MOLTOX®

Molecular Toxicology, Inc.

Ames FT™

“471”

Mutagenicity Test Kit

Instruction Manual

31-301

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INTRODUCTION

MOLTOX® FT™ tests measure the ability of test material treatments to induce reversion of histidine-requiring *Salmonella typhimurium* strains or *Escherichia coli* tryptophan auxotrophs to their respective prototrophic conditions (1, 2, 3).

Fluctuation tests (FT tests) were originally developed by Luria and Delbruck to show that bacterial variants arise by random mutation rather than by adaptation to selective agents (4). Subsequently, their methods have been adapted to questions of chemical mutagenic potential and applied to yeast (5), mammalian cells (6) and bacteria (7). The mutagen screening methods used in MOLTOX® FT™ assays were derived from those reported by Gatehouse (8, 9).

MOLTOX® FT™ assays are performed by exposing approximately 10^7 cells to various concentrations of test agent, positive control chemicals or solvent (negative control) for 90 minutes in a chemically defined medium containing a concentration of histidine (and tryptophan for *E. coli* strains) sufficient to allow for about two population doublings. After the treatment period, the cultures are diluted in a chemically defined medium that is histidine-free (and tryptophan-free) and contains a pH indicator. Fifty microliter aliquots of these treated cell dilutions are dispensed into 48 wells of a 384-well plate for each treatment condition. After two days incubation, his+ revertants (or trp+ revertants for *E. coli* WP2) may proliferate, acidifying the culture media resulting in a color change (purple to yellow) in individual wells. Wells exhibiting evidence of bacterial growth (turbidity/colony growth) and/or color change (to yellow) contain revertant cell populations and are counted. The number of wells with revertants for the test material-treatments are compared to the number observed in the untreated or solvent-treated negative controls.

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BEFORE YOU BEGIN

MOLTOX® FT™ products are intended for research purposes only; they are not for use in humans or animals.

The Salmonella and Escherichia strains provided in MOLTOX® FT™ kits are attenuated but potentially etiologic agents and as such should be handled under Biosafety Level 2 conditions. Positive control chemicals are provided in very small quantities; nevertheless, they are mutagens/carcinogens and must be handled in strict accordance with your institutions chemical hygiene plan. Aseptic technique and standard microbiological and chemical safety precautions should be employed. Do not eat, drink, smoke, apply cosmetics, etc., while working in the laboratory area. Wear appropriate personal protection equipment (safety glasses, nitrile gloves, lab coat) when handling kit reagents and bacterial strains. Never pipette by mouth.

Additional items you will need to complete the assay:

Sterile Pipette Tips (20-200 and 200-1000 µL)

Micropipettors Single channel (2-20 µL, 20-200 µL and 100-1000 µL)

and 8-channel (30-300 µL)

Sterile Pipettes (1, 5, 10 mL)

37°C bacteriological incubator

Shaker, variable speed

Spectrophotometer/colorimeter (600 nm)

Sterile 15 and 50 mL centrifuge tubes

Sterile 125 mL culture flasks with closures

Sterile 24 and 384 well plates

Sterile Test tubes/microfuge tubes/96 well plates for dilutions

Solvents including sterile purified water, DMSO, acetone and ethanol

Quart size sealable plastic bags (e.g., Ziplock®)

Sterile 25 mL reagent reservoirs

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31-301 MOLTOX® FT™ "471" Mutagenicity Assay Kit		
11-406.3L	30% PB/BNFS9 Mix for Ames FT™ Kits, 3.25 mL	2 each
22-147	Ampicillin, 55 mg/vial	1 each
26-710.1	FT™ Exposure Media, 100 mL	1 each
26-711.5	FT™ Reversion Indicator Media, 500 mL	2 each
26-712.1	FT™ Growth Media, 100 mL	1 each
60-157	2-Aminoanthracene, 100 µg/vial	1 each
60-157.2	2-Aminoanthracene, 2 mg/vial	1 each
60-158	9-Aminoacridine hydrochloride, 500 µg/vial	1 each
60-159	4-Nitroquinoline-*N*-oxide, 50 µg/vial	1each
60-160	N^4-aminocytidine, 2.5 mg/vial	1each
60-161	2-Nitrofluorene, 50 µg/vial	1 each
71-098.2L	TA98 discs	1 each
71-100.2L	TA100 discs	1 each
71-1535.2L	TA1535 discs	1 each
71-1537.2L	TA1537 discs	1 each
72-188.2L	EC WP2 *uvrA* discs	1 each

MATERIALS

Tester Strains: Salmonella strain master cultures were the generous gift of the B.N. Ames Berkeley laboratory. *E. coli* WP2 were obtained from the National Collection of Industrial, Food and Marine Bacteria, Ltd. (NCIMB), UK. *Salmonella typhimurium his-* and *Escherichia coli WP2 trp-* strains are supplied as STDisc™ and ECDisc™ freeze dried inocula, respectively. Inocula should be refrigerated.

CATALOG NO.	TESTER STRAINS	GENOTYPE	REVERSION EVENT
71-098L	*S. typhimurium* TA98 [1,2]	*hisD3052, rfa, uvrB,* pKM101	frame shift
71-100L	*S. typhimurium* TA100 [1,2]	*hisG46, rfa,uvrB,* pKM101	base-pair substitution
71-1535L	*S. typhimurium* TA1535 [1,2]	*hisG46, rfa, uvrB*	base-pair substitution
71-1537L	*S. typhimurium* TA1537 [1,2]	*hisC3076,rfa, uvrB*	frame shift
72-188L	*E. coli* WP2 *uvrA* [3]	*trpE65, uvrA*	transition/transversion

[1]These strains contain a deletion mutation through the *uvrB*-bio genes; they exhibit an error-prone DNA repair mechanism and are biotin dependent.
[2]Salmonella strains (Ames strains) are as described by Maron and Ames and Levin, et. al. (1,10,11).
[3]*E. coli* WP2 strain is characterized by Mortelmans and Riccio (3).

pKM101 plasmid containing strains exhibit ampicillin resistance.

Metabolic Activation System: MOLTOX® 30% PB/BNF S9 Mix is a freeze-dried product that contains Phenobarbital/β-Naphthoflavone induced Sprague Dawley rat liver S9 and cofactors as described by Matsushima, et al., (In Vitro Metabolic Activation in Mutagenesis Testing (F.J. de Serres, ed.), Elsevier, 1976, p 85). Reconstitute to the label volume using ice cold, sterile, purified water – maintain on ice.

CATALOG NO.	FILL SIZE	DESCRIPTION
11-406.3L*	3.25 mL	30% PB/BNF S9 Mix for MOLTOX® FT™ Tests

*Store as is in a standard freezer. Once reconstituted, place on ice and use within a few hours. Do not refreeze, thaw, and use reconstituted S9 Mix.

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Growth Media: Use for overnight culture of Salmonella and *E. coli* strains.

CATALOG NO.	FILL SIZE	DESCRIPTION
26-712.1*	100 mL	MOLTOX® FT™ Growth Media

*Store at room temperature

Exposure Media: Chemically defined media for treatments of Salmonella and *E. coli* strains with control and test agents. Supplemented with limiting (0.05 mM) L-histidine and L-tryptophan and excess D-biotin.

CATALOG NO.	FILL SIZE	DESCRIPTION
26-710.1*	100 mL	MOLTOX® FT™ Exposure Media

*Store at room temperature

Reversion Indicator Media: Chemically defined L-histidine- and L-tryptophan-free media for growth and expression of Salmonella and *E. coli* revertants. Contains a pH indicator.

CATALOG NO.	FILL SIZE	DESCRIPTION
26-711.5*	500 mL	MOLTOX® FT™ Reversion Indicator Media

*Store at room temperature

Ampicillin: Use to ensure pKM101 plasmid stability and prevent contamination.

CATALOG NO.	FILL SIZE	DESCRIPTION
22-147**	55 mg	Ampicillin sodium salt

**Refrigerate

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CONTROLCHEM™ Positive Control Chemicals: Used to determine and confirm assay sensitivity and target strain response characteristics. **WARNING: These positive control chemicals are mutagens/carcinogens and must be handled accordingly**. **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.**

**Refrigerate

CAT. NO.	CONTROLCHEM™	SOLVENT	STRAIN
60-157** 60-157.2**	2-Aminoanthracene (100 µg) 2-Aminoanthracene (2 mg)	DMSO DMSO	Salmonella strains + S9 *E. coli* strains + S9
60-158**	9-Aminoacridine HCl (500 µg)	Purified water	TA1537
60-159**	4-Nitroquinoline-*N*-oxide (50 µg)	DMSO	TA100; EC WP2 *uvrA*
60-160**	N^4-Aminocytidine (2.5 mg)	Purified water	TA100; TA1535
60-161**	2-Nitrofluorene (50 µg)	DMSO	TA98

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MOLTOX® FT™ "471" MUTAGENICITY TEST PROCEDURE

ASEPTIC TECHNIQUE MUST BE USED

A. Culture Tester Strains

Add 1.1 mL of sterile purified water to the 22-147, Ampicillin, vial. Cap and mix well; the concentration is now 50 mg/mL. After reconstituting, store in the refrigerator for up to 2 months.

Add an appropriate volume of 26-712 growth medium to the required number of culture flasks or tubes (e.g. 20-25 mL for 125 mL flasks or 10-15 mL for 50 mL centrifuge tubes). To those flasks that will be inoculated with pKM101 plasmid strain (see page 5), add a volume of reconstituted Ampicillin to equal 25 µg/mL finished media. (i.e. 0.5 µL Ampicillin/mL broth). Inoculate with the desired STDisc™ or ECDisc™ tester strain by transferring a single disc to each culture vessel using aseptic technique. The freshly inoculated cultures should be incubated stationary overnight at approximately 37°C. In the AM, incubate with shaking (125 - 150 rpm; avoid foaming) at 37°C until a density of approximately 1 - 2 x $10E^9$ CFU/mL is achieved (OD_{650} 1.0 - 1.4)

B. Prepare Positive Control and Test Chemicals

1. Positive Controls

Dissolve the positive control chemical(s) recommended for the tester strain(s) employed using sterile purified water or DMSO, as required (see Page 7). CONTROLCHEM™ packaging is designed such that the target concentration will be attained by dissolving in 1 mL solvent. For example, in an experiment in which TA98 is used as a tester strain, the appropriate -S9 positive control material is 2-Nitrofluorene (60-161). Accordingly, the contents of one vial of 2-Nitrofluorene would be dissolved in 1 mL DMSO yielding a dosing solution of 50 µg/mL. Due to the dilutions that occur over the course of the assay, dose concentrations are 25X the test concentration. Therefore, in this example, the treatment concentration is 2 µg/mL. For the *E. coli* WP2 *uvrA* strain +S9, the correct 2-Aminoanthracene is 60-157.2; the treatment concentration is 80 µg/mL.

2. Test Material

Test material solubility in water, DMSO, or another solvent compatible with the assay system (e.g., acetone, ethanol, DMF) should be determined. In general, the final concentration in the well should not exceed approximately 5 mg/ml (or 5 µL/mL). Dosing solutions must be prepared such that their maximum concentration is 25X the desired final concentration. Six concentrations should be prepared; these may be serial 1:2's, half log, or another dilution scheme thought likely to reveal a dose-response. Sterile tubes or 96 - well plates may be employed for the dilutions – a minimum of 60 µL of each dilution will be required for dosing.

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C. Treatments

24 - well plates are used for the treatments Each test material concentration should be tested in triplicate in the presence and absence of S9. Accordingly, a single test material will require dosing 18 wells for each condition; the remaining 6 wells should be prepared for the respective positive and negative controls. Thus, an assay utilizing a single target cell strain will require two 24 - well plates, one with and one without S9.

1. Dose the 24 - Well Plates

Pipette 10 µL of each test material dilution into 3 - wells (18 wells total). Similarly, pipet positive and negative controls into 3 wells each. Repeat with a second plate.

a. Without S9

Measure 7.2 mL Exposure Media (26-710) into a sterile 15 mL centrifuge tube. Add 800 µL of the overnight culture. Mix gently by inversion. Add 240 µL to each of the 24 wells of the first (-S9) plate.

b. With S9

Reconstitute the 30% S9 Mix (11-406L) by adding the appropriate volume of cold, sterile, purified water. Mix gently until dissolved. Store in an ice water bath until used in the assay.

Measure 6 mL of Exposure Media (26-710) into a sterile tube as before. Add 1.2 mL 30% S9 Mix and 800 µl overnight culture. Mix and add 240 µL to each of the 24 wells of the second (+S9) plate.

2. Incubate

a. Incubate the plates with gentle shaking at approximately 37°C for 90 minutes.

b. After the 90 minute incubation, add 2.75 mL Reversion Indicator Media (26-711) to each well. Mix thoroughly to assure homogeneity.

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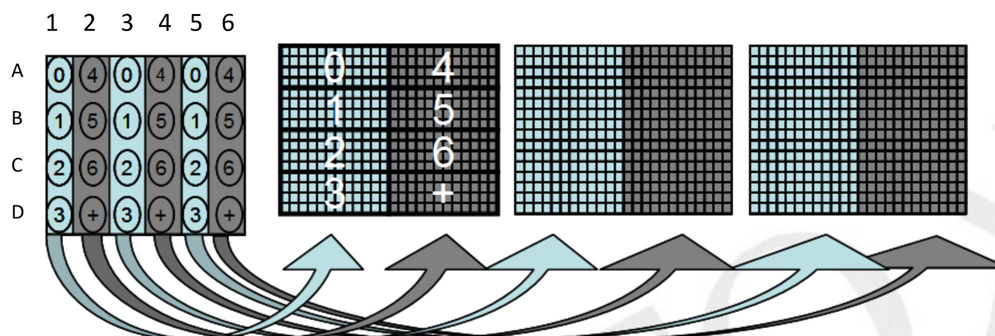
D. Select for Revertants

The estimations of mutant frequency are performed in 384 - well plates. Each well of each of the 24 - well Exposure Plates is transferred to 48 wells of a 384 - well revertant selection plate. Thus, in the present example, a total of six 384 - well plates will be required - 3 each for the -S9 and +S9 conditions. If you have little or no experience using 384 - well plates, we strongly recommend that you practice the following steps before starting the assay.

1. Pipette 50 µL of the homogenous mixture from C. 2. b, above, into 384 - well plates so that A1 of the 24 - well plate corresponds to rows A-D and columns 1-12 of the 384 - well plate, B1 corresponds to rows E-H and columns 1-12, and so on.



2. Place each 384 - well plate into a plastic bag (e.g., Ziploc®) and incubate at 37°C for approximately 48 hours.

Analysis

After the 48 hour incubation period, some number of the initially purple wells will have changed color to yellow. This color change is the result of media acidification arising due to the growth of Salmonella *his+* or *E. coli trp*+ revertants. The ratio of the number of wells retaining their initial purple color and the number of yellow wells is proportional to the number of reversions to wild type that arose either spontaneously or due to the treatments (Note: wells exhibiting intermediate color and wells with colonial growth should also be scored as positives). For purposes of evaluation, the significance of an observed increase in the number of yellow wells arising due to a given treatment can be estimated by determining a fold increase value. For example, if 25 positive (yellow) wells were observed in the positive control in a particular experiment but only 5 positives among the corresponding negative control wells, the fold increase would be 5. Generally, an increase ≥2-fold in a treatment group in relation to the corresponding negative control is thought to be biologically significant; i.e., it is thought to represent the induction of a significant number of mutagenic events in the treated population. Activity in the fluctuation test may also be evaluated using one or another commonly employed statistical method such as the chi-square test, conditional binomial test or Fisher exact test (7, 9, 12, 13, 14). If the average number of positive wells in the negative control (i.e. spontaneous rate) is less than 1.0, use the value 1.0 for calculations.

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