 ECDiscs™, TRI PC™ plates, and LS9.

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 bacteriocidal activities, hazardous qualities and stability.
 - b. Determine the solubility of your test chemical in the appropriate solvent (water and DMSO are preferred solvents - see Green & Muriel or check the Merck Index). In many cases, you may find it necessary to dose with suspensions rather than true solutions. If an organic solvent is used (e.g. DMSO, acetone), the test material may precipitate upon addition to the aqueous top agar.

- c. Decide on the doses that you wish to test. In general, the upper dose should not exceed 5 mg/plate (50 mg/ml assuming a 100 μ l/plate dosing volume). Select 5 to 7 doses separated by factors of 2, 3 (or half logs) or 5. There are sufficient plates in your kit for a 7 dose duplicate plate (including controls) assay 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 that 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?
 3. Assemble the supplies and equipment needed to perform the test on the day before.

Items you will need to complete the assay that are **not** supplied in the kit:

| | |
|---|---------------------------------|
| 2 - Sterile Erlenmeyer flasks | Sterile inoculating loops |
| Sharpie or wax pencil | Sterile swabs (optional) |
| 200 μ l and 1000 μ l sterile pipette tips | Ice |
| 1 ml and 5 sterile pipets | Ice bucket |
| Pipet-aid or rubber bulb | Latex gloves |
| Test tube rack | 13 x 100 mm sterile disposable |
| Dimethyl Sulfoxide (DMSO) | test tubes |
| Sterile water | Microwave or boiling water bath |
| Vortex mixer | Sterile forceps (optional) |
| 45°C water bath or heating block | |
| 37°C incubator with shaker | |
| Positive displacement pipette aid (e.g. Pipette-man, Eppendorf) | |
| Automatic colony counter or magnifying counter | |

- a. Remove the TRI 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 - 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 ECDiscs™

Your kit is supplied with dried/stabilized strains in disc format. Each disc contains sufficient viable cells to serve as the inoculum for a 20-25 ml culture. ECDiscs™ 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 Green & Muriel paper. To prepare strain cultures for use in the assay:

PREPARE THESE STEPS LATE IN THE DAY BEFORE THE ASSAY

ASEPTIC TECHNIQUE MUST BE USED

1. Label sterile Erlenmeyer flasks with the strain number in accordance with your experimental design.
2. Using aseptic technique, carefully decant approximately 20-25 ml of Oxoid #2 nutrient broth into Erlenmeyer flasks.
3. Remove the ECDisc™ 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 ECDisc™ vial.

6. After the flasks are inoculated, place them on your shaker and incubate at 37°C shaker operating at approximately 150 rpm. Incubation must not exceed 16 hours. For best viability, connect your incubator to a simple timer (available from any hardware store) and set for 10 hours. In many cases, it may be desirable to grow up your cells on the day of the assay (rather than incubating “overnight”). To do this, it is necessary to increase the size of the initial inoculum; for example, if you inoculate with 6-7 discs your culture should reach an absorbance of 1.0 to 1.2 (@ 660 nm) within approximately 5-6 hours. *Please note: It is important that your culture has not overgrown - the target cells perform best when they have just reached a density of approximately $1-2 \times 10^9$ per ml (i.e., when they have reached an absorbance of approximately 1.2 to 1.4 at 660 nm).*
7. After incubation (e.g., on the morning of the assay) remove the flask cultures and place them in the refrigerator until you begin the assay.

PERFORM THESE STEPS ON THE DAY OF THE ASSAY

ASEPTIC TECHNIQUE MUST BE USED

C. Treatments and Plating

1. Melt the tryptophan 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 activation system, remove the tear-off seal from one or both LS9 vials. Rehydrate each vial with 2.1 ml ice cold sterile water and mix to homogeneity. Your NADPH REGENSYS can be used at 5% or 10% S9. For 5%, add 2 ml rehydrated LS9 and 2 ml sterile water to the Reagent A bottle. For 10% S9, add a total of 4 ml rehydrated LS9. Keep on ice. Just before use, add the contents of the Reagent B tube (NADP), mix thoroughly, and hold on ice.
3. Open the CONTROLCHEM™ packages:

Latex or vinyl plastic gloves must be worn when handling these chemicals.

Add one ml of the appropriate solvent to each of the CONTROLCHEM™ tubes.

| <u>Mutagen</u> | <u>Amount</u> | <u>Strain</u> | <u>Solvent</u> |
|--|---------------|---------------|----------------|
| Methymethane-Sulfonate | 25 µl | all | DMSO |
| 2-Aminoanthracene (activation control) | 100 µg | 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 3b. Place rack in 45°C water bath or heating block and pipette 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 WP2 *trp* 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 WP2 *trp* 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* 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 WP2 *trp* culture as described in step 8b. (above).

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

REPEAT THE ABOVE PROCEDURES FOR WP2 *trp uvrA*

9. Treat the Positive Control Cultures:

a. Set up 4 tubes for each strain. Two 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:

| <u>Chemical</u> | <u>Strain</u> | <u>Dose/Plate</u> |
|------------------------|---------------|-------------------|
| Methylmethanesulfonate | both | 2.5 ul |
| 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 and strains as was previously described.

10. Inoculate the TRI PC™ Plates:

a. Using a sterile loop or swab, wet with the appropriate culture, squeeze out excess nutrient broth against side of flask and inoculate each of the four sectors of a TRI PC™ plate using a “Z” inoculation pattern.

b. Repeat for each strain.

11. Determine the Titer of the Strain Cultures

a. Arrange sets of 3 sterile tubes with closures for each strain. Pipette 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 - use a vortex mixer at low speed or mix 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 - 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, 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 5×10^{-6} and 5×10^{-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 continue incubation 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 condition, 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, Artek). 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 dissecting microscope or similar instrument.

A normal background consists of microscopic colonies containing 2-8 cells.

If a plate contains many very small, just macroscopic, microcolonies, that 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 ECDisc™ QC sheets - values of the positive control chemicals shown in the Green & Muriel paper 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 to 2×10^9 “viable” cells per ml. Very much lower or higher initial titers may result in reduced frequencies and background or increased backgrounds, respectively.

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

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 tryptophan. The genetic reversion, from auxotrophy to prototrophy, which enabled those cells to grow in the absence of exogenous trp might have arisen spontaneously or as the result of a mutation induced by the treatments. 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 from the “normal” range of spontaneous reversion values may signal systematic problems with the assay; expect about 40-100 revertants per plate.

2. Diagnostic Positive Control Counts

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

| <u>Strain</u> | <u>Chemical</u> | <u>Number of Colonies/Plate</u> |
|---------------|-----------------|-------------------------------------|
| WP2 | MMS | 200 -400 |
| WP2, UVRA | MMS | 300-500 |

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

| <u>Sector</u> | <u>Observation</u> | <u>Phenotype</u> |
|---------------|--------------------------|---|
| I | No growth (both strains) | <i>trp</i> - |
| II | Profuse growth (both) | Viability check |
| III | No growth | Ampicillin sensitivity |
| I | | (some WP2 strains contain pKM101, an <i>amp</i> ^R plasmid) |

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 nonmutagenic 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.

