Assessment of Tobacco and Nicotine Products from Several Product Categories in the Ames Assay

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Introduction

- Necessity for *in vitro* assessment of the mutagenic potential of tobacco and nicotine consumer products
  - Ames has been a “gold standard” assay for the pharmaceutical and tobacco industries
  - Ames data has been an integral part of PMTA submissions
- Challenges of tobacco testing in the Ames Assay
  - Variety of tobacco and nicotine product types
  - Sample preparation methodologies
  - How to compare results between product categories
  - Wide range of responses and levels of toxicity

The data in this presentation were obtained over a series of independent studies, following similar study designs and protocols

Test Items and Sample Preparation

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Test Items

1. Combustible Cigarette (CC)
2. Heated Tobacco Product (HTP)
3. Electronic Nicotine Delivery System (ENDS)
4. Smokeless Tobacco Product (SNUS)
5. Modern Oral Pouched Nicotine Product (MO)
6. Nicotine Replacement Therapy Product (NRT)

Test Items

Samples prepared from tobacco products for testing in the Ames assay

- Aerosol generating tobacco products
  - CC & HTP: pad-collected Total Particulate Matter (TPM)
  - ENDS: pad-collected Aerosol Collected Material (ACM)
  - CC, HTP & ENDS: solvent-captured Gas Vapor Phase (GVP)
- Oral products
  - Complete Artificial Saliva (CAS) extracts

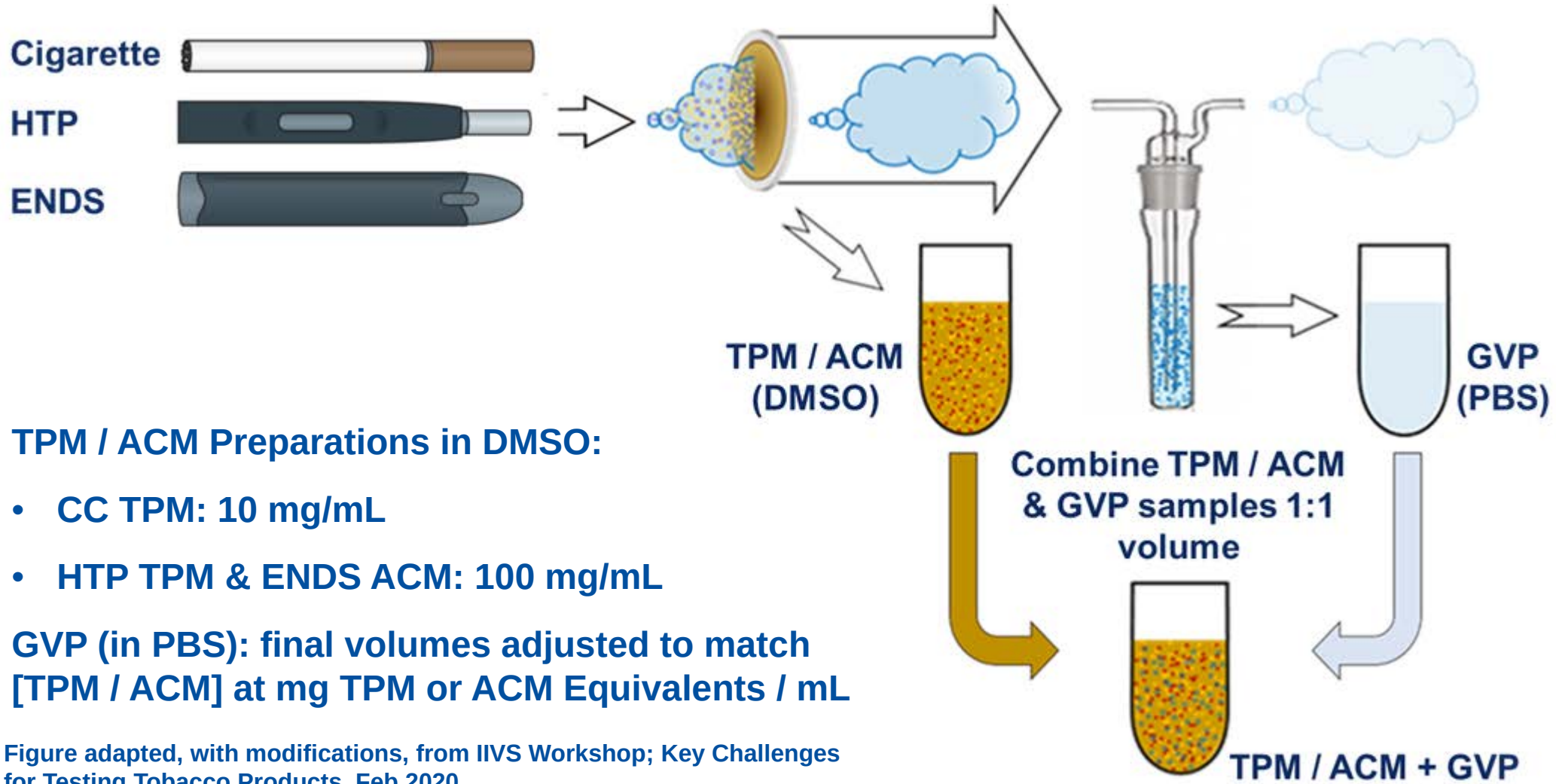
Nicotine levels in the prepared samples were determined allowing normalization across different tobacco product categories and different studies

Aerosol Sample Collection Methodology

Aerosol Generating Products (CC, HTP, ENDS)

- Puffing parameters:
  - CC: 55 mL puff, 2 second puff, 30 second puff interval; 100% vent blocking (ISO 20778, 2018)
  - HTP: ISO 20778 (2018), no vent blocking
  - ENDS: 80 mL puff, 4 sec puff, 15 second puff interval
- GVP collected concurrently with TPM / ACM
- TPM / ACM and GVP preparations either tested separately (ENDS) or combined (CC, HTP) 1:1 v:v (HC T-502, 2017)
  - GVP and TPM + GVP samples tested within 1 hour of final preparation

Aerosol Sample Collection Methodology



TPM / ACM Preparations in DMSO:

- **CC TPM: 10 mg/mL**
- **HTP TPM & ENDS ACM: 100 mg/mL**

GVP (in PBS): final volumes adjusted to match [TPM / ACM] at mg TPM or ACM Equivalents / mL

Figure adapted, with modifications, from IIVS Workshop; Key Challenges for Testing Tobacco Products, Feb 2020.

CAS Extraction Methodology

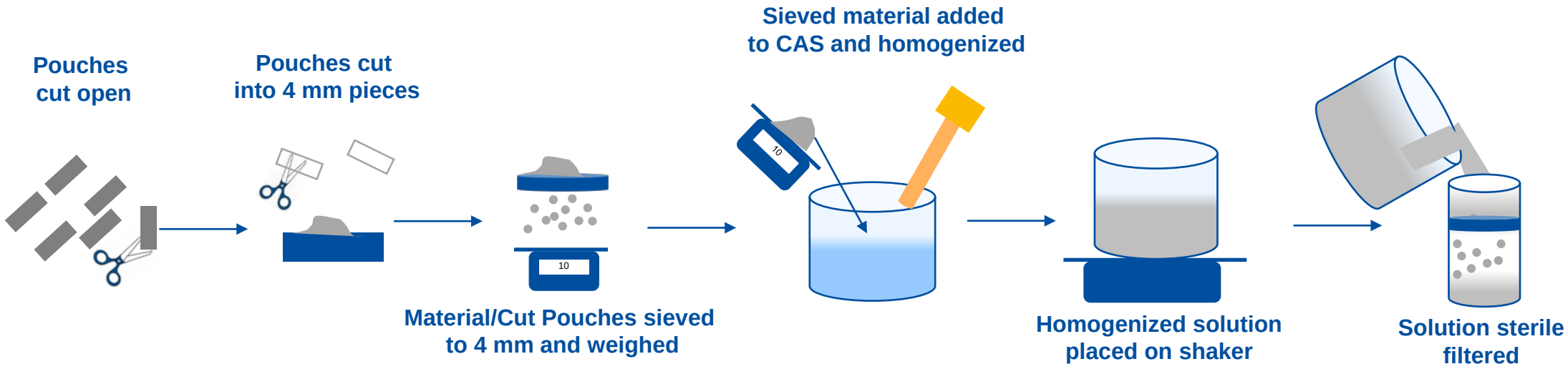
SNUS, MO, and NRT

- Complete Artificial Saliva with enzymes
  - Alpha-amylase (100,000 units / L)
  - Lysozyme (750 units / L)
  - Acid-phosphatase (4 units / L)
- Extract concentrations
  - SNUS & MO: 300 mg / mL
  - NRT: 200 mg / mL

Chou, C. C., and Hee, S. S. (1994) Bioassay-driven analysis of chewing tobacco extracts. *Environ. Toxicol. Chem*. 13, 1177-86.

CAS Extraction Methodology

Extracts of pouched test articles prepared in complete artificial saliva (CAS)



Keyser, B. M. (2022) Cytotoxicity, oxidative stress, and inflammatory response of smokeless tobacco extracts and cytotoxicity of combustible cigarette whole smoke in a 3D oral organotypic buccal cell model, *Toxicology Mechanisms and Methods*, 32:5, 352-361

Miller-Holt, J. et. al. (2022) *In vitro* evaluation of mutagenic, cytotoxic, genotoxic and oral irritation potential of nicotine pouch products. *Tox Reports* 9, 1316 – 1324.

Ames Assay

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Ames Assay

- Preincubation (OECD 471, HC T-501)
- *Salmonella* tester strains (±S9):
  - TA98, TA100, TA102, TA1535 & TA1537
  - Aroclor 1254-induced Sprague Dawley Rat liver S9
- Individual strains mixed with test item, 5% S9-mix (+S9) or PBS (-S9) and incubated 20 ± 2 minutes at 37 ± 1°C
  - Highest tested concentrations of either TPM, ACM, GVP or CAS extract per plate tested: 1 mg (CC), 5 mg (ENDS), 10 mg (HTP, NRT), 15 mg (MO, SNUS)
- Molten top agar added to exposure mix and poured on minimal agar plates and incubated 48 – 72 hours at 37 ± 1°C

Ames Assay

Assay acceptance criteria

- Confirmation of tester strain genotypic characteristics
- Average spontaneous solvent control revertant counts fall within the expected historical range
- Average positive control response must be >3X's the spontaneous revertant count

Criteria for determining a positive mutagenic response

- A concentration-related increase in revertant numbers is observed over the tested dose range
- A statistically significant increase (Dunnett's Test, $p < 0.01$) in the mean revertant number is observed for at least one test concentration over solvent control
- Revertant counts above the historical solvent control range (i.e., Poisson 95% CI)
- Test item is considered mutagenic if ALL the above criteria are met

Results

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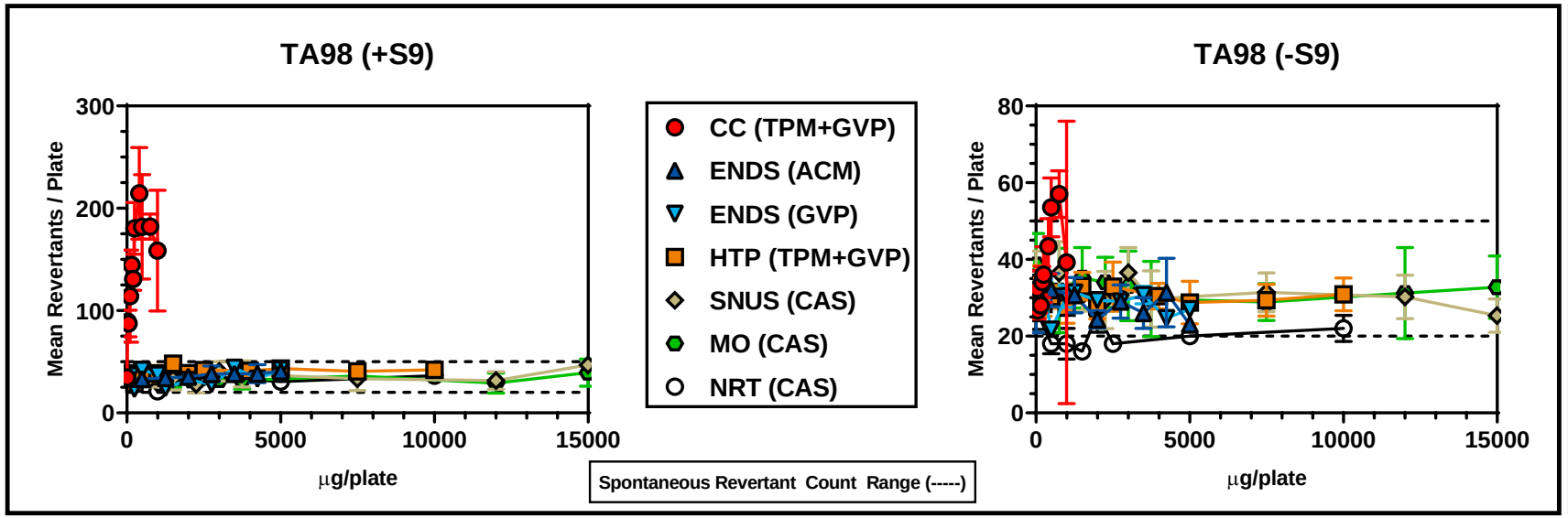
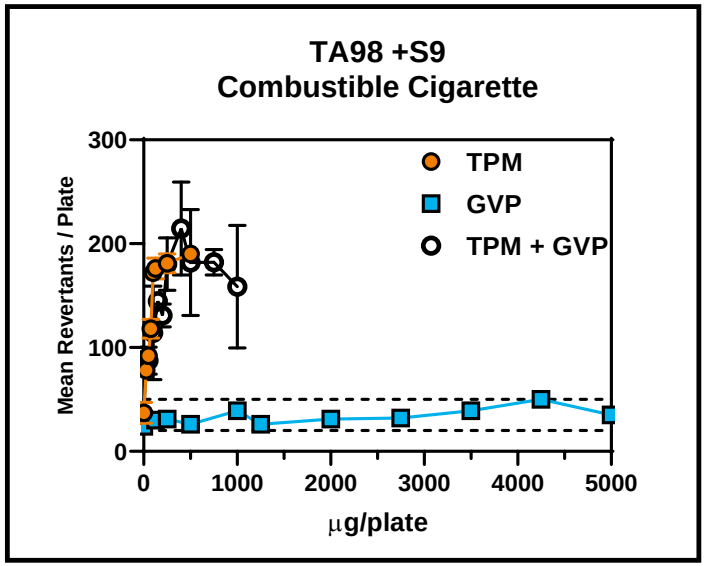
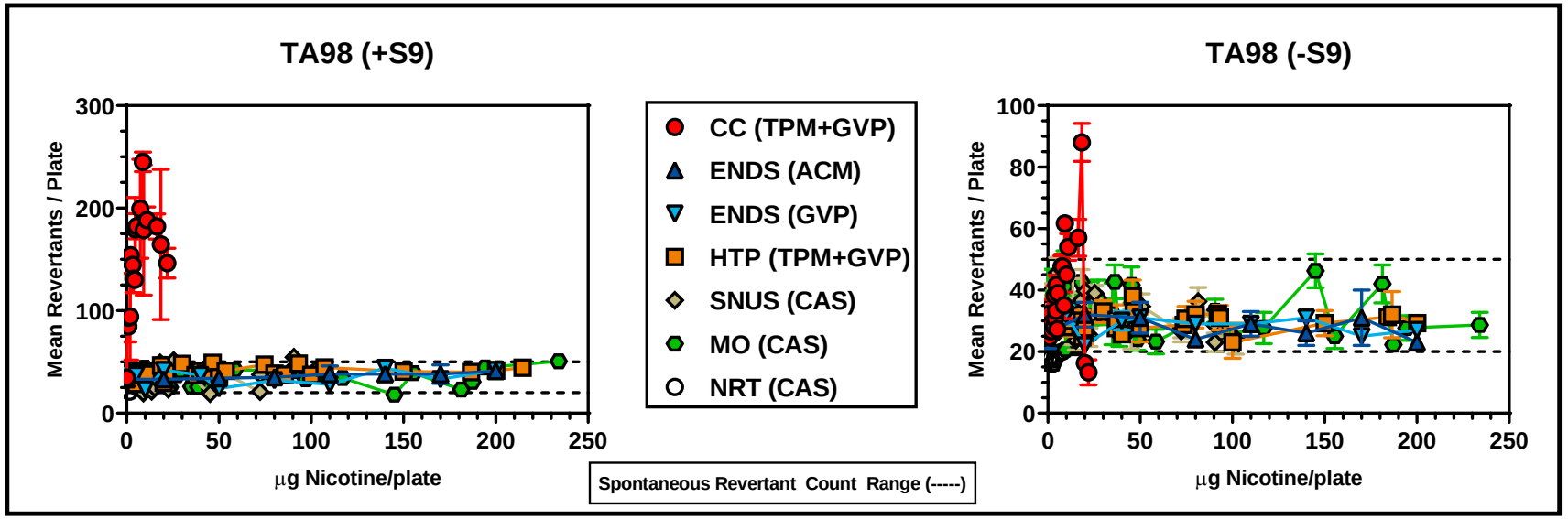
Ames Results

- Results presented from *Salmonella* tester strains TA98, TA100 and TA1537
- All test items were negative in *Salmonella* tester strains TA102 (±S9) and TA1535 (±S9); data not shown
- Toxicity: thinning of background lawn or decrease in revertant counts
  - CC (TPM + GVP): observed in top 3 doses
  - HTP (TPM + GVP) and ENDS (ACM & GVP): observed in top 2 doses
  - SNUS, MO, and NRT (CAS): observed in the top 2 doses
- Data displayed using either the mass of sample (µg / plate) or the level of nicotine (µg nicotine / plate)

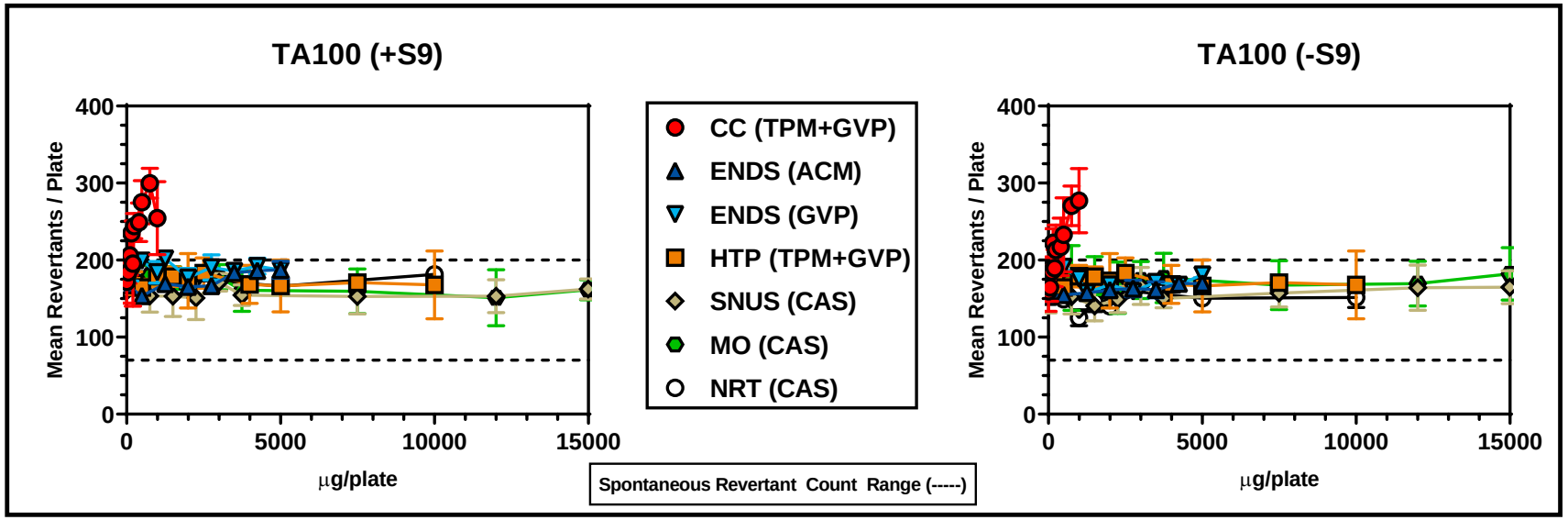
Ames Results: TA98

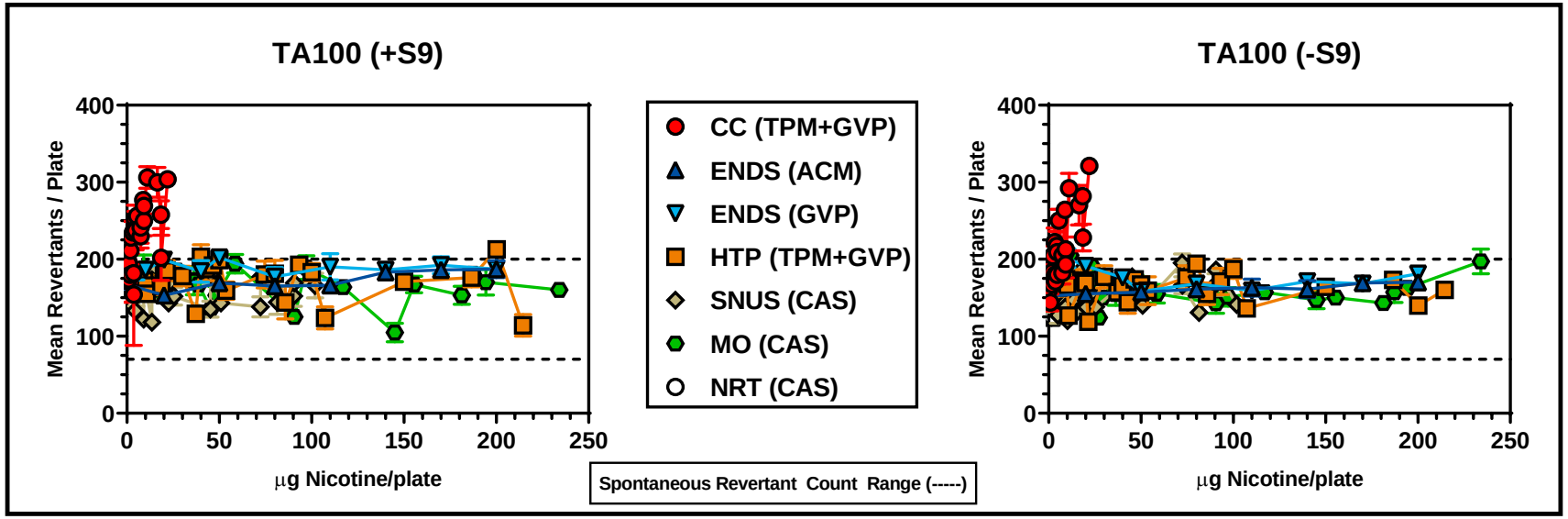




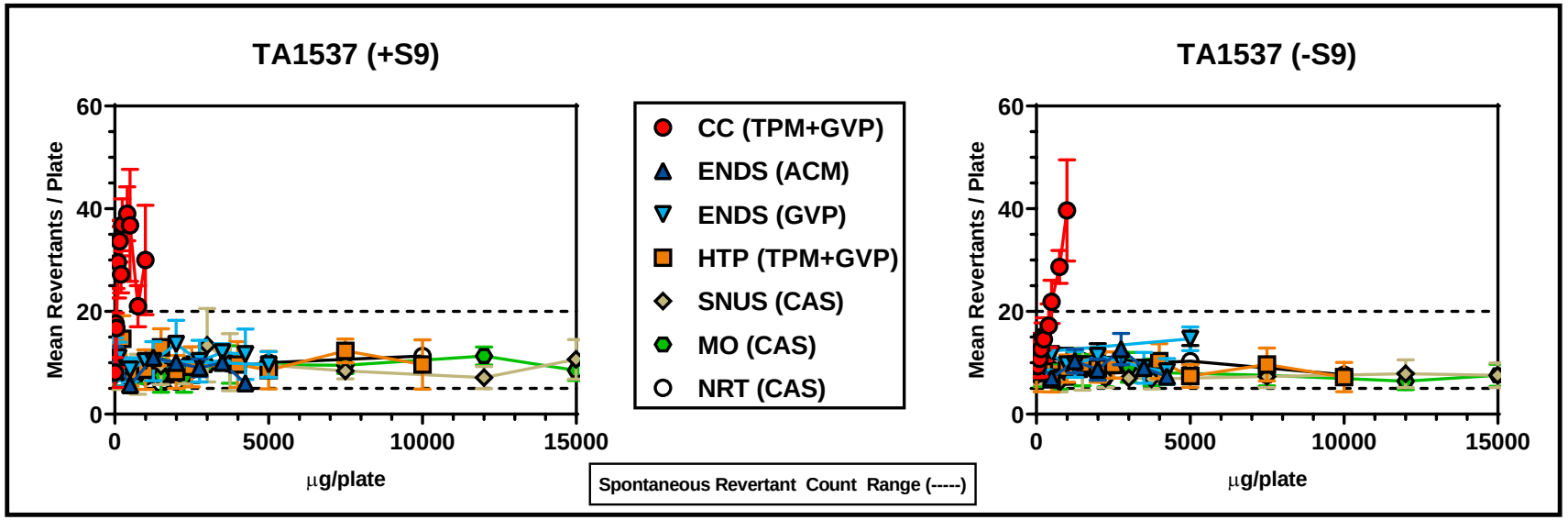


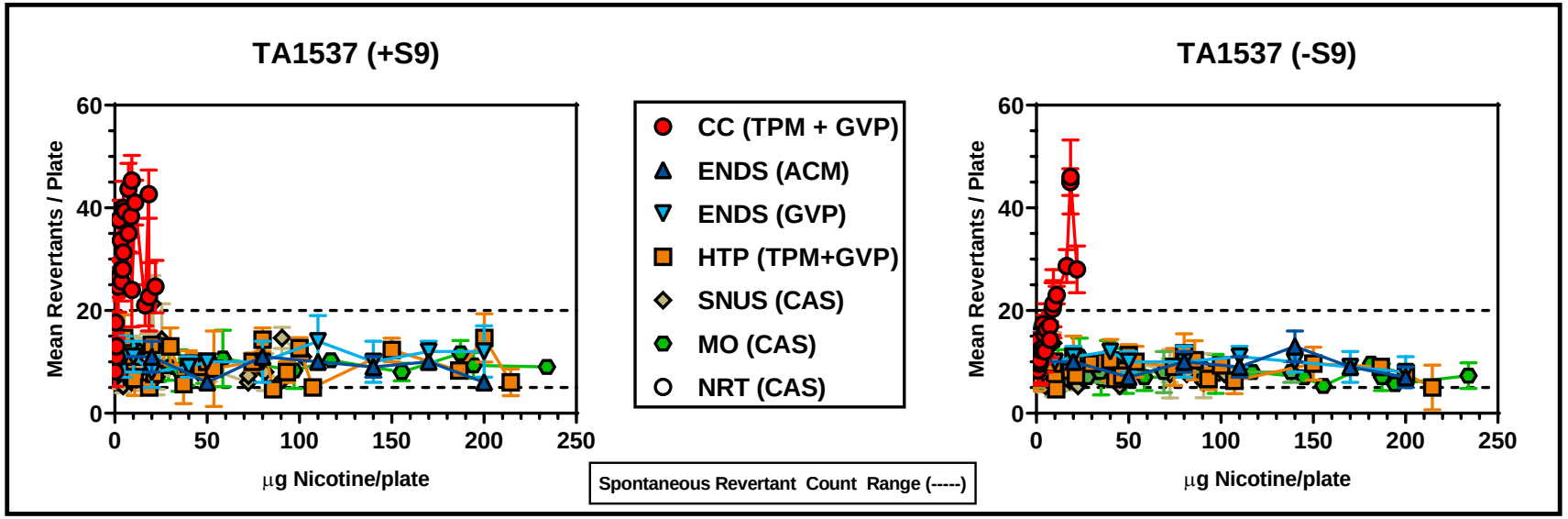
Ames Results: TA100





Ames Results: TA1537





Conclusions and Summary

- Observed indications of toxicity at higher doses suggest the tested dose ranges for all test items were appropriate
- HTP (TPM + GVP): negative in all strains (±S9) when tested up to 10 mg (~200 µg nicotine)
- ENDS (ACM and GVP): negative in all strains (±S9) when tested up to 5 mg (~200 µg nicotine)
- SNUS (CAS): negative in all strains (±S9) when tested up to 15 mg (~100 µg nicotine)
- MO (CAS): negative in all strains (±S9) when tested up to 15 mg (~240 µg nicotine)
- NRT (CAS): negative in all strains (±S9) when tested up to 10 mg (~16 µg nicotine)
- CC (TPM + GVP): tested up to 1 mg (~20 µg nicotine)
  - mutagenic in strains TA98 (±S9), TA100 (±S9) and TA1537 (±S9)
  - non-mutagenic in strains TA102 (±S9) and TA1535 (±S9)
- CC induced mutagenicity at nicotine doses approximately 5-12 X's lower than the maximum non-mutagenic doses of HTP, ENDS, SNUS and MO

Recommendations

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Recommendations

- Standard sample preparation methods
  - Which methods are appropriate for tobacco product types?
  - Sample characterization and stability
  - Comparison within and across studies
- Dosimetry measures across tobacco product categories
  - Comparison on a per mass basis is feasible, but dose ranges vary considerably with the different tobacco product types
  - Normalizing to nicotine allows comparison across studies and product types

Questions?

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