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Designing your Bacterial Reverse Mutation Assay

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Topics for Discussion

- Definitions
- Historical Perspectives and Background
- Test System Theory
- Exposure Methods
- Study Design
- Scoring
- Criteria for a Valid Test
- Evaluation Criteria
- Sample Data
- Clarifying Equivocal Responses
- You Make the Call
- Summary of Guideline Requirements
- Miniaturized Versions for M7

Definitions

- Mutagen – a substance that under the correct metabolic and cellular conditions has the ability to alter a cell's genetic information (DNA) in a heritable manner.
- Carcinogen – a substance that under the correct metabolic and cellular conditions has the ability to transform a normal cell to a neoplastic cell, i.e., a cancer cell.
- Test Article – the chemical under test in the assay.

Historical Perspectives and Background

- Synonyms
 - > Bacterial Reverse Mutation Assay
 - > *Salmonella typhimurium* and/or *Escherichia coli* Mutagenicity Assay
 - > Ames Test
- Purpose
 - > To evaluate a chemical's genotoxicity by measuring its ability to induce reverse mutations at selected loci of several bacterial strains
- Relevance
 - > Sensitive to a wide range of mutagenic and carcinogenic chemicals
 - > Required for product safety testing
- Developed in early 1970s as a quick and inexpensive screen
- Mutagens versus Carcinogens
- Genetic Disease
 - > Hemophilia, phenylketonuria, albinism and sickle cell anemia

Test System – How it works

- Histidine (*Salmonella typhimurium*) or tryptophan (*Escherichia coli*) auxotrophs - cannot synthesize histidine or tryptophan
- Point mutation in one of the his or trp genes disables histidine or tryptophan biosynthesis
- Each strain only detects a certain type of damage - typically 5 strains needed to detect the different types of mutational events
- 100,000,000 individuals at risk per plate
- Reverse mutation process



Test System – Mutational Events

- Frameshift mutation – insertion or deletion of a base(s)
- Basepair mutation – substitution of one base for another

DNA

Protein

Frameshift

CAT/CAT/CAT/CAT/CAT

VAL-VAL-VAL-VAL-VAL

CAT/CAT/GCA/TCA/T

VAL-VAL-ARG-SER-SER

Basepair

CAT/CAT/CAT/CAT

VAL-VAL-VAL-VAL

CAT/CAT/CTT/CAT

VAL-VAL-GLU-VAL

Test System - Genotypic Components to Increase Sensitivity

Mutations are rare events and even the simplest organisms have very effective DNA repair systems. To enhance strains sensitivity, a number of mutations have been introduced:

- *uvrA* / *uvrB* Mutation
 - > Partial deletion of the *uvrA* or *uvrB* gene
 - > Deficient DNA excision repair system
 - > Sensitivity to ultraviolet (UV) light
- pKM101 Plasmid
 - > Contains error-prone DNA repair system
 - > Damage is repaired incorrectly
 - > Resistance to ampicillin
- *rfa* Mutation
 - > Partial loss of lipopolysaccharide (LPS) cell wall in *Salmonella*
 - > Increased permeability to large molecules
 - > Sensitivity to crystal violet

Test System – Metabolic Activation

- Applicable to in vitro genetic toxicology assays
- Pro-mutagens and pro-carcinogens (e.g., polyaromatic hydrocarbons, azo dyes and amines) require conversion to their active metabolites
- In vitro test systems generally lack in vivo metabolic properties
- Phenobarbital and β -naphthoflavone induced rat liver S9 (PBNF)
 - > Used in Japan
 - > Equivalent to Aroclor[®]-induced S9
- S9 Homogenate characterized by several mutagens prior to use
 - > Benzo(a) pyrene, 2-aminoanthracene and/or dimethyl benz(a)anthracene are most commonly used
- S9 mix (Cytochrome P450 microsomal enzymes)

Exposure Methods

Plate Incorporation	Pre-Incubation
Most Common Exposure Method	Considered Most Sensitive Exposure Method
Not sensitive to all mutagens (e.g. nitrosamines (R-N-N=O), Divalent metals (Cd^{2+} , Co^{2+} , Fe^{2+} , Mn^{2+} , Zn^{2+}), Aldehydes (RH-C=O , $\text{H}_2\text{-C=O}$), Azo dyes (R-N=N-R), Pyrollizidine alkaloids, Allyl compounds ($\text{H}_2\text{C=CH-CH}_2\text{-R}$) or Nitro compounds (R- NO_2))	More susceptible to toxicity
Tester strain + Activation Mix (sham or S9 mix) + Test Article + Selective Top Agar overlayed on bottom agar plate.	Tester strain + Activation Mix + Test Article heated to 37°C , with shaking for 20 to 60 minutes. Top Agar added to mixture and overlayed on bottom agar plate.
Incubate 48-72 hours	Incubation 48-72 hours
Assess toxicity and precipitate formation	Assess toxicity and precipitate formation
Score by machine or manual counting	Score by machine or manual counting

Vehicle Selection

- Water - test up to 50 mg/mL or 100 mM, whichever is lower
- Dimethyl sulfoxide, ethanol, acetone - test up to 500 mg/mL or 1000 mM, whichever is lower, for compatibility between all *in vitro* assays
- Others as needed
 - acetonitrile, dimethyl formamide, methanol, polyethylene glycol, propylene glycol, N-methyl-2-pyrrolidone, ethyleneglycoldimethylether, dimethylacetamide
 - pH 3 to 11 physiological buffers
- Prefer soluble mixture but workable suspension acceptable
- Select the same vehicle for all GLP-regulated *in vitro* studies, if possible

GLP=Good Laboratory Practice

Study Design – Toxicity Test

Preliminary Toxicity Test (if toxicity profile unknown)

- Evaluate Toxicity
- Typically TA98, TA100, TA1535, TA1537, WP2 *uvrA*
- May use a subset of strains (TA100 and WP2 *uvrA*) to conserve test article
- With and without S9 activation
- 10 doses (5 mg to 6.7 μ g per plate at quarter-log intervals) plus vehicle controls
- single plate per dose
- Use either plate incorporation or preincubation
- Follow-up with a mutagenicity test

Study Design – Mutagenicity Test

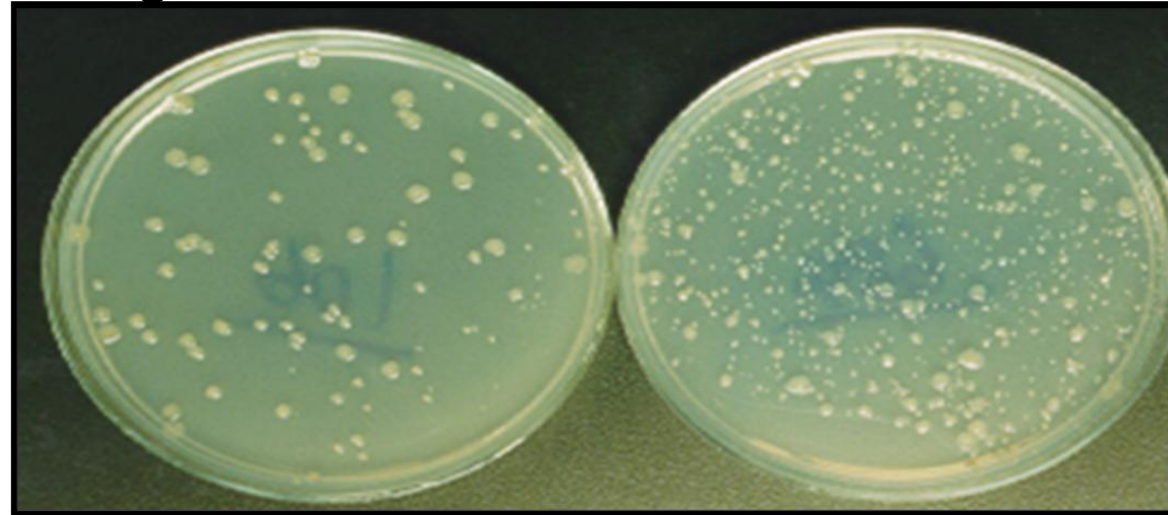
- Evaluate mutagenicity
- Typically TA98, TA100, TA1535, TA1537, WP2 *uvrA*
- With and without S9 activation
- Test up to 5 mg per plate, or a toxic or mutagenic dose
- 5 to 7 doses, at half-log intervals, plus vehicle and positive controls
- Maximum half-log intervals between doses, but closer as warranted
- 3X replicate-plates per dose
- Use either plate incorporation or preincubation
- Tester strain genotype confirmation
- Equivocal results clarified using modified experimental conditions

Sample Plates

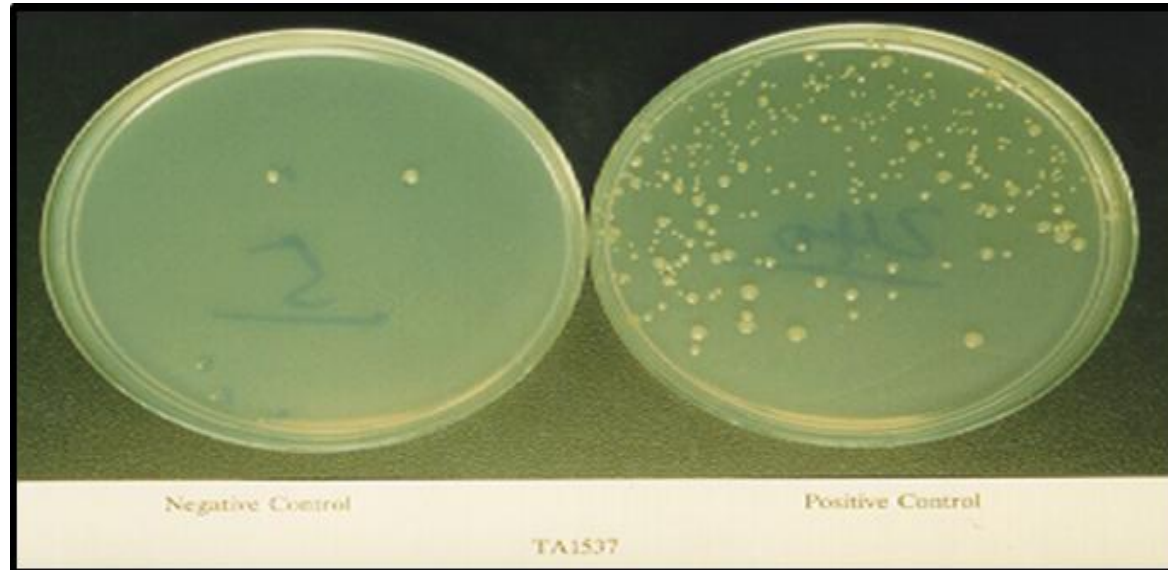
TA100

Negative Control

Positive Control

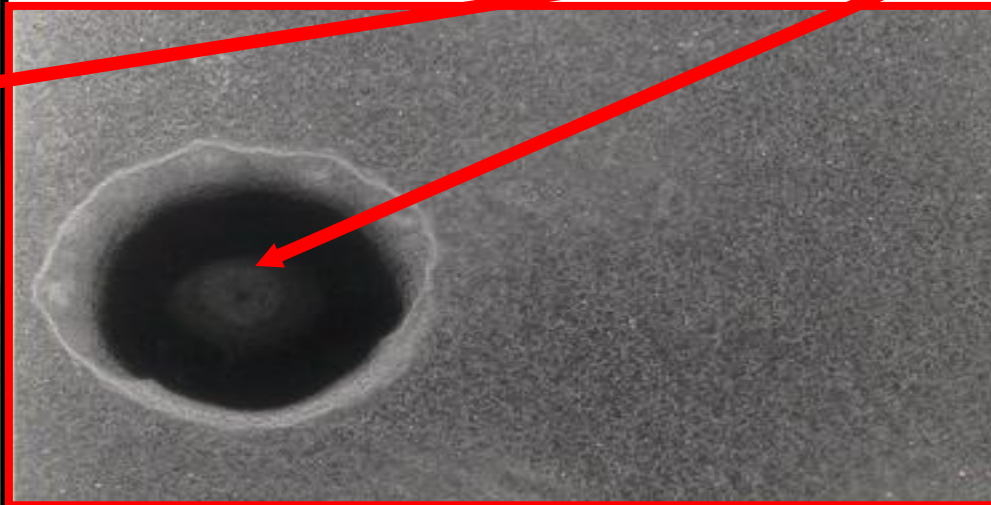


TA1537



Scoring: Background Lawns

- Observe the background lawn under a dissecting scope to assess toxicity and determine true revertant colonies (~20X magnification)
 - Normal – Code 1
- The background lawn is the result of colonies that stop growing when the histidine or tryptophan is exhausted.

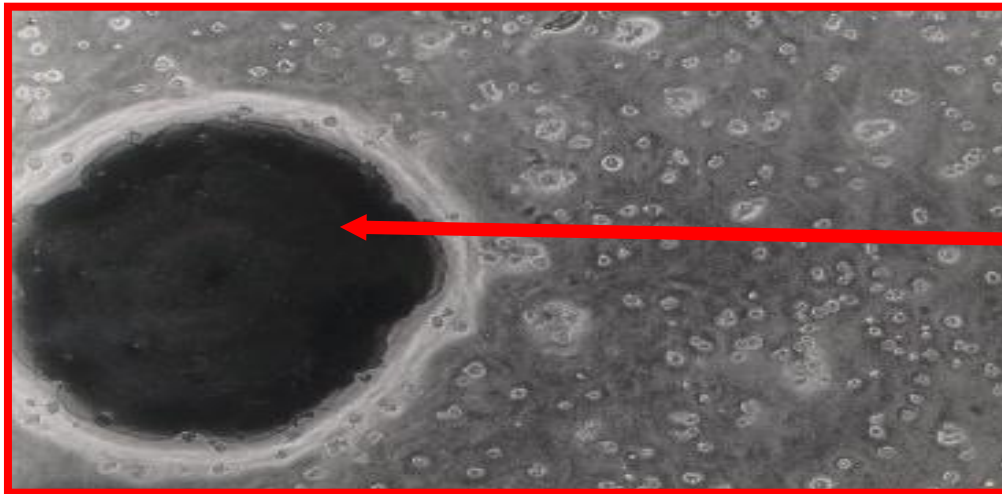


Revertant

Scoring: Background Lawns

Moderate Reduction – Code 3

- Large number of bacteria killed
- Allows surviving colonies to grow larger
- Results in moderate thinning of the background colonies with a microscopic enlargement visible to the aided eye.

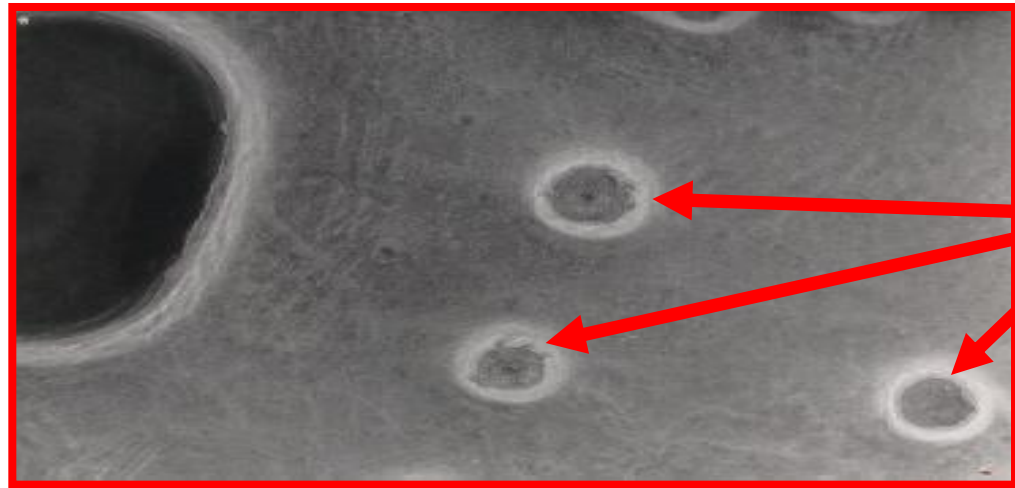


Revertant

Scoring:Background Lawns

Extreme Reduction – Code 4

- Majority of bacteria killed
- Allows surviving colonies to grow much larger
- Results in severe thinning of the background colonies with a macroscopic enlargement visible to the unaided eye



Enlarged background colonies (pinpoint colonies)

Scoring of Plates – Background Lawn and Precipitate Evaluation

Code	Description
5	Absent – no background lawn
6	Obscured microscopically by precipitate - background lawn cannot be accurately evaluated
p	Precipitate^a – Distinguished by precipitate on the plate that is visible to the naked eye and requires plate to be counted manually.

^a Precipitate does not otherwise affect the validity of a study, only determines how the affected plates are scored (either by hand or by machine)

Evaluation: Toxicity Definition

- A dose level is considered toxic if one or both of the following criteria are met:
- At least a moderate background lawn reduction (code 3, 4, or 5)
- A greater than 50%, abrupt, dose-dependent reduction in the mean number of revertants per plate as compared to the mean vehicle control value

Evaluation: Criteria for a Valid Test

- Tester Strain Genotypes
 - > Ampicillin, crystal violet, tetracycline and UV sensitivity or resistance, as appropriate
- Tester Strain Titters
 - > Must be at least 3.0×10^8 cells/mL to yield $\geq 3.0 \times 10^7$ cells/plate
- Sterility Checks
 - > Sham & S9 mixes and the test article dilutions
 - > Confirm these components are not contaminated with microbial organisms
- Spontaneous Revertant Frequencies
 - > Mean vehicle control values within 95% vehicle control limits (99% with Study Director approval)

95% Control Limits (99% Upper Limit)					
	TA98	TA100	TA1535	TA1537	WP2 <i>uvrA</i>
-S9	6-26 (31)	66-122 (136)	3-19 (23)	1-13 (16)	11-39 (46)
+S9	10-38 (45)	66-138 (156)	3-23 (28)	1-17 (21)	12-44 (52)
With Study Director justification, values including the 99% control limit and above are acceptable.					

Evaluation: Criteria for a Valid Test

Positive Control Values

- Mean values must be at least 3.0–times greater than respective, mean vehicle control values and outside 95% vehicle control limits

Toxicity

- Must have at least 3 nontoxic dose levels
- Must demonstrate toxicity or mutagenicity at top dose or have tested up to 5 mg/plate

Precipitate

- Must demonstrate precipitate at top dose or test up to 5 mg/plate

Evaluation: Criteria for a Positive Response

- A dose-related increase in the mean revertants per plate compared to mean vehicle control
- Over two increasing concentrations of test article in one tester strain either with or without S9 activation
- Peak of the dose response is $\geq 3.0X$ for TA1535 and TA1537 and outside 95% vehicle control limits or
- Peak of the dose response is $\geq 2.0X$ for TA98, TA100, and WP2 *uvrA* and outside 95% vehicle control limits
 - > TA102 and WP2 *uvrA* (pKM101)
- Note: Statistical Analysis may be used for studies performed for Europe
 - > But no standard method is currently recognized

Evaluation: Criteria for an Equivocal Response

- A non-dose responsive increase in a revertant count that is greater than the acceptable vehicle value (range), or
- Does not achieve the respective fold increase cited
- **Negative:** if it is neither positive nor equivocal

Sample Data:Preliminary Toxicity Data

	Toxic at ≥ 3333 $\mu\text{g}/\text{plate}$ without S9				Non-toxic			
<u>μg per plate</u>	<u>TA100 +S9</u>		<u>TA100 -S9</u>		<u>WP2 <i>uvrA</i> +S9</u>		<u>WP2 <i>uvrA</i> -S9</u>	
	<u>Rev/plate</u>	<u>Bkgd</u>	<u>Rev/plate</u>	<u>Bkgd</u>	<u>Rev/plate</u>	<u>Bkgd</u>	<u>Rev/plate</u>	<u>Bkgd</u>
Vehicle	101	1	118	1	21	1	26	1
6.7	102	1	107	1	22	1	22	1
10	107	1	112	1	17	1	33	1
33	104	1	105	1	24	1	31	1
67	93	1	121	1	23	1	24	1
100	104	1	116	1	14	1	29	1
333	118	1	105	1	28	1	19	1
667	100	1	109	1	30	1	19	1
1000	106	1	100	1	26	1	20	1
3333	119	1	79	3	19	1	28	1
5000	100	1	0	4	20	1	25	1

Selected Doses: 5000, 1500, 500, 150, 50 μg per plate

Sample Data: Mutagenicity Data

	<u>TA100 -S9</u>	
<u>µg/plate</u>	<u>Rev/plate</u>	<u>Bkgd</u>
Vehicle	105 ± 8	1
50	113 ± 12	1
150	114 ± 6	1
500	116 ± 8	1
1500	107 ± 9	1
5000	43 ± 5	3
Positive	510 ± 43	1
<u>Historical Values (95% CL)</u>		
Vehicle	94 ± 14 (66-122)	
Positive	681 ± 176	

Check Criteria for Valid Test

- Ampicillin resistant, Crystal Violet sensitive
- Titer $\geq 0.3 \times 10^9$ cells/mL
- Sterilities free of contamination
- Vehicle control mean within 95% vehicle control (VC) limit
- Mean positive control $\geq 3.0X$ mean vehicle control and >95% VC limit
- One toxic dose (5000-µg) and 4 nontoxic doses
- Tested to 5 mg/plate; no precipitate

Data Not Shown

Response: no dose response
max increase = 1.1-fold, and not outside 95% VC limit

Conclusion: Negative

Sample Data: Mutagenicity Data

TA1537 +S9		
<u>µg/plate</u>	<u>Rev/plate</u>	<u>Bkgd</u>
Vehicle	7 ± 3	1
50	6 ± 2	1
150	9 ± 3	1
500	15 ± 4	1
1500	30 ± 7	1
5000↓	54 ± 3	1
Positive	131 ± 24	1
<u>Historical Values (95% CL)</u>		
Vehicle	9±4 (1–17)	
Positive	72±52	

Check Criteria for Valid Test

- Ampicillin and Crystal Violet sensitive
- Titer $\geq 0.3 \times 10^9$ cells/mL
- Sterilities free of contamination
- Vehicle control mean within 95% vehicle control (VC) limit
- Mean positive control $\geq 3.0X$ mean vehicle control and >95% VC limit
- No toxic dose (at least 3 nontoxic doses)
- Tested to 5 mg/plate; no precipitate

} Data Not Shown

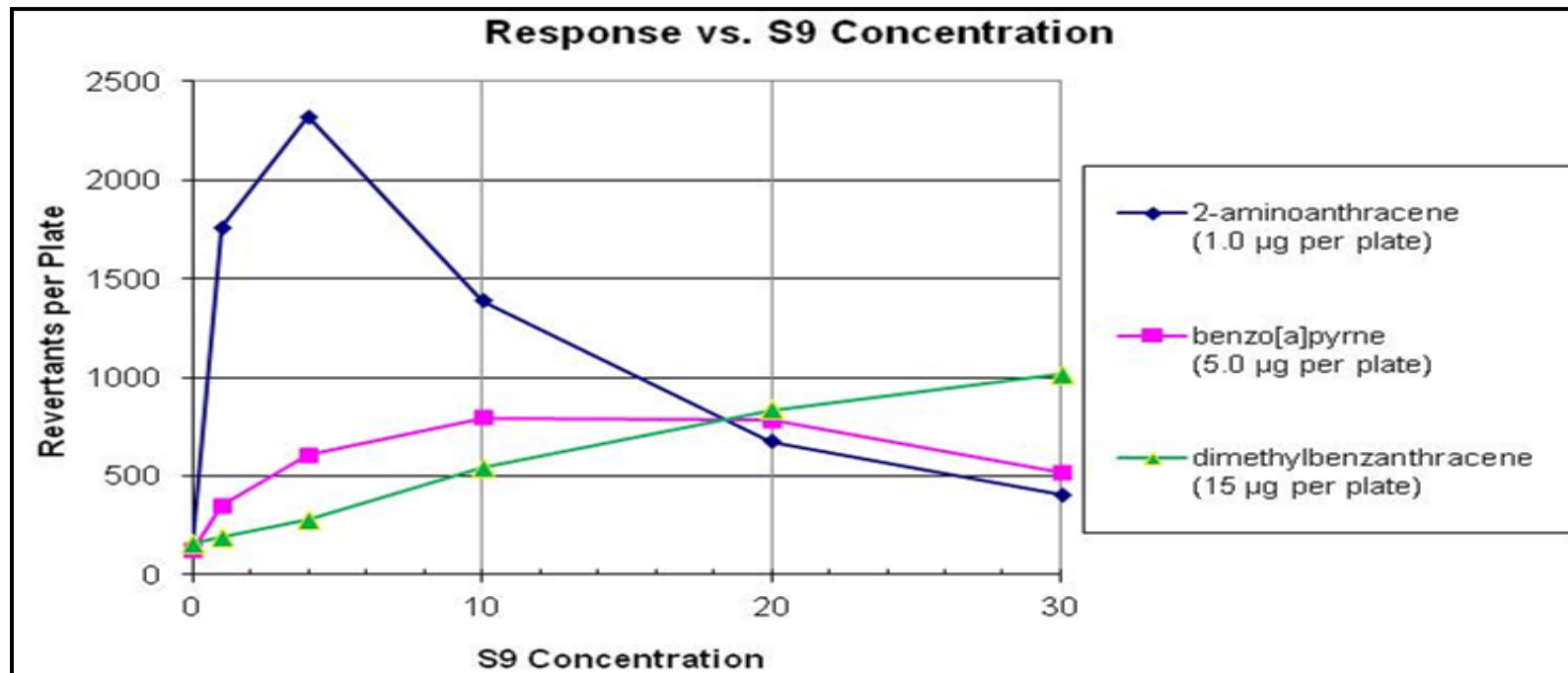
Response: dose response over at least 2 increasing concentrations
max increase = 7.7-fold and outside 95% VC limit

Conclusion: Positive

Clarifying Equivocal Responses

Summary of Options to Clarify a Response

- Modify the dose levels (most common)
- Include alternate exposure method (plate incorporation or preincubation)
- Modify the S9 concentration, if response is with S9



You Make the Call

$\mu\text{g}/\text{plate}$	TA98				TA1537				WP2 <i>uvrA</i>			
	Rev/plate	Bkgd	FI		Rev/plate	Bkgd	FI		Rev/plate	Bkgd	FI	
VC	20 $\pm$ 5	1	-		7 $\pm$ 5	1	-		21 $\pm$ 5	1	-	
1.5	21 $\pm$ 7	1	1.1		8 $\pm$ 7	1	1.1		24 $\pm$ 7	1	1.1	
5.0	25 $\pm$ 6	1	1.3		9 $\pm$ 6	1	1.3		19 $\pm$ 6	1	0.9	
15	26 $\pm$ 8	1	1.3		5 $\pm$ 8	1	0.7		30 $\pm$ 8	1	1.4	
50	22 $\pm$ 10	1	1.1		7 $\pm$ 10	1	1.0		45 $\pm$ 10	1	2.1	
150	15 $\pm$ 7	1	0.8		4 $\pm$ 7	1	0.6		60 $\pm$ 7	1	2.9	
500	10 $\pm$ 5	2	0.5		8 $\pm$ 5	1	1.1		132 $\pm$ 5	1	6.3	
1500	5 $\pm$ 2	3	0.3		9 $\pm$ 2	1	1.3		15 $\pm$ 2	3	0.7	
5000	0 $\pm$ 0	5	0.0		NT $\pm$ -	-	-		0 $\pm$ 0	5	0.0	
PC	300 $\pm$ 20	1	15.0		62 $\pm$ 8	1	8.9		180 $\pm$ 20	1	8.6	
FI=Fold Increase; VC=Vehicle Control; PC=Positive Control; NT=Not tested Bkgd: 1=Normal; 2=Slight reduction; 3=Moderate reduction; 4=Extreme reduction; 5=Absent												
Evaluation												
VC	(6-26) $\checkmark$				(1-13) $\checkmark$				(11-39) $\checkmark$			
PC	($\geq 3\text{X VC}$ & >26) $\checkmark$				($\geq 3\text{X VC}$ & >13) $\checkmark$				($\geq 3\text{X VC}$ & >39) $\checkmark$			
Tox	(≥ 3 nontoxic) $\checkmark$				(≥ 3 nontoxic) $\checkmark$				(≥ 3 nontoxic) $\checkmark$			
Valid	Yes				No (Not tested to 5 mg/plate)				Yes			
Call	Negative				Need to retest				Positive (6.3-fold)			

Key Guideline Requirements

Tester Strain Genotypes						
Per OECD 471 and ICH, Select at Least One Strain in each of the Five Categories World-wide acceptance (US, Europe, Japan & OECD member countries) adopted in 1997						
Strain Categories	Strain	Mutation	Detector	Additional Mutations		
				LPS	Repair	R-factor (pKM101)
1	TA98	<i>hisD3052</i>	FS	<i>rfa</i>	$\Delta uvrB$	+R
2	TA100	<i>hisG46</i>	BP	<i>rfa</i>	$\Delta uvrB$	+R
3	TA1535	<i>hisG46</i>	BP	<i>rfa</i>	$\Delta uvrB$	-
4	TA1537	<i>hisC3076</i>	FS	<i>rfa</i>	$\Delta uvrB$	-
	TA97	<i>hisD6610</i>	FS	<i>rfa</i>	$\Delta uvrB$	+R
	TA97a	<i>hisD6610</i>	FS	<i>rfa</i>	$\Delta uvrB$	+R
5	TA102	<i>hisG428</i> (pAQ1)	BP, CL	<i>rfa</i>	-	+R
	WP2 <i>uvrA</i>	<i>trpE</i>	BP	-	$\Delta uvrA$	-
	WP2 <i>uvrA</i> (pKM101)	<i>trpE</i>	BP	-	$\Delta uvrA$	+R
Extra	WP2 (pKM101)	<i>trpE</i>	BP, CL	-	-	+R
FS=frameshift; BP=Base pair; CL=crosslinker Use TA102 or WP2 (pKM101) to detect crosslinkers						

Key Guideline Requirements

- Test with and without S9 activation at a minimum of 5 dose levels
- Maximum dose level of 5 mg per plate unless:
 - > Toxic or mutagenic - test up to toxic or mutagenic dose
 - > Non-mutagenic, nontoxic and insoluble - test up to precipitating dose
 - > Don't exceed half-log intervals between doses, but closer as warranted
- Confirmatory Assay
 - > ICH S2(R1) guideline no longer requires a confirmatory trial
 - > Equivocal results clarified using modified conditions (OECD & ICH)
 - > Clear positive results do not require confirmation (OECD & ICH)
 - > Negative results need not be repeated when justified (OECD)
- Precipitate evaluated by unaided eye

Summary

Advantages	Disadvantages
Detects 90% of carcinogens (McCann & Ames 1976)	Not appropriate for test articles that interfere with mammalian cell replication (<i>e.g.</i> topoisomerases)
Detects 83% of carcinogens (McCann & Ames 1981)	Antibiotics (toxic at $\geq \sim 15 \mu\text{g}$ per plate) – require ICH S2(R1) option 1 (at least one <i>in vitro</i> mammalian cell assay)
58% sensitivity to rodent carcinogens and 73% specificity for non-carcinogens (Ashby <i>et al.</i> 1989)	Histidine or tryptophan containing test articles will interfere with the selective assay conditions

Summary: Chemical Class Considerations

Chemical Class	Ames Plating Methodology Considerations
Proteins (containing histidine and/or tryptophan)	Treat and plate method if shown to interfere with the selective conditions of the assay; this is not an automatic requirement for proteins and can be assessed in a simple test.
Antibiotics / Bacteriocides	Plate incorporation or preincubation with extra dose levels
Gases (below ~20°C) / Vapors	Desiccator method
Volatiles	Preincubation method with screw-capped tubes
Nitrosamines, divalent metals, aldehydes, nitro compounds, pyrrolizidine alkaloids, allyl compounds	Preincubation method
Azo dyes and diazo compounds	Reductive S9 using preincubation method in addition to the standard metabolic activation conditions, if negative under standard activation conditions
Botanical compounds	Conduct sterility test prior to running assay to test for contamination
Chemicals specifically active in TA102 such as peroxides, aldehydes, psoralens, DNA-crosslinkers or potential crosslinkers	Use TA102 in place of or in addition to WP2 <i>uvrA</i> or WP2 <i>uvrA</i> (pKM101)
Medical Devices	Test both aqueous (polar) & organic ("nonpolar") extracts

ICH M7(R1) Note 2 – Approval of Mini Versions

- For impurities that are not feasible to isolate or synthesize or when compound quantity is limited, it may not be possible to achieve the highest test concentrations recommended for an ICH-compliant (OECD471) bacterial mutagenicity assay according to the current testing guidelines.
- In this case, bacterial mutagenicity testing could be carried out using a miniaturized assay format with proven high concordance to the ICH-compliant assay to enable testing at higher concentrations with justification.

Assessment and Control of DNA Reactive (Mutagenic) Impurities in Pharmaceuticals to Limit Potential Carcinogenic Risk, ICH M7 (R1), Step 4, 31 May 2017

ICH M7(R1) Note 2 – Approval of Mini Versions

Study Design	Test Article Amount (mg)*	Maximum Dose	Strains	Cells Exposed (x 10 ⁸)	Replicates (plates or wells)
Standard	350	5000 µg/plate	ICH Compliant	1.0	3
Modified Standard (2X)	225	5000 µg/plate	ICH Compliant	1.0	2
Mini-well (3X)	75	1000 µg/well +	ICH Compliant	0.2	3
Mini-well (2X)	50	1000 µg/well +	ICH Compliant	0.2	2
Micro-well (3X)	19	250 µg/well +	ICH Compliant	0.05	3
Micro-well (2X)	13	250 µg/well +	ICH Compliant	0.05	2
Abbreviated (3X)	13	5000 µg/plate	TA98 (FS), TA100 (BP)	1.0	3
Abbreviated Standard (2X)	90	5000 µg/plate	TA98 (FS), TA100 (BP)	1.0	2
= consistent with standard ICH design					
= modification of standard ICH design					
FS=frameshift detector; BP=base pair detector					
Amounts exclude that needed for solubility assessments					
* Assumes no toxicity trial, half-log dose spacing, no adjustment for purity or active ingredient and no samples for formulation analysis					
+ Equivalent to 5000 µg/plate in the standard assay					

Conclusions

- Standard Ames assays are expected but Alternative Ames assays have been submitted to FDA and accepted for the purpose of impurity qualification
- Miniaturized Ames designed discussed here have high concordance with standard Ames assay (it's anticipated that closer alignment to ICH is preferred)
- Recommend that sponsors provide rationale to support alternate approach (e.g., difficult synthesis, limited amounts, suitability of selected assay)
- Recommend that reports include sufficient detail of methods and results to evaluate adequacy of study

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