

MOLTOX[®]

Molecular Toxicology, Inc.

μ Ames Mutagenicity Test Kit Procedures



μAmes Procedure & Records

Inventory check	ID numbers (if required)	Init./date
Forceps, sterile (several pairs or flame between plates) or sterile inoculating loops	—	
Incubator, 37°C, with shaker		
Micropipettes		
Microscope		
Microwave/boiling water bath		
Pipet aid	—	
Refrigerator		
Spectrophotometer		
<i>Study Design</i> workbook – initiated and saved under study code number	—	
Vortex mixer		
Water bath, 45°C		
Desirable equipment	—	
Heating block, 45°C		
Illuminator with magnifier (e.g. Quebec counter)	—	
Multi-dose/repeater micropipette		
Tally counter, bench top		

Procedure	Study No.	Init./Date
Consumables check (refer to Study Design to calculate amounts):	Lot No.'s	—
Bacterial strains lyophilized	Entered later	
TA1535		
TA1537		
TA98		
TA100		
EC WP2 <i>uvrA</i>		
DMSO (dimethyl sulfoxide, anhydrous) *		
Incubation pouches (re-sealable perforated bags)	—	
MGA ,24-well plates		
Microcentrifuge tubes, sterile	—	
Micropipette tips, sterile *	—	
Mutazyme (S9 mix 10%)		
Nutrient broth – 1 bottle per strain		
Phenotype Test Plates (6-well) – 1/strain		
Phenotype Test Packet discs – 1/type/strain		
Phosphate buffer 0.1 M, pH 7.4		
Ames CONTROLCHEM™ Packet—1		
Solvent for test article*		
Spectrophotometer cuvettes	—	
Top agar – 3 ml/24-well plate; 4 ml/Phenotype Test Plate		
Tubes (e.g. 13×100 mm diameter glass) with caps autoclaved *	—	
Tubes to dispense bacteria, sterile (e.g. 50 mL centrifuge tube) *	—	
Water, sterile, deionized		
* Not supplied with the kit		

Procedure	Study No.	Init./Date
<p>Planning</p> <p>Enter the study number in the top row of this table then print out the procedures and/or save them electronically under the Study Number.</p> <p>We strongly recommend you read through the entire procedure and perform a dry run to ensure you understand the procedures. If unclear, please email MOLTOX[®] with any questions. Much of the preparation work (up to weighing out of your test article) can be performed ahead of time.</p>		
<p>Study Design</p> <p>All spreadsheets are supplied as tabs in the Excel <i>Study Design</i> workbook. These can also be used with Google and OpenOffice applications (double checking formatting). The <i>Study Design</i> should be saved with a unique identifier, e.g. your own study number.</p>		
<p>Using the Study Design workbook</p> <p>Open the first tab (<i>Input</i>) of the <i>Study Design</i> workbook. Following the instructions given in the sheet, enter the required information in the lavender cells. Go to the second tab (<i>Formulation</i>) and, following the instructions given in the sheet, enter the required information. Save the workbook under the study number name and print out the following sheets: <i>Formulation, Growth Estimate, μ control, and μ treated.</i></p> <p><i>The electronic versions of the worksheets can be used without printing if you prefer electronic record keeping and it's acceptable at your facility. In this case one may wish to employ the split screen Excel function for ease of viewing.</i></p>		
<p>Procedures</p> <p>All procedures involving bacteria should be carried out using aseptic technique.</p>		—
<p>Label the 24-well plates in accordance with the μ control and μ treated worksheets using an indelible marker (e.g. Sharpie). Label one 6-well phenotype plate and one sterile test tube per strain with the strain identity. Label all plates on the side of the base rather than the lid to avoid potential mix-ups.</p>		
<p>Place the deionized water sterile on ice in the refrigerator. This will be used to reconstitute the Mutazyme.</p>		

Procedure		Study No.	Init./Date
<p>Culture initiation</p> <p>In the evening prior to the test, allow a vial of each lyophilized strain to come to room temperature for at least 30 minutes prior to opening. This avoids condensation formation in the vial. Standard strains included in the kit are TA1535, TA1537, TA98, TA100 and E. coli WP2 <i>uvrA</i> are listed below: alternate strains including TA97a, TA102, and E. coli WP2 <i>uvrA</i> pKM101 can be purchased separately but will work with the components included in this kit.</p>			—
Strain	MOLTOX [®] P/N	Lot number	
TA1535	71-1535L		
TA1537	71-1537L		
TA98	71-098L		
TA100	71-100L		
Ecu (E. coli WP2 <i>uvrA</i>)	72-188L		
<p>For each strain, label one nutrient broth bottle with the strain number and date. Transfer one lyophilized bacterial disc into the culture bottle using sterile forceps. or inoculating loop. Immediately replace and tightly fasten the lid on the vials and return them to the refrigerator. Tighten then loosen the caps on the inoculated cultures by 1/2 turn to allow gas exchange then place in a 37°C incubator stationary overnight.</p>			
<p>Day of the test, Day 0</p> <p>Early the next morning, incubate the nutrient broth cultures at 37°C with shaking at a speed below that causing foaming (typically 125 rpm). <i>Cultures should reach the required growth phase/density later in the morning (typically after about 2 hours).</i> Refer to the <i>Growth Estimate</i> tab for recording of incubation conditions, tracking of OD, etc.</p> <p>Incubation start time: <input type="text"/> Shaker speed: <input type="text"/></p>			
<p>Preparation on day of the test, Day 0</p> <p>If desired, the characteristics of the strains can be confirmed using the 6-well Phenotype Test plates and Phenotype Test Packet. If stored in the refrigerator, remove the labelled 6-well phenotype plates and the 24-well plates from the refrigerator and allow them to warm to room temperature prior to use. This will help prevent irregularities in the depth of the top agar when it is added; irregularities are caused by rapid gelling of the top agar before it has time to spread. Remove antibiotic disc vials from the freezer and allow to warm to room temperature before opening.</p>			
<p>Verify the water bath/heating block is set to 45°C.</p>			

Procedure	Study No.	Init./Date
Slightly loosen the lid of the top agar bottle then melt the agar in a boiling water bath or microwave. Wearing oven gloves/mitts, tighten the lid then invert several times to ensure complete mixing. DO NOT shake (this causes excess pressure build-up within the bottle). If the agar does not appear completely homogenous, re-heat and mix again. Place the agar in a water bath set at 45°C to equilibrate; maintain at 45°C during use.		
Prepare formulations of the positive controls and test article as per the <i>Formulation</i> spreadsheet.		—
Prepare the 10% S9 mix by reconstituting the Mutazyme with the ice-cold deionized water sterile - add a total 20 mL water to one bottle of 11-402L Mutazyme. Place the S9 mix in a beaker of wet ice and store in the refrigerator when not in use. Replenish ice as necessary.		
Dispense 3 mL aliquots of molten top agar into sterile glass tubes and place in the 45°C heating block. 1 tube per plate is needed plus spare tubes in case of error.		
Dispense aliquots of buffer, S9 mixes, solvent and positive control solutions to the sterility plate (plate ST).		See μ control worksheet
Add 130 μ L of molten top agar from one of the 3 mL tubes to the appropriate (dosed) wells of the sterility plate then set the plate aside on a level surface for the agar to gel. Discard the tube.		

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<p>Check growth of the 25 mL suspension cultures. As a quick visual check of density, the bacteria will have reached an appropriate density once the culture obscures print when held against a printed page. If print is legible continue incubation.</p> <p>Once each culture becomes opaque remove it from the incubator. Use a serological pipet to aseptically remove 1.0 mL of the culture into a 1 mL cuvette to measure OD (650nm). Use an aliquot of uninoculated broth as the blank. Record incubation time and OD on <i>Growth record</i> sheet. If the culture has not reached an OD₆₅₀ of 1.0 continue incubation until this density is achieved.</p> <p><i>In the case of GLP experiments, the density of the cultures should be quantified and confirmed as $1-2 \times 10^9$ bacteria per mL. This can be done by:</i></p> <p><i>Prepare a 1 in 10 dilution of the liquid cultures in saline or, preferably, BCF (bacterial counting fluid: contact MOLTOX® for recipe) followed by a direct microscope count using a bacterial counting chamber, e.g. Helber Thoma. This is our recommended method.</i></p> <p><i>Perform two 1/1000 serial dilutions of the bacterial suspension followed by plating 100 µL of the final dilution on nutrient agar and subsequent incubation. This is the conventional method but has the disadvantage that results will not be available until the next day. Culture density is calculated by colonies/plate/$1 \times 10^{-7} = X \times 10^9$ cells/ml.</i></p> <p>When the culture has reached the appropriate density, pipette into a sterile container, e.g. a 50 mL centrifuge tube. Hold the cultures at room temperature before use and while dispensing.</p>		Refer to <i>Growth Estimate Sheet</i>
<p>Phenotype test, if performed</p> <p>For each strain being used, dispense 4 mL molten top agar into a sterile glass tube labelled with strain identity and place in the 45°C heating block.</p> <p>Orientate the 6-well phenotype plates so well No. 1 is on the top left. For each strain in turn, transfer 100 µL of the suspension culture to the corresponding tube of top agar. Mix using a vortex mixer then dispense 0.5 mL to each of the 6 wells of the corresponding phenotype plate.</p> <p>Ensure the top agar covers the surface of each well by rotating and gently tilting the plate after each addition. Leave the plate on a level surface for the agar to gel.</p> <p>Use sterile forceps to place one C, A, T, and M (crystal violet, ampicillin, tetracycline and mitomycin C) phenotype disc approximately in the center of wells 1, 2, 3 & 4 respectively in each of the 6-well phenotype plates. Recap the vials and return any unused discs to the freezer. Crystal violet discs can be stored at room temperature if desired.</p>		
<p>Dosing The layout of the solvent and treated types of plate is shown in Appendix 1 of the manual. Some 'blank' wells remain untreated and do not require any additions or scoring at the end of the test.</p>		-

Procedure	Study No.	Init./Date	
<p>A. Dispense aliquots of the vehicle to all the control plates then dose the positive control wells with the appropriate agent. Evenly distribute by tilting/rotating the plate while you are dosing.</p>		See μ control worksheet	
<p>B. Dose all plates listed in the μ treated worksheet with each dose level of the test agent in turn.</p>		See μ treated worksheet	
<p>C. Immediately after dosing the plates proceed as below for each plate in turn:</p> <p>To one 3.0 mL top agar tube add buffer or S9 as appropriate plus bacterial culture:</p> <p>OS9: 750 μL Phosphate buffer + 150 μL of the relevant bacterial strain. OR +S9: 750 μL S9 mix + 150 μL of the relevant bacterial strain.</p> <p>Mix by swirling or vortex. Immediately dispense 130 μL top agar/bacterial mix into the wells of the plate, evenly distribute by tilting/rotating the plate then leave plate on a level surface. Proceed to the next plate.</p>		See μ control and μ treated worksheets	
<p>Plate Incubation</p> <p>Once the agar has gelled, invert the plates, including sterility and phenotype plates, and place them in the perforated re-sealable plastic bags supplied with the kit (or Tupperware-type boxes with the lid loosely fitted) to minimize evaporation. Transfer the boxed or bagged plates to an incubator set to 37°C.</p>			
Incubator ID:		Incubation start time:	

Procedure		Study No.		Init./Date
<p><i>Phenotype plates</i></p> <p>Remove the 6-well phenotype plate from the incubator after 20 - 24 hours incubation.</p>				
Target finish:		Actual finish:		
<p>If necessary, store phenotype plates REFRIGERATED for up to 3 days before scoring them.</p>				
Refrigerator ID:				
<p>Estimate the diameter of the zone of inhibition in wells 1 to 4 using a ruler. Very occasionally, the zone may extend to the edge of the plate (no growth evident) in which case the diameter should be recorded as >33 mm. Record the density of growth in wells 5 and 6. See Appendix 1 of the Manual for examples.</p> <p><i>To facilitate observation of bacterial growth in the wells, hold the plate 3 or 4 cm above a printed page; strong growth will make the characters unreadable, weak growth will make them hazy; in the absence of significant growth, the print will be clear.</i></p>				Enter results in <i>Phenotype</i> worksheet
<p>Remove the 24-well plates from the incubator after 65 hours (3 overnight incubations)</p>				
Target finish:		Actual finish:		
<p>If necessary, store 24-well plates REFRIGERATED for up to 3 days before scoring them.</p> <p>Refrigerator ID: _____</p>				

Procedure	Study No.	Init./Date
<p>Count the number of revertants in each well. Enter results directly into the appropriate sheets in the <i>Study Design</i> (electronic spreadsheet or paper copy). A bench-top or hand-held tally counter will facilitate counting (Denominator-type two channel). High counts (above approximately 100) should be estimated and noted as such in the results spreadsheet: record counts in categories of greater than 100, 150, 200 and 300 as 101, 151, 201 and 301 respectively to facilitate data handling and tabulation.</p> <p>Assess low counts accurately, taking care to include colonies around the edge of the plate. These bacteria are motile; any colony that is smeared out due to wetness on the surface or edge of the well is counted as a single colony.</p> <p><i>Inexperienced personnel may have trouble distinguishing colonies from precipitate; colonies are white and generally rounded with a smooth outline; precipitate is generally jagged and may be colored. Precipitated liquids can appear similar to colonies.</i></p> <p><i>Occasionally a ring of bacterial growth will appear around the edge of the well. This is caused by a revertant colony touching a meniscus of fluid at the edge of the well and growing around it. These rings do not have any significance and can be ignored.</i></p> <p>Where appropriate, evaluate the quality of the background lawn for the wells. If in doubt about revertant colonies, the lawn, or precipitate, use a low-power microscope to confirm the presence of the lawn. Record any relevant comments about the plates, e.g. precipitate, in the worksheet. If obscured by precipitate, the background lawn can generally be assumed to be normal if the colony counts are close to expected based on concurrent and laboratory historical vehicle control data.</p>		See μ control and μ treated worksheets
<p>Re-save the <i>Study Design</i> under the Study number (e.g. <i>ABC123 Study Design</i>). <i>The TABULATED RESULTS worksheet contains two sets of tables. The first table shows average revertants per well for each strain for each test article dose, vehicle control, and positive control, both without and with S9. The 2nd table shows fold increase over the vehicle control (i.e. spontaneous reversion). Cells are automatically highlighted and text colored red if the fold increase is equal to or greater than 2. (indicating mutagenic activity).</i></p>		See <i>Study Design</i>
<p>Store plates refrigerated. Refrigerator ID: _____</p>		
<p>Discard the plates following review by the scientist. Scientist authorization (Initials/Date): _____</p>		

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