MOLTOX®

Molecular Toxicology, Inc.

# µAmes
# Mutagenicity Test Kit
# Procedures

| 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 | | |
| *Study Design* 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 *uvrA* | | |
| 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 buffer0.1 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 |
|---|---|---|
| **Planning**<br>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.<br>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. | | |
| **Study Design**<br>All spreadsheets are supplied as tabs in the Excel *Study Design* workbook. These can also be used with Google and OpenOffice applications (double checking formatting). The *Study Design* should be saved with a unique identifier, e.g. your own study number. | | |
| **Using the Study Design workbook**<br>Open the first tab (*Input*) of the *Study Design* workbook. Following the instructions given in the sheet, enter the required information in the lavender cells. Go to the second tab (*Formulation*) 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: *Formulation*, *Growth Estimate*, *μ control*, and *μ treated*.<br>*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.*<br>*Note that not all wells on the sterility and control plates will be used.* | | |
| **Procedures**<br>All procedures involving bacteria should be carried out using aseptic technique. | | – |
| 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. | | |
| Place the deionized water sterile on ice in the refrigerator. This will be used to reconstitute the Mutazyme. | | |

| Procedure | Study No. | Init./Date |
|---|---|---|
| **Culture initiation**<br>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 uvrA are listed below: alternate strains including TA97a, TA102, and E .coli WP2 *uvrA* pKM101 can be purchased separately but will work with the components included in this kit. | | – |

| Strain | MOLTOX® P/N | Lot number |
|---|---|---|
| TA1535 | 71-1535L | |
| TA1537 | 71-1537L | |
| TA98 | 71-098L | |
| TA100 | 71-100L | |
| Ecu (E. coli WP2 *uvrA*) | 72-188L | |

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.

**Day of the test, Day 0**

Early the next morning, incubate the nutrient broth cultures at 37˚C with shaking at a speed below that causing foaming (typically 125 rpm). *Cultures should reach the required growth phase/density later in the morning (typically after about 2 hours)*. Refer to the *Growth Estimate* tab for recording of incubation conditions, tracking of OD, etc.

Incubation start time: Shaker speed:

**Preparation on day of the test, Day 0**

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.

Verify the water bath/heating block is set to 45˚C.

| **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 *Formulation* 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 11-404L 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 µL 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. | | |

| Procedure | Study No. | Init./Date |
|---|---|---|
| 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.<br><br>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 *Growth record* sheet. If the culture has not reached an OD650 of 1.0 continue incubation until this density is achieved.<br><br>*In the case of GLP experiments, the density of the cultures should be quantified and confirmed as 1-2 × $10^9$ bacteria per mL. This can be done by:*<br><br>*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.*<br><br>*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 x $10^{-7}$ = X x $10^9$ cells/ml.*<br><br>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. | | Refer to *Growth Estimate* Sheet |
| **Phenotype test, if performed**<br><br>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.<br><br>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. 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.<br><br>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. | | |
| **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. | | – |

| **Procedure** | **Study No.** | **Init./Date** |
|---|---|---|
| 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. | | See *μ control* worksheet |
| B. Dose all plates listed in the *μ treated* worksheet with each dose level of the test agent in turn. | | See *μ treated* worksheet |
| C. Immediately after dosing the plates proceed as below for each plate in turn:<br>To one 3.0 mL top agar tube add buffer or S9 as appropriate plus bacterial culture:<br><br>0S9: 750 µL Phosphate buffer + 150 µL of the relevant bacterial strain.<br>OR<br>+S9: 750 µL S9 mix + 150 µL of the relevant bacterial strain.<br><br>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. | | See *μ control* and *μ treated* worksheets |
| **Plate Incubation**<br>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. | | |

| | | | |
|---|---|---|---|
| Incubator ID: | | Incubation start time: | |

| Procedure | Study No. | Init./Date |
|---|---|---|
| *Phenotype plates*<br>Remove the 6-well phenotype plate from the incubator after 20 - 24 hours incubation.<br>Target finish: \| Actual finish: | | |
| If necessary, store phenotype plates REFRIGERATED for up to 3 days before scoring them.<br>Refrigerator ID: | | |
| 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.<br>*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.* | | Enter results in *Phenotype* worksheet |
| Remove the 24-well plates from the incubator after 65 hours (3 overnight incubations)<br>Target finish: \| Actual finish: | | |
| If necessary, store 24-well plates REFRIGERATED for up to 3 days before scoring them.<br>Refrigerator ID: _____ | | |

| **Procedure** | **Study No.** | **Init./Date** |
|---|---|---|
| Count the number of revertants in each well. Enter results directly into the appropriate sheets in the *Study Design* (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.<br><br>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.<br><br>*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.*<br><br>*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.*<br><br>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. | | See *μ control* and *μ treated* worksheets |
| Re-save the *Study Design* under the Study number (e.g. *ABC123 Study Design*). *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).* | | See *Study Design* |
| Store plates refrigerated. Refrigerator ID: | | |

| | |
|---|---|
| Discard the plates following review by the scientist.<br>Scientist authorization (Initials/Date): _____ | |

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