

*Which Ames is Which?
Lessons Learned from Some Recent
Studies on Mainstream Smoke
Condensate Mutagenicity Using the
Ames Assay*

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Outline of Presentation

- Background
- Ames assays on tobacco smoke
- Strain/activation versus potency
- Recent data for KY1R4F
- TA102 and mainstream smoke condensate
- Comparisons of MSC Mutagenicities
- Factors affecting study results
- Monitoring assay performance
- Nonlinear regression
- Concluding remarks
- Acknowledgements

Background (1)

- The Ames assay is one of a series of short-term *in vitro* assays for genetic toxicology
- Ames assays are used to measure the mutagenic potential of a test substance
- In studies of the relationships among several *in vitro* assays and rodent carcinogenicity, the Ames assay gave the best concordance (Haseman *et al.*, *J. Amer. Stat. Assn.*, 1990; Tennant *et al.*, *Science*, 1987)

Background (2)

- Ames assay uses one or more strains of *Salmonella typhimurium* bacteria
 - Strains have preexisting mutations that leave the bacteria unable to synthesize histidine
 - Such bacteria will not grow unless there are additional mutations that permit the bacteria to synthesize histidine
 - Different strains respond to different mutagens
 - Extent of bacterial growth depends on the strain, mutagen, and assay conditions

Background (3)

- Two classes of mutagens have been detected with the Ames assay
 - Direct-acting mutagens that do not require any metabolic activation to show a positive result
 - Indirect-acting mutagens (promutagens) that require exogenous metabolism to show a positive result
 - Most commonly used system for achieving metabolic activation is to add the supernatant of an Arochlor-induced, rat-liver homogenate (S9) to the test system

Background (4)

- Ames assays of various chemicals showed
 - Strains TA98 and TA100 with and without S9 identified 90% of mutagens (Zeiger *et al.*, *Environ. Mutagen.*, 1985)
 - Strains TA98, TA100, TA97 or TA1537 “are always considered necessary” with TA1535 and TA102 in special cases (e.g., TA102 for compounds that may cross-link DNA) (Mortelmans and Zeiger, *Mutat. Res.*, 2000)
 - “unless required by specific guidelines, it is recommended that for general screening purposes a tier approach be used using strains TA98 & TA100 with & without metabolic activation” (Mortelmans & Zeiger)

Ames assays on tobacco smoke (1)

- In many publications, test substance has been mainstream smoke condensate (MSC)
 - Collected on Cambridge pad and extracted into DMSO (contemporary practice)
 - Collected in glass impaction trap and extracted into DMSO (used by some labs)
 - Condensed in cold trap cooled with liquid air, with final extract in DMSO (Kier *et al.*, *Proc. Nat. Acad. Sci. USA*, 1974)
 - Collected in solvent traps with final extract in DMSO (Chortyk and Chamberlain, *Arch. Environ. Health.*, 1990)

Ames assays on tobacco smoke (2)

- First reported by Ames and coworkers (Kier *et al.*, *Proc. Nat. Acad. Sci. USA*, 1974)
 - TA1535, TA1536, TA1537, TA1538; all \pm S9
 - MSC from control, control + 10% $\text{Mg}(\text{NO}_3)_2$, control + filter with 300 mg charcoal cigarettes
 - All MSC samples active with TA1538+S9 and inactive with TA1536, TA1537; both \pm S9
 - MSC from nitrated tobacco sample active with TA1535 \pm S9, TA1538-S9
 - With TA1538+S9, most of activity from KY1A1 MSC was in basic and weakly acidic fractions

Ames assays on tobacco smoke (3)

- By 1980, some drivers of Ames activity in MSC had been discovered
 - Burley MSC more active than Bright MSC with TA1538+S9, TA98+S9
 - Leaf total N2, protein N2, and soluble N2 well correlated with MSC activity with TA98+S9
 - Protein pyrolysis products shown to be mutagenic with TA98+S9, TA100+S9
 - Starch pyrolysis products shown to be active with TA100-S9
 - MSC active with TA100±S9

Ames assays on tobacco smoke (4)

- More recent literature tends to show two types of studies using the Ames assay
 - Research on novel products or ingredients has generally used five strains \pm S9 per OECD Guideline 471 [e.g., TA98 \pm S9, TA100 \pm S9, TA102 \pm S9, TA1535 \pm S9 and TA1537 \pm S9 (or TA97 \pm S9)]
 - Research on effects of smoking conditions or other purposes has generally used TA98+S9 alone or TA98+S9 and TA100+S9

Strain/activation versus potency

- Of the 10 combinations of strain \pm S9, MSC is most potent with TA98+S9, least potent with TA1535-S9 and TA1537-S9
 - Definitions of potency from Margolin *et al.*, *Environ. Health Perspect.*, 1994 Suppl 1.
 - Slope of regression line from Bernstein's point-rejection method
 - Mutagenic effect per unit dose adjusted for concomitant toxicity (used in SALM program)
 - Maximum observed slope of dose-response curve
- Higher potency = higher reproducibility?

Recent data for KY1R4F (1)

Source	TA98+S9	TA100+S9	TA102+S9	TA1535+S9	TA1537+S9
Tewes	3462±289	2052±203	n.q.	n.q.	323±68
Roemer	3433±101	1790±55	n.q.	n.q.	392±18
Study 1	2331±88	736±86	617±104	81±27	379±35
Study 2	3043±184	735±66	585±74	67±5	428±26
Study 3	3027±231	725±113	535±87	58±5	388±28

Tewes *et al.*, *J. Appl. Toxicol.*, 2003; Roemer *et al.*, *Food Chem. Toxicol.*, 2002; $n = 4$, both studies
 Study 1, $n = 5$ over 5 days; Study 2 and 3, $n = 3$ with replicate assays on same day; slopes calculated
 over 0 to 125 μg TPM/plate dose range; mutagenicity values confirmed with SALM program
 n.q. = not quantifiable

Recent data for KY1R4F (2)

Source	TA98-S9	TA100-S9	TA102-S9	TA1535-S9	TA1537-S9
Tewes	19±4	176±26	n.q.	n.q.	n.q.
Roemer	16±1	106±6	n.q.	n.q.	n.q.
Study 1	72±35	508±81	292±162	67±29	108±63
Study 2	149±11	459±44	595±121	60±7	n.q.
Study 3	99±17	388±47	n.q.	34±20	n.q.

Tewes *et al.*, *J. Appl. Toxicol.*, 2003; Roemer *et al.*, *Food Chem. Toxicol.*, 2002; $n = 4$, both studies
 Study 1, $n = 5$ over 5 days; Study 2 and 3, $n = 3$ with replicate assays on same day; slopes calculated
 over 0 to 125 mg TPM/plate dose range, mutagenicity values confirmed with SALM program
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Comments on Studies 1, 2, and 3

- Smoke collection under ISO conditions
- Preincubation method used
- TPM doses
 - Seven nonzero doses: 25, 50, 75, 100, 125, 250, 500 μg TPM per plate
 - Concurrent zero-dose controls run with each replicate assay
 - Triplicate plates per dose per replicate assay
- Statistical analysis
 - Slopes calculated from pooled replicates over dose range from 0 to 125 μg TPM per plate
 - Standard deviations calculated from pooled standard errors

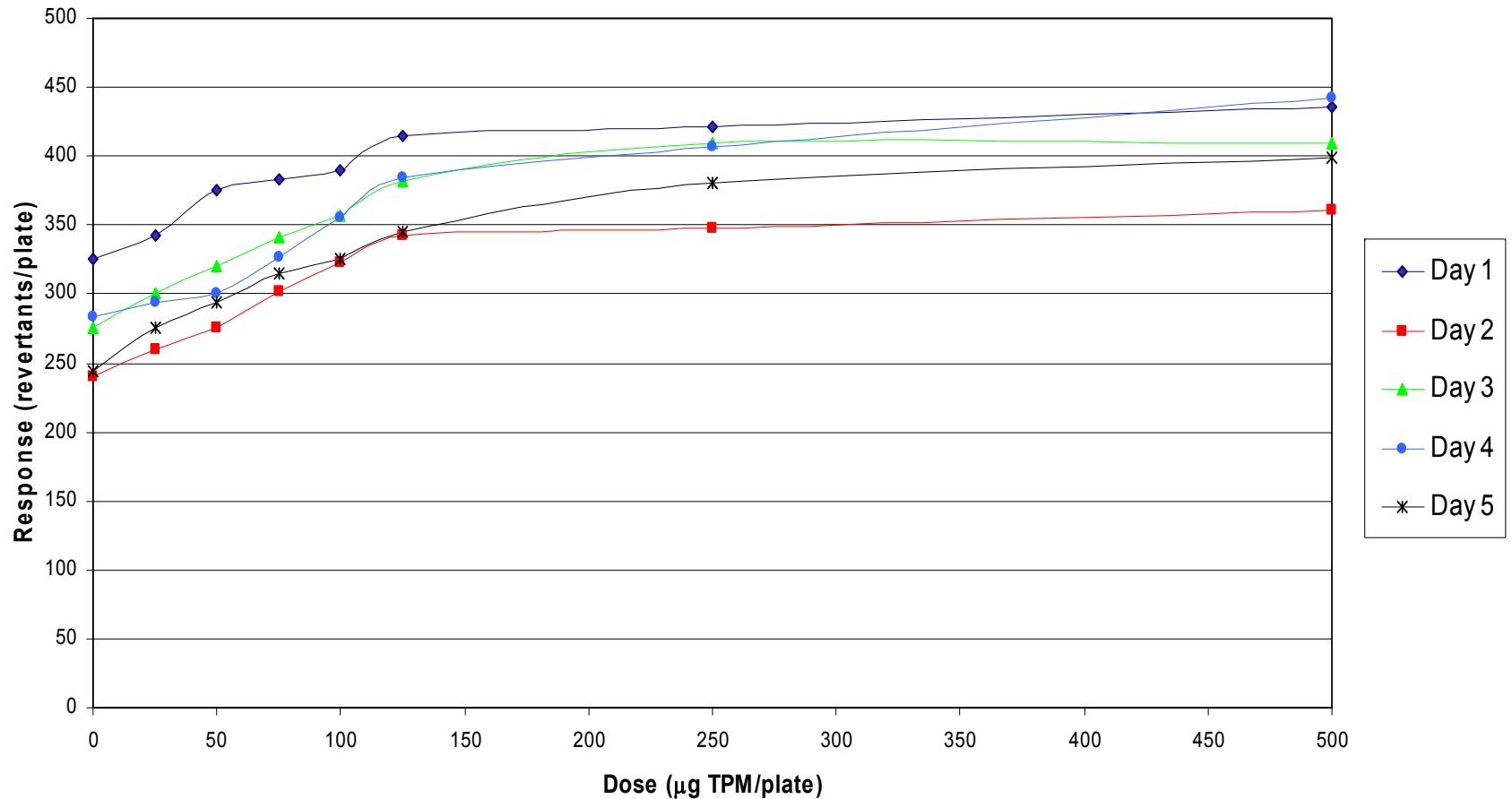
Does MSC show activity with TA102?

- TA102 difficult strain to use in assays
 - Spontaneous revertants high and variable
 - Need to use concurrent zero-dose control plates
 - Dose-response curves show more variability than TA100
 - Linear portion of dose-response curves tends to fall in range of 25 to 100 μg TPM/plate especially for TA102-S9
 - MSC mutagenic potency higher with S9 than without

TA102 and MSC

- Dicarbonyls and hydroquinone present in MSC should show activity with TA102±S9
 - Based on single-compound data, only methyl glyoxal and hydroquinone at levels in KY1R4F MSC to be in responsive range
 - However, no reports of systematic studies with TA102 on MSC including stability of MSC solutions
- By analogy with mutagenicity of coffee aroma, responses observed may be result of several dicarbonyl compounds (Aeschbacher et al., *Food Chem. Toxicol.*, 1989)

KY1R4F MSC and TA102+S9



Comparisons of MSC Mutagenicities (1)

- Types comparisons
 - Comparisons among different products with MSC collected under the same protocol
 - Chepiga *et al.*, *Food Chem. Toxicol.*, 2000
 - Lauterbach *et al.*, *TSRC*, 2001
 - Comparisons among products with MSC collected with different smoking protocols
 - Rickert *et al.*, *CORESTA*, 2002
 - Appleton *et al.*, *TSRC*, 2003
 - Roemer *et al.*, *Toxicology*, 2004

Comparisons of MSC Mutagenicities (2)

- MSCs from most conventional products have similar mutagenicity
- Relatively small differences among mutagenic potencies in a single study can be statistically significant
 - Are the differences truly real and repeatable if the study were performed again?
 - Or, did the differences occur because of the circumstances in a particular study?

Factors affecting study results (1)

- Experimental design
 - Number and timing of replicates for MSC collection and for assays
 - Number and levels of nonzero doses
 - Concurrent versus average zero-doses
- Statistical analyses
 - Method used to determine potency
 - Method used for determination of variance
 - Use of within study variances
 - Use of historical variances

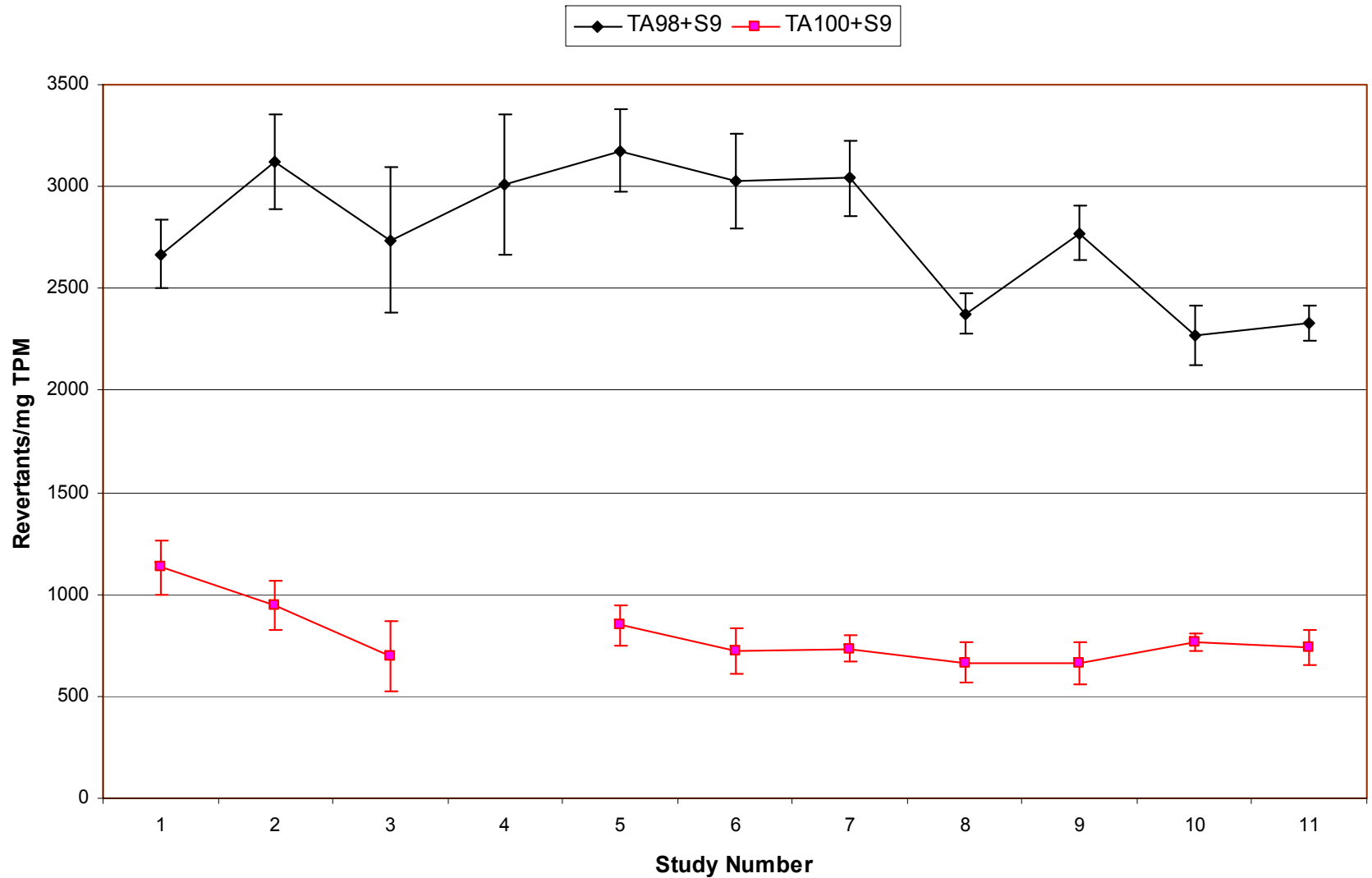
Factors affecting study results (2)

- Relative amounts of water, nicotine, humectants, and other nonmutagenic compounds in each MSC
- Unanticipated changes in the metabolic activation system
- Laboratory error
- Random error

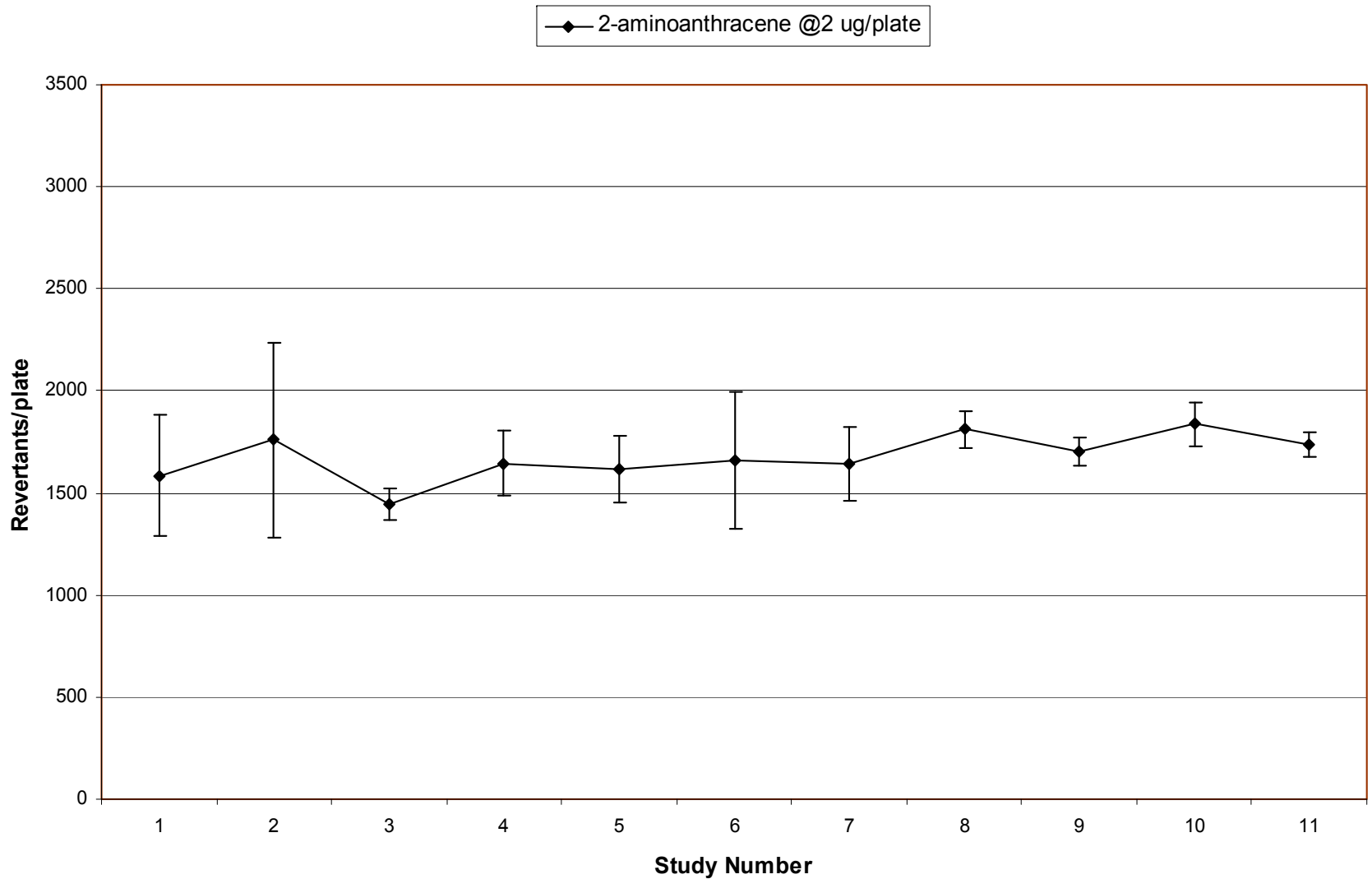
Monitoring Assay Performance

- Tests to ensure strain integrity
- Use of positive controls
 - Single compounds that are mutagenic under the conditions of the assay without S9
 - Single compounds that are mutagenic only in presence of S9
- Use of reference cigarettes such as KY1R4F
 - MSC collected under same conditions as for test articles
 - Assay results should be representative of total system performance

Results for KY1R4F MSC ISO smoking



Results for TA98 positive control



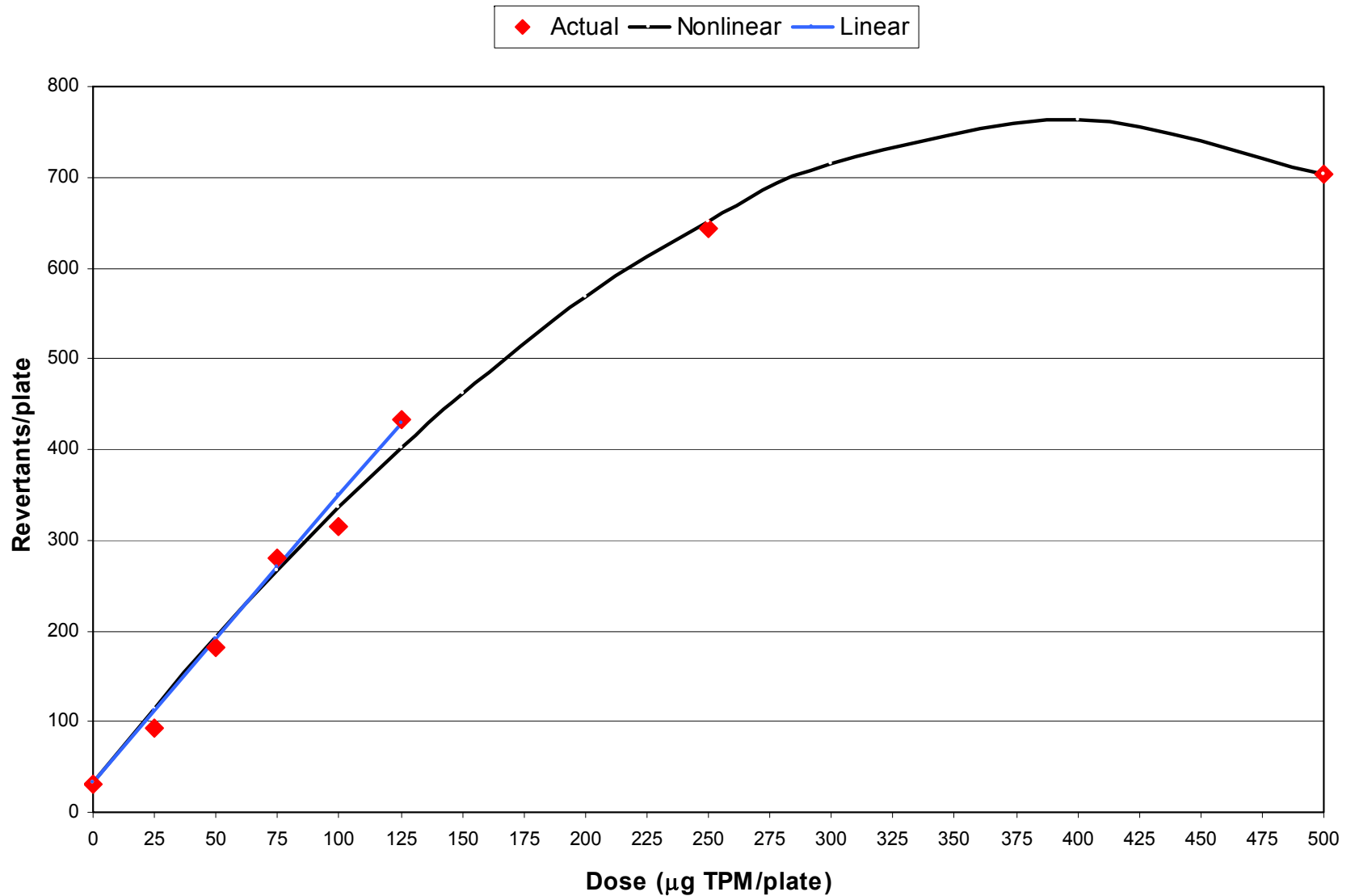
Caveat

- Satisfactory performance on tests for strain integrity and positive control may not be reflective of performance with MSC even for studies that are run in same time frame
- Results may have been reflective of USEPA statement that “2-aminoanthracene should not be used as the sole indicator of the efficacy of the S9-mix” (OECD Guideline 471, 1997)

Nonlinear regression

- Under most conditions, MSC from conventional cigarette products gives nonlinear dose-response curves
- Nonlinearity arises from competing mechanisms of mutagenicity and toxicity
- Most approaches used for MSC have involved statistical methods to determine the slope of the linear part of the dose response curve
 - Assumptions in determining linearity
 - Assumption that toxicity is insignificant

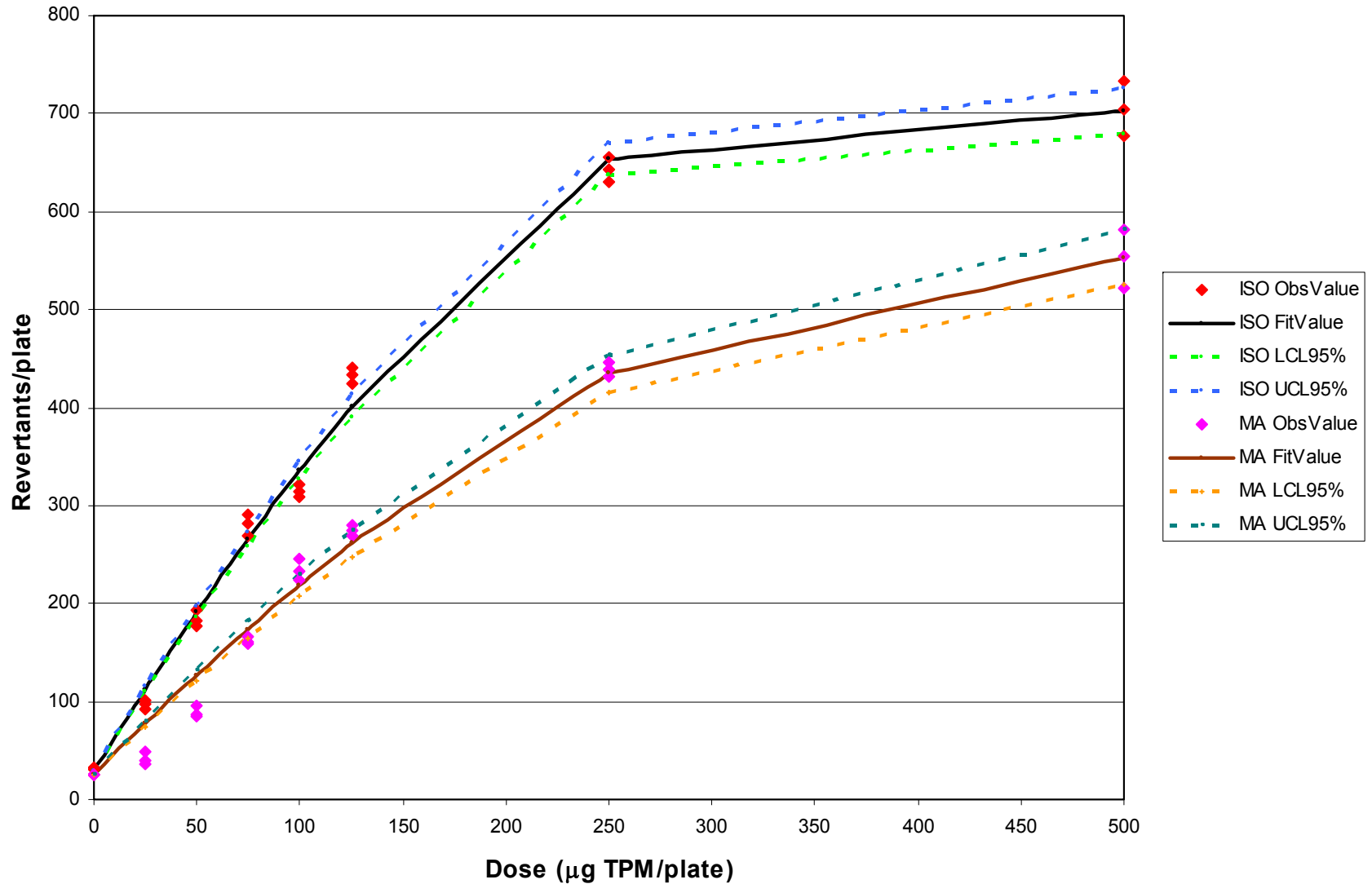
KY1R4F MSC ISO TA98+S9



Equations for nonlinear regression

- $R = (C + \beta * D) * \text{EXP}(\gamma * D)$
- $R = (C + \beta * D) * (2 - \text{EXP}(\gamma * D))$
 - Where
 - D = TPM doses ($\mu\text{g TPM/plate}$)
 - R = Average revertants/plate at dose D
 - C = Average revertants/plate at zero dose
 - β and γ are values estimated by nonlinear regression program (Statgraphics 4.1 with initial estimates from SALM program)
 - β is also an estimate of mutagenicity adjusted for toxicity
 - Equations developed by Kim and Margolin, *Mutat. Res.*, 1999, for SALM program

KY1R4F MSC ISO vs. MA TA98+S9



Concluding remarks (1)

- The Ames assay continues to be one of the most widely used *in vitro* toxicological assays for MSC
- Most all frequently used combinations of Ames strains with/without metabolic activation will show a positive response with MSC
 - For combinations that show weak response, dosing regimen is critical
 - Biological relevance is best understood when combined with other types of assessments

Concluding remarks (2)

- For studies involving MSC from closely related products and/or different smoking conditions, experimental design, monitoring of assay performance, choice of statistical methods are important for success
- The use of nonlinear regression for the determination of mutagenic potency may improve the reliability of the results

Acknowledgements

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