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Molecular Toxicology, Inc.

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μAmes
Mutagenicity Test Kit
Instruction Manual

31-500

INTRODUCTION

The Ames Bacterial Reverse Mutation Test is used to evaluate mutagenicity which, in humans and other animals, is associated with various long-term adverse health effects including cancer and reproductive damage. Regulatory bodies around the world normally require Ames testing to determine genotoxicity of any new materials (including chemicals, pharmaceuticals, agrochemicals and medical devices) before they are made publicly available or introduced into the environment. Consequently, the bacterial reverse mutation test described in OECD guideline 471 is probably the most widely, and in some cases, the only, employed regulatory toxicity test. Because of the importance placed on the results of the eventual GLP study many laboratories employ a screening version of the assay's early stage product development. The microAmes (µAmes) version of the test accurately predicts the outcome of the subsequent GLP study with similar sensitivity, uses a very low amount of test article, and can be performed by an individual on a single day without special equipment. Since both the standard and µAmes method use nearly the same procedures, results from the micro method can be extrapolated to those expected for the standard Ames test. These factors make the µAmes ideal for preliminary screening and for general genotoxicity testing in non-GLP situations. Although the µAmes test isn't designed for regulatory submission, it may be acceptable provided appropriate justification is presented as OECD Guideline 471 allows for variations in the test when appropriate.

The µAmes uses the same plate incorporation method as the standard test but all volumes are reduced by a factor of 20. It can be modified in the same ways as the parent test, e.g. using the pre-incubation method. To compensate for any loss of sensitivity, the micro method increases the number of vehicle control replicates to more accurately assess the spontaneous incidence of mutation. In addition, the bacterial density, growth phase and exposure period are tightly controlled to make any comparison with laboratory historical control data more reliable. Validation experiments performed at MOLTOX® and elsewhere confirm that the sensitivities of the two methods are comparable. The 24 - well format incorporates minor adjustments to the procedure, pre-characterized purified bacterial strains and aliquoted control materials to substantially reduce the testing workload.

This µAmes kit was manufactured using the highest quality components; material preparation, strain characterization and procedures for its use closely follow the formative guidelines (see References). Many of the materials supplied have been specifically developed by MOLTOX® to facilitate testing in your laboratory and are accompanied by GLP level Quality Control and Formulation Statements. Each batch of materials is thoroughly tested for performance in the Ames test.

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The μAmes Kit is intended for use by individuals who have limited experience with microbiology and aseptic technique as well as those experienced in genetic toxicology testing. The bacterial strains included (*S. typhimurium* strains TA1535, TA1537, TA98, TA100 and *E. coli* WP2 *uvrA*) are those most widely used in routine testing as prescribed by OECD and most regulatory bodies. Although attenuated, the bacteria are potentially pathogenic and should be handled in accordance with biohazard level 2 guidelines. Since the media and positive controls are appropriate for all Salmonella and *E. coli* strains, alternate strains can be substituted or added without modification to other components or procedures.

The μAmes method involves the same considerations as the standard method in terms of study design, metabolic activation, dosing and plating, phenotype confirmation, colony counting and data interpretation. We have supplied detailed procedures to successfully conduct a valid assay. The materials contained in the kit and the procedures described are amenable to appropriate modification. If you would like any technical assistance in any phase of the assay, please contact our experienced scientists at (828) 264-9099, email us at info@moltox.com or visit our website www.moltox.com. The advice given in this manual follows appropriate aspects of OECD guideline 471 which we suggest you consult prior to doing any lab work; www.oecd.org/chemicalsafety/risk-assessment/1948418.pdf.

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µAmes Mutagenicity Assay Kit Components

31-500 µAmes Mutagenicity Assay Kit			Storage
71-300	Bacterial Strain Packet	1 each	
71-1535.2L	TA1535; 2 discs/vial		R
71-1537.2L	TA1537; 2 discs/vial		R
71-098.2L	TA98; 2 discs/vial		R
71-100.2L	TA100; 2 discs/vial		R
72-188.2L	*E. coli* WP2 *uvrA*; 2 discs/vial		R
26-505.025	Oxoid Nutrient Broth, No. 2; 25 mL/bottle	6 each	R
21-100.1	Nutrient Agar; 10/sleeve	2 each	R
21-40S294	Minimal Glucose Agar, 24-well plates; 2/sleeve	11 each	R
21-40S296	Phenotype Test Plates, 6-well plates; 2/sleeve	3 each	R
26-300	Phenotype Test Packet	1 each	
26-810	Ampicillin, 2 µg/disc; 10 discs/vial		F
26-811	Tetracycline, 1 µg/disc; 10 discs/vial		F
26-812	Mitomycin C, 0.5 µg/disc; 10 discs/vial		F
26-813	Crystal Violet, 10 µg/disc; 10 discs/vial		RT or F
26-721.1	Top Agar, 0.05 mM His/Bio/Tryp; 100 mL/bottle	1 each	RT
11-404L	Mutazyme™, 10%, Lyophilized PB/BNF Rat Liver S9 Mix; 20 ml/vial	1 each	F
26-682	Water, deionized, sterile; 25 mL/bottle	1 each	RT
26-543.039	Phosphate buffer, 0.1 M, pH 7.4; 100 mL/bottle	1 each	RT
60-300	Ames ControlChem Packet	1 each	
60-103.1	Sodium Azide, 10 µg/vial		R
60-107.21	2-aminoanthracene, 20 µg/vial		R
60-107.2	2-aminoanthracene, 200 µg/vial		R
60-111	2-nitrofluorene, 20 µg/vial		R
60-114.6	Benzo(a)pyrene, 60 µg/vial		R
60-121.3	4-nitroquinoline-*N*-oxide, 10 µg/vial		R
60-147.5	9-aminoacridine HCl, 500 µg/vial		R
PS-112-CS	Inoculating loops, 10/pk	1 each	RT
90-002	Cuvettes, spectrophotometer, 1 mL; 10/pack	1 each	RT
90-003	Microcentrifuge tubes, 2 mL; 10/pack	1 each	RT
———	Incubation bags	14 each	RT

R	Store refrigerated (4 - 8°C)

RT	Store room temperature

F	Store frozen (approx. -20°C)

This kit contains more than enough reagents to test 1 chemical at 8 dose levels in triplicate, with and without S9, with concurrent positive (in triplicate) and solvent/vehicle (12 replicate wells) controls. It can be used with any *E. Coli* or Salmonella tester strains including TA97a and using appropriate positive controls, with TA102 if required. Larger experiments and subsequent testing will require additional materials which can be purchased individually from

Additional items you will need to complete the assay:

Biosafety laminar flow containment cabinet, type B2*

Light box with magnifier or dark field colony counter*

Hand-held or benchtop tally counter*

Microwave or boiling water bath

Dimethyl sulfoxide (DMSO), pure, anhydrous

Incubator, 37°C, with shaker

Micropipettes & sterile tips

Microscope (ideally phase and inverted but standard is adequate)

Permanent marker pen, e.g. Sharpie-type

Personal protective clothing - nitrile gloves, disposable Tyvec-type coverall/jumpsuit and NIOSH-type dust-stopper face mask as required

Pipets-1, 5 and 10 mL sterile and pipet controller (Pipet aid)

Refrigerator

Forceps (Sterile, 5 pairs. /Alternatively, utilize 70% ethanol between uses)

Spectrometer (or bacterial counting chamber)

Test tube racks

Test tubes 13 × 100 mm, sterile, disposable

Tubes (micro centrifuge or similar)

Vortex mixer

Water bath or heating block, 45°C

* Desirable for routine testing but not essential

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SUMMARY OF PROCEDURES

Advance	Read through the manual and the procedures	
	Perform solubility test	
	Prepare and save *Study Design* workbook available online	
	Check you have all the required equipment, components, including enough 24-well plates, buffer, S9, test agent and top agar to comply with the study design	
	Label racks and plates, sterilize tubes	
Day -1	Late pm, inoculate suspension cultures of each strain, incubate at 37°C	
Day 0	Shake suspension cultures	Set aside vehicle for control & dilutions
	Grow bacteria to 10^9/mL	Formulate test agent & dilutions
	Perform phenotype check*	Formulate positive controls
		Prepare S9 mix
		Prepare sterility check plates
		Dose 24 well plates with formulations
		Mix S9/buffer + bacteria + top agar then plate
	Invert and incubate plates	
Day 1	Remove and score phenotype plates (if used)	
Day 3	After 65 hours incubation, check background lawn & enumerate revertant colonies	
Day 3+	Check plates if any results are questionable	
	If results OK, discard plates and any retained formulations	

* Optional

THE ASSAY

The µAmes kit contains Salmonella tester strains TA1535, TA1537, TA98, and TA100 together with *E. coli* strain WP2 *uvrA*. Each Salmonella strain was constructed with a different lesion in the histidine operon rendering it incapable of synthesizing this amino acid, i.e. they are histidine auxotrophs. The strains were constructed with a deep rough mutation (*rfa*) that increases the bacterial wall permeability to higher molecular weight material. In addition, a deletion of the DNA repair-coding gene (*uvrB*) promotes error-prone repair and enhances sensitivity to mutagens. The *uvrB* lesion extends through the gene for biotin Synthesis thus biotin is required for growth. Strains TA98 and TA100 carry the R-factor plasmid pKM101 which increases the activity of error-prone DNA repair systems and confers resistance to ampicillin; these strains are sensitive to a number of mutagens that are detected weakly or not at all by their parent strains TA1538 and TA1535 respectively. *E. coli* WP2 *uvrA* carries a mutation site within the *trpE* gene which blocks tryptophan biosynthesis and, like the Salmonella strains, is excision repair deficient. The individual strains differ in their lesions and in their response to different classes of mutagens so are generally employed in combination. Other Salmonella and *E. coli* tester strains (e.g. TA97*a*, TA102, *E. coli* WP2 *uvrA* pKM101) can be used with the reagents supplied in this kit without modification of the methods with the inclusion of an additional positive control chemical (Mitomycin C) with strain TA102.

The bacteria are exposed to the test agent in the presence of a small amount of the required amino acids (histidine and tryptophan) which allows a limited growth period during which DNA damage can be induced and 'fixed' in the form of mutation. The millions of microscopic colonies formed by the non-reverted bacteria give a hazy appearance to the agar referred to as the background lawn. The condition of the background lawn can give important information about general toxic effects. We recommend you use a low-power microscope (40x) to confirm its condition in cases where its integrity is in doubt, especially in the presence of precipitation. Mutations to histidine or tryptophan independence that occur in this limited growth period lead to prototrophic mutant colonies (revertants) that carry on growing even though the medium has been depleted of the required amino acid. A substantial dose-related increase in revertant colony numbers is indicative of mutagenic activity.

The procedures employed closely parallel those used for standard OECD Ames tests with minor variations. We suggest that you follow the instructions carefully; even those experienced in the Ames test will find useful information. The most common version of the test (plate incorporation) is described but may be modified where appropriate (e.g. pre-incubation method). The procedures follow appropriate aspects of GLP Regulations and Guidances.

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Planning

1. 20 mg of test agent is required to perform the test and any necessary solubility testing.
2. Search for chemical and biological information concerning the test material. Although not normally required, if the material is likely to be highly toxic to bacteria a preliminary dose range-finding test is recommended.
3. Consider the potential presence of test material mutagenic impurities which could confound results.
4. Amino acids or peptides in the test material can supply histidine or tryptophan leading to overgrowth of the background lawn and spurious slight increases in revertant colony counts. The cause is usually obvious due to the formation of a more luxuriant background lawn.
5. What is the material's aqueous solubility? Aqueous solvents are preferred but if solubility is lower than 10 mg/mL in water, saline, buffer, dilute acid or alkali, consider using a relatively non-toxic water-miscible organic solvent (e.g. DMSO). Consider chemical stability in solution.
6. Usually a solubility test is required prior to the study to establish or confirm an appropriate solvent. Determine the solubility of the test chemical in a water-miscible relatively non-toxic solvent. Anhydrous DMSO is the most commonly chosen solvent for organic chemicals that aren't water-soluble; methanol, ethanol, dimethylformamide and tetrahydrofuran may be used occasionally - see Maron & Ames (1983). A positive-displacement pipette is needed for more volatile solvents, such as acetone, for accurate dosing. Dichloromethane should be avoided because it is a bacterial mutagen. Very insoluble compounds may require solubilization in a non-miscible solvent before diluting to an appropriate concentration in DMSO for dosing. Such solvents are generally toxic to the bacteria (particularly if the pre-incubation modification is employed), so their final concentration in the top agar should be considered. Very high concentrations of solvent can inhibit some S9 mix enzymes. In certain circumstances, it may be appropriate to dose extracts of the test material or to prepare a fine suspension in an aqueous suspending agent such as aqueous 1% methylcellulose. The test material may precipitate upon addition to the aqueous top agar limiting exposure which can lead to unusual dose-response curves.

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7. Consider potential microbial contamination of the test article. This is generally not a problem with purified chemicals (especially those dissolved in organic solvents), but filter-sterilization of aqueous solutions of biologically-derived or environmental samples may be a wise precaution.
8. The conventional dose volume is 5 µL per well (equivalent to 100 µL per plate in the standard assay). In order to achieve the desired top concentration (250 µg/well = 5000 µg/plate) the dose volume can be increased to 50 µL per well or more for aqueous solutions or suspensions before interference agar gelling interference; the volume should be reduced accordingly if toxic solvents are used.
9. The suggested study design uses 8 doses separated by an approximately half log10 ($\sqrt{10}$) interval, i.e. 250, 80, 25, 8, 2.5, 0.8, 0.25 & 0.08 µg/well as indicated in the suggested study design in Appendix 1. This will nearly always result in an adequate number of non-toxic dose levels to evaluate mutagenicity and give an idea of the dose-response curve. A positive response is indicated by a substantial dose-proportionate increase in revertant colony numbers (see below). The dose-response is not necessarily expected to be linear and may be affected by toxicity or precipitation at higher doses.
10. It is recommended that you retain dose formulations in case repeat testing is necessary, e.g. unexpected and excessive toxicity. Confirmatory testing, if needed to clarify borderline or unclear results, will often employ a narrower dose interval. (e.g. 2-fold)
11. Prepare, assemble and label (as appropriate) the supplies and equipment needed at least one day before use. If this is the first time performing the µAmes test it will take most of the day. We strongly recommend you go through a dry run of the test in advance. If questions arise, contact MOLTOX® prior to beginning the test.

Records

1. Procedural records may be made directly into an electronic file or, using indelible ink, onto a paper file – see µAmes procedure. It is recommended that GLP recordkeeping procedures be followed for tests that may be used to support regulatory approval.
2. As with any genetic toxicity test, if using the µAmes method on a regular basis we recommend maintaining a laboratory historical negative/solvent and positive control database to facilitate interpretation of data.

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Validity of the study

1. Where performed, the phenotypic check results should meet the criteria listed in Appendix 1. Failing this check is atypical.
2. The spontaneous mean revertant colony counts for each strain (i.e. values obtained for the vehicle) should lie close to or within the current historical control range of the laboratory. Where an adequate laboratory historical control database has not been established, vehicle control values are expected to lie close to those listed in Appendix 1.
3. Where part of the study is invalid based on phenotypic characteristics, unusual vehicle control results or absence of a clear response to the positive control agent, it may be appropriate to repeat that part of the study.

Interpretation of results - toxicity

1. Toxic effects of the test item are normally indicated by the partial or complete absence of a background lawn (colony counts, if any, should not be reported in this case) or a substantial dose-related reduction in revertant colony counts compared with lower dose levels, concurrent vehicle control and expected values. If precipitation obscures background lawn observations, the lawn can be considered normal and intact if the revertant colony counts are within the expected range based on results for lower dose levels and historical control counts.

Criteria for negative/positive/equivocal outcome

1. The mean number of revertant colonies for all treatment groups is compared to those obtained in the vehicle control.
2. 'Fold Responses' are calculated using the following calculation:

   Fold Response = (mean revertant colony count + 0.5) ÷ (mean vehicle control count + 0.5)

Note: In the µAmes test a standard value of 0.5 is added to both the control and treated revertant colony count to account for low values obtained for the vehicle control with some of the strains.

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3. The mutagenic activity of the test item is assessed via the following criteria: Results are considered positive (i.e., indicative of mutagenic potential) if:

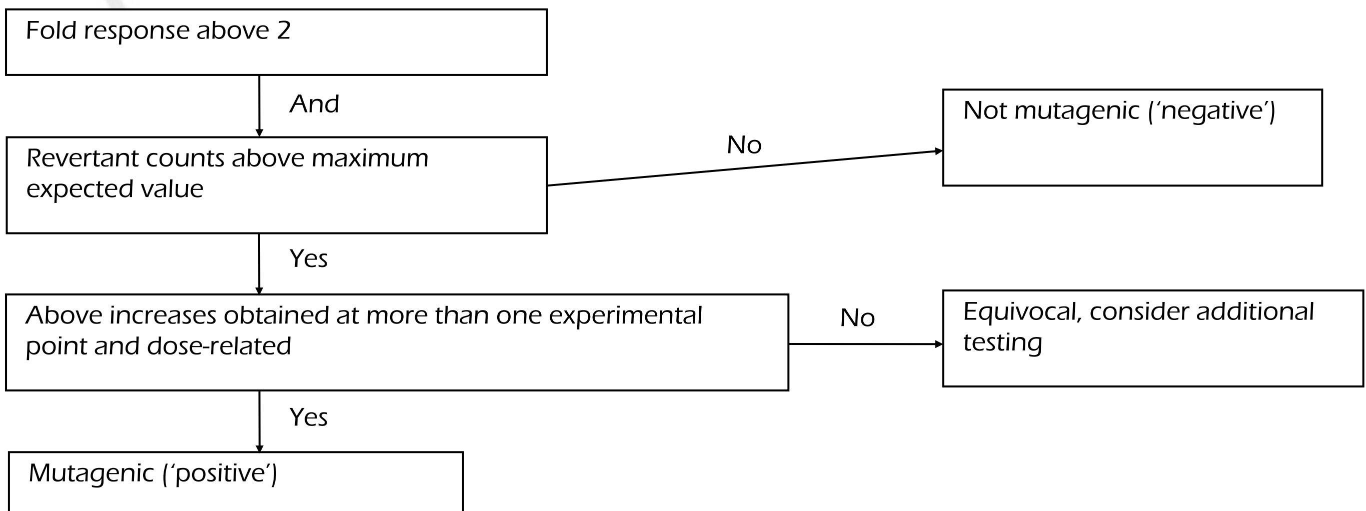
   The results for the test item show a substantial increase in revertant colony counts, i.e., "fold response", of at least 2.0 with mean value(s) outside the expected range for vehicle controls as described in the Validity section above. This two-fold rule is conventional but somewhat arbitrary. Isolated responses in only a single replicate well should be viewed with suspicion; generally they would be regarded as outliers and reported in parentheses with justification for their exclusion. Otherwise results are considered negative.

   The above increase must be dose related and/or reproducible, i.e., increases must be obtained at more than one experimental point (at least one strain, more than one dose level, more than one occasion or with different methodologies).

   If the second criterion is not met the results may be classified as equivocal. Further testing may be appropriate using a modified study design, e.g., a narrower dose interval with the appropriate strain. If no substantial increase is obtained in the confirmatory test, the results should be considered negative.

   If a treatment-related increase in revertant colony counts is obtained on more than one occasion but the increase does not meet the 2-fold criterion described above, it may be appropriate to consider the outcome as borderline or equivocal.

   Note that the same assessment criteria described above are also appropriate for the standard Ames test and are summarized in the decision tree below:



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SETTING UP AND ESTABLISHING A GENETOX ASSAY - GENERAL ADVICE

These suggested phases apply to any genetic toxicology assay that's to be developed for routine use. Following this model will reduce the time needed to develop the assay and future issues that may arise.

Set up

a) Read the main papers describing the procedure - see "references".

b) Ensure you are comfortable with the procedures described. Consider a dry run.

c) Write up the procedures used in each test you perform. Include results, conclusion and recommendations after the experiment is complete to avoid loss of details.

d) Contact an experienced scientist in the field for advice and support. If practical, view an experiment in progress.

Validation

a) Examine the plates carefully and confirm the quality of the background lawn using a microscope. Poor growth of the lawn may indicate a technical issue.

b) Generate negative and positive control results to confirm procedures. Confirm results obtained are acceptable and within the expected range.

c) Confirm the specificity and sensitivity of the test using an adequate range of compounds with and without S9.

d) Confirm reliability and reproducibility of the test using appropriate vehicles/solvents and standard positive controls.

e) Use the above results to establish a negative and positive control database. The database should include parameters one might like to analyze retrospectively, e.g. vehicle, dose volume, date of test, method (plate incorporation/preincubation) and S9 details.

f) Generate protocols and reports for each of the validation experiments. If the assay is to be used in a GLP environment each of the experiments should be inspected and reviewed by QA.

g) Prepare standard template protocols and reports for use in routine testing later.

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APPENDIX 1

Suggested Study Design

Dose level/ Treat-ment	Final conc. (µg/well)	Number of replicates -S9	Number of replicates +S9	Number of strains
Vehicle (solvent)	-	12	12	5
1/ Test material	0.08	3	3	5
2/ Test material	0.25	3	3	5
3/ Test material	0.8	3	3	5
4/ Test material	2.5	3	3	5
5/ Test material	80	3	3	5
6/ Test material	25	3	3	5
7/ Test material	80	3	3	5
8/ Test material	250 [A]	3	3	5
Positive control	[B]	3	3	5

[A] equivalent to the OECD 471 limit dose of 5000 µg/plate in the standard test

[B] dose depends on the test organism, the positive control chemicals and methodology used

- S9 with buffer, no S9

+S9 with S9

Expected Vehicle/Negative Control Counts

Strain	Mean per 12 wells	Max
TA1535	0.8	2.5
TA1537	0.5	1.2
TA97a	7.5	11.0
TA98	1.4	3.0
TA100	6.0	14.2
TA102	20.5	41
WP2 *uvrA*	2.2	4.0
WP2 *uvrA* pKM101	13.2	23.4

Values based on results obtained at MOLTOX®; results in other laboratories may differ somewhat. We recommend each laboratory establish and maintain its own historical control database. Strains not included in the kit are indicated in italics. Mean values (revertants/well) are approx. 1/20th of those seen in the conventional Ames bacterial mutation test (revertants/plate).

Positive controLs are expected to cause a substantial increase in revertant colony counts; i.e. revertant colony count > 2× (mean vehicle control count + 0.5).

6-Well Phenotype Test Plates

	Expected results					
	Zone of inhibition (mm)				Growth	
Strain	**C**	**A**	**T**	**M**	**Trp ⁻**	**His ⁻**
TA1535	X	X	X	X	++	-
TA1537	X	X	X	X	++	-
TA1538	X	X	X	X	++	-
TA97a	X		X	X	++	-
TA98	X		X	X	++	-
TA100	X		X	X	++	-
TA102	X				++	-
EC WP2		X	X		-/+	++
EC WP2 *uvrA*		X	X	X	-/+	++
EC WP2 pKM101			X		-/+	++
EC WP2 *uvrA* pKM101			X	X	-/+	++

Key:

- C Crystal violet
- A Ampicillin
- T Tetracycline
- M Mitomycin C
- X Zone > 12 mm
- \- no growth
- -/+ no or weak growth
- \+ weak growth
- ++ strong growth

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Figure 1 - STANDARD STUDY DESIGN – Negative/Positive control plate

Each plate contains 12 wells treated with the vehicle/solvent (NC) and 3 wells treated with the positive control (PC) against one bacterial strain and one S9 condition. With a standard study design of 5 bacterial strains, with and without S9, a total of 10 control plates plus a sterility plate are generated.

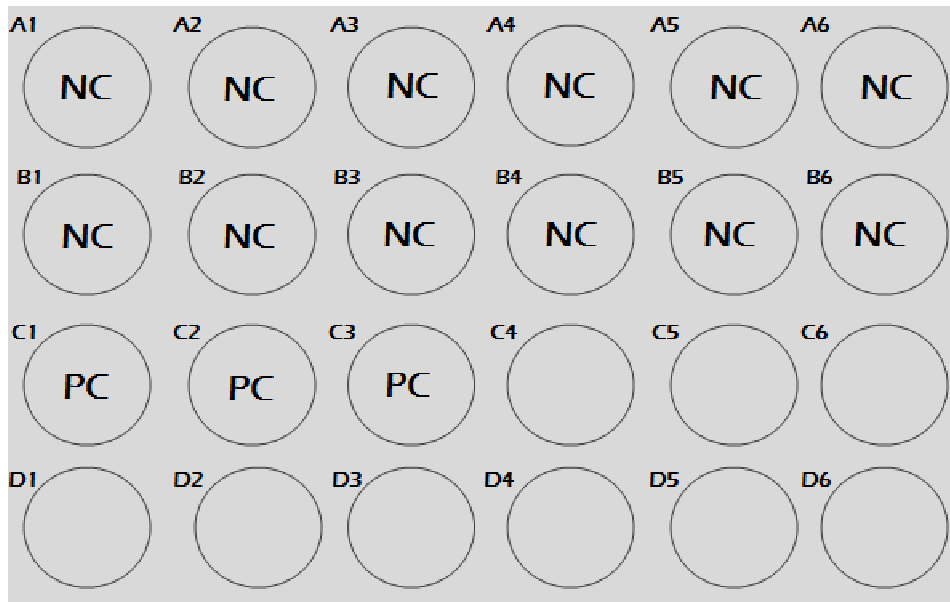


Figure 2 - STANDARD STUDY DESIGN – Treated plate

Each plate can be used to test 8 dose levels in triplicate with one bacterial strain and one S9 condition. A suggested layout is shown below. With a standard study design of 5 bacterial strains and with and without S9 there will be a total of 10 treated plates per test article are generated.

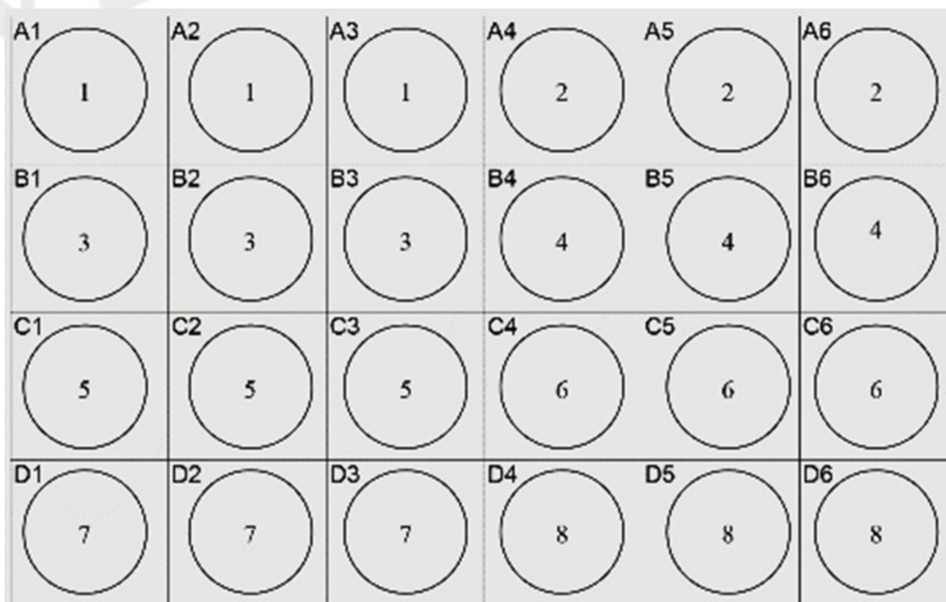


Figure 3 - STANDARD STUDY DESIGN – Additional solvent controls

Each plate contains 12 wells treated with the solvent plus buffer (S0), the remaining 12 wells are treated with solvent plus S9 mix (S+). Therefore, each additional solvent tested needs one extra plate per strain.

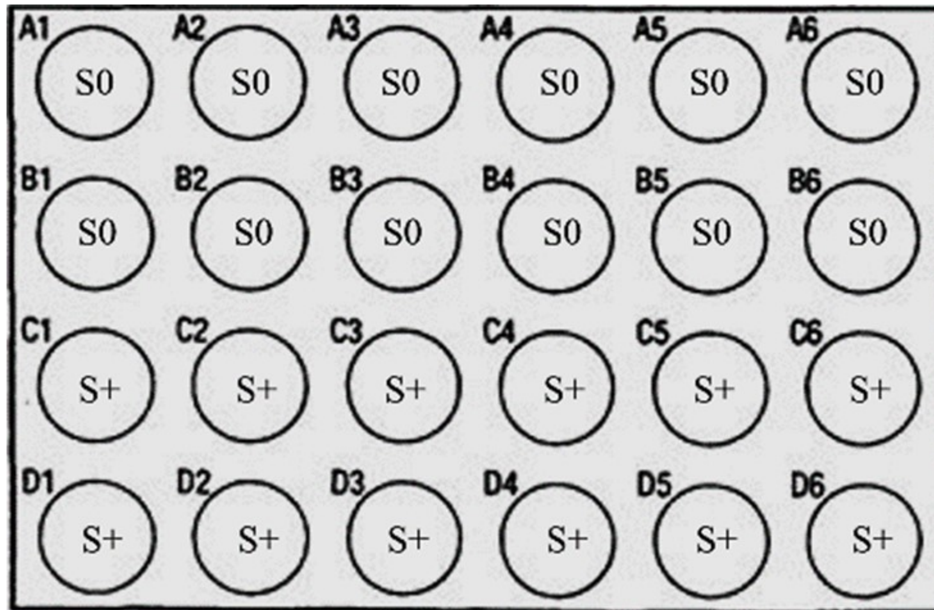
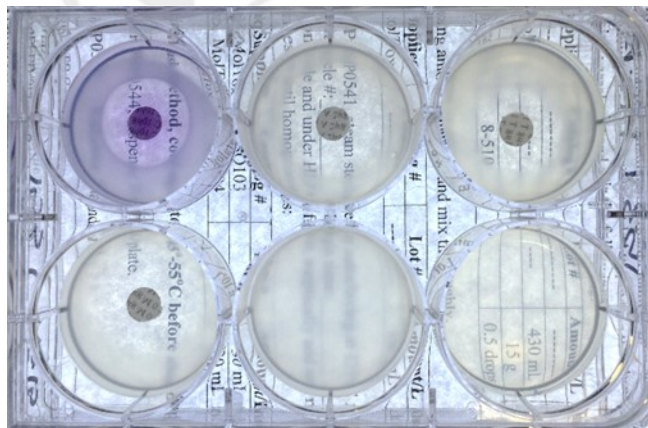
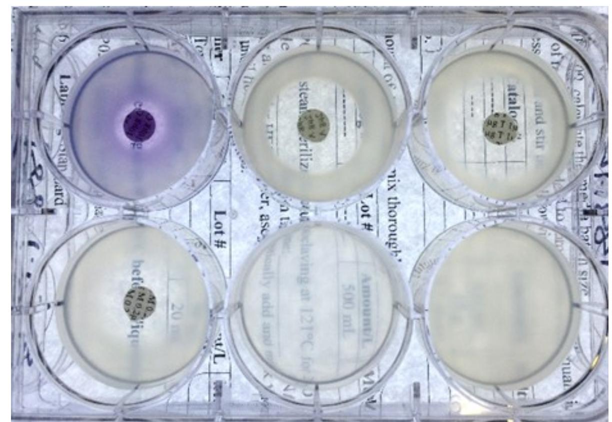


Figure 4 - Phenotype test plates with C (crystal violet), A (Ampicillin), T(Tetracycline), M (Mitomycin C) discs in wells 1 - 4, trp- and his- wells 5 and 6, respectively

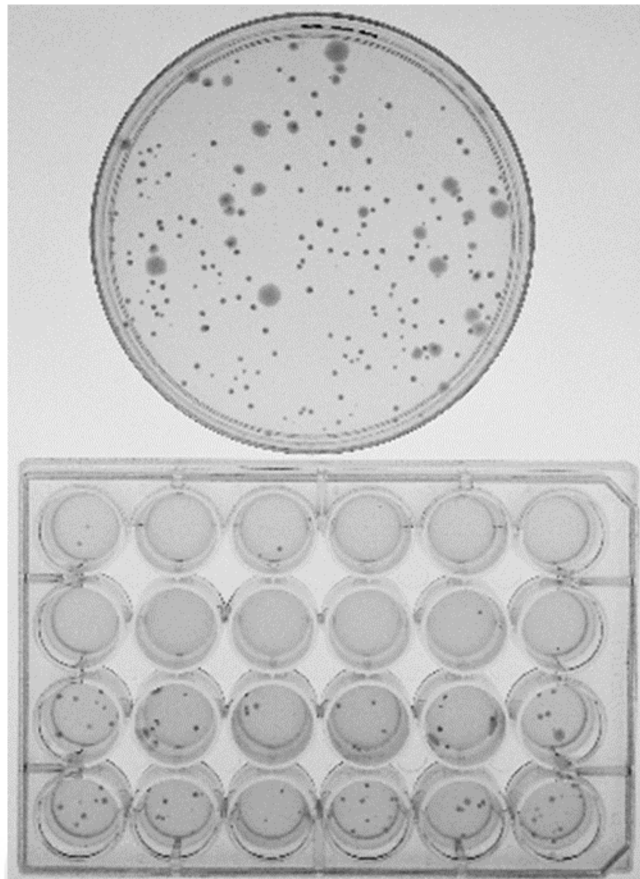


TA1537: C sensitive, A sensitive, T & M sensitive, his+ strong growth, his$^-$ poor/no growth.



EC WP2 *uvrA*: C slight sensitivity, A sensitive, T & M sensitive, trp- weak growth, trp+ strong growth

Figure 5 - Comparison of a single standard plate vs 24 well plate format showing a dose response



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