Toxicological Assessment of Oral Tobacco and Nicotine Products Relative to Combustibles Using ToxTracker® Assay

Utkarsh Doshi, Principal Scientist 2, ALCS, Richmond, VA

IIVS Workshop 11, November 12, 2025

Outline

Introduction

Oral tobacco and nicotine-containing products are generally considered to present lower risk compared to combustible products due to their composition, lower HPHCs and absence of combustion.

NEVERTHELESS, evaluating the risk of oral products containing GRAS (generally recognized as safe) ingredients that elicit genotoxic responses in regulatory in vitro assays, yet show no corresponding in vivo effects, **remains a considerable challenge**

The novel mechanistic genotoxicity assay, ToxTracker®—which has recently been accepted into the OECD test guidelines programme—**demonstrates high sensitivity and specificity in detecting in vivo genotoxicants** and can distinguish between direct and indirect toxicity mechanisms.

The study evaluates whether oral tobacco and nicotine containing products exhibit lower toxicity or indirect genotoxic mechanisms compared to combustibles, when assessed **using the ToxTracker® assay**

GRAS=Generally recognized as safe, OECD= Organisation for Economic Co-operation and Development

ToxTracker® Assay

ToxTracker® is a mammalian stem cell-based reporter assay that predicts genotoxic properties of compounds

The assay uses a panel of six mouse embryonic stem cell lines that contain different reporters (green fluorescent protein signals) corresponding to four distinct biological responses (DNA damage, oxidative stress, protein damage, or general cellular stress)

Specificity of the ToxTracker® Reporters	
Biomarker Reporter Gene	**Biological Response**
Bscl2	DNA damage
Rtkn	
Srxn1	Oxidative stress
Blvrb	
Ddit3	Protein damage
Btg2	Cellular stress

By evaluating the induction of various reporter genes that play a pivotal role in relevant cellular pathways, **the assay provides insights into chemical mode of action, thereby allowing differentiation between direct-acting genotoxicants and cytotoxic chemicals, which is not possible with traditional genotoxicity assays**

Materials & Methods

Test Articles

Combustible
Reference (3R4F, 1R6F)

Snus
Reference (CRP 1.1) &
4 Commercial Products:
Original | Mint | White
Wintergreen

Moist Smokeless Tobacco (MST)
Reference (CRP 2.1) &
1 Commercial Product:
Wintergreen

Nicotine Pouches (NP) (2-7 mg nicotine)
4 Commercial Brand A:
Cinnamon | Peppermint
Wintergreen | Mango
6 Commercial Brand B:
Cinnamon | Coffee | Peppermint
Wintergreen | Citrus | Dragon fruit
2 Commercial Brand C:
Cinnamon | Coffee

Test Materials

Condensates (combustibles)
- 20 cigarettes per collection (ISO 20778)
- TPM collected on 92 mm pad, GVP in impinger with ethanol at 0˚C
- Pad extracted in ethanol (impinger solution) to prepare whole smoke condensate

Artificial Saliva Extracts (oral products)
- 5 gm in 50 mL (10% w/v)
- 2 hrs. at 37˚C at 250 rpm
- Centrifuged for 10 min
- Filtered twice via 0.2 µm filter

ToxTracker® Assay

Dose Range Finding
- 20 concentrations
  - 1% v/v (ethanol) or
  - 20% v/v (artificial saliva) as the top dose)
- 24 hrs. treatment ± S9

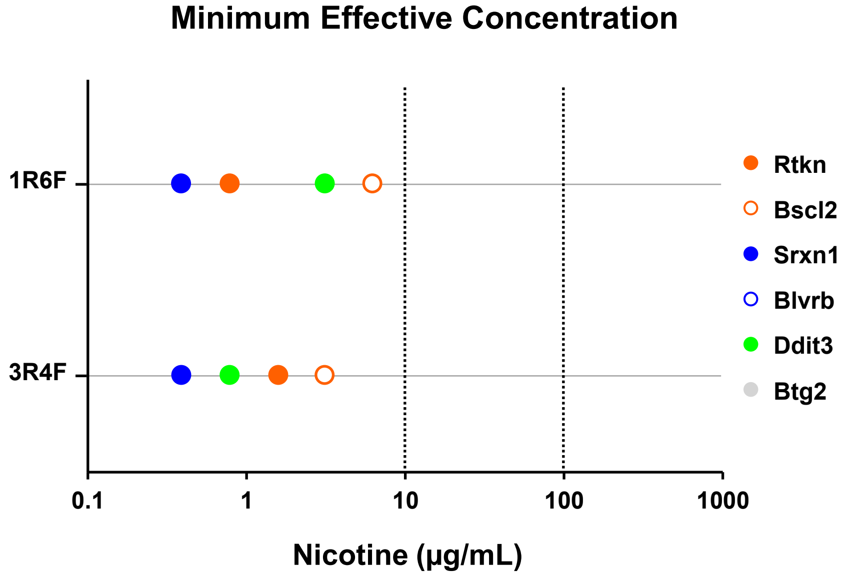
Definitive Study
- 5 concentrations based on range finding study
- 24 hrs. treatment ± S9

Aneugenic Clastogenic Evaluation
- Cell-cycle analysis
  - 4 hrs. & 24 hrs. ± S9
- Aneuploidy
  - 24 hrs. ± S9

Results: Combustibles

Category	Test Article	Metabolism	DNA Damage: Bscl2	DNA Damage: Rtkn	Protein Misfolding: Ddit3	Cell Stress: Btg2	Oxidative Stress: Srxn1	Oxidative Stress: Blvrb	Aneugenic Response
Combustible	3R4F	-S9	Positive	Positive	Positive	Positive	Positive	Positive	Negative
		+S9	Positive	Positive	Positive	Positive	Positive	Positive	Negative
	1R6F	-S9	Equivocal/Weak Positive	Positive	Positive	Positive	Positive	Positive	Negative
		+S9	Equivocal/Weak Positive	Positive	Positive	Positive	Positive	Positive	Negative

Positive ≥2-fold · Equivocal/Weak Positive 1.5–<2-fold · Negative <1.5-fold



Both combustible cigarettes showed activation of all 6 biomarkers, including both DNA damage markers (Rtkn & Bscl2)

No aneuploidy was observed at concentrations tested

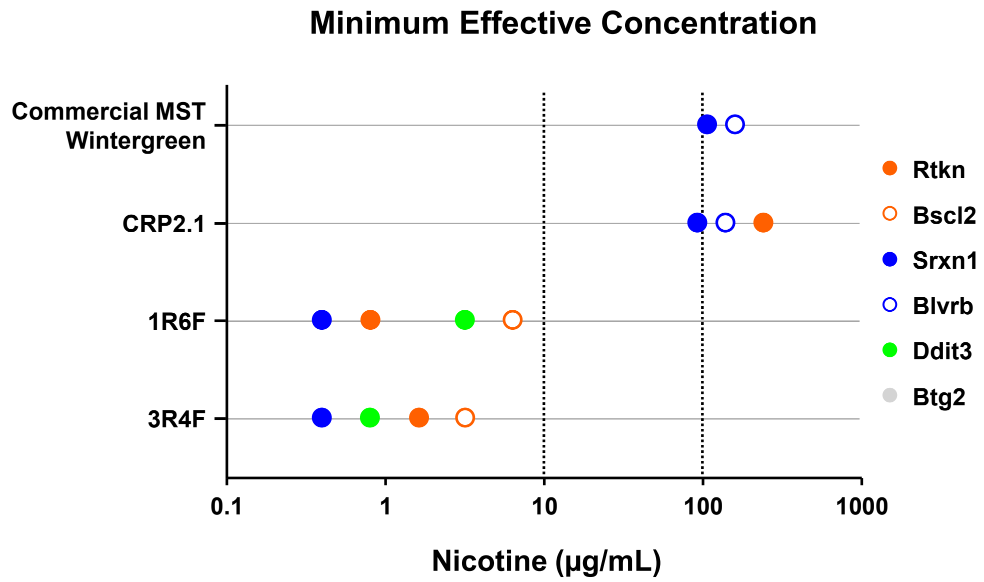
The minimum effective concentration (MEC) driving reporter activation in ToxTracker, when normalized to nicotine in the extract, ranged from 0.39–6.25 µg/mL

MEC is the lowest nicotine concentration in the extract that shows ≥ 1.5-fold induction in any ToxTracker biomarker

Results: Oral Products: Moist Smokeless

Category	Test Article	Metabolism	DNA Damage: Bscl2	DNA Damage: Rtkn	Protein Misfolding: Ddit3	Cell Stress: Btg2	Oxidative Stress: Srxn1	Oxidative Stress: Blvrb	Aneugenic Response
MST	CRP 2.1	-S9							
		+S9							
	Commercial MST Wintergreen	-S9							
		+S9							

Positive ≥2-fold   Equivocal/Weak Positive 1.5–<2-fold   Negative <1.5-fold



Both reference and commercial MST induced oxidative stress biomarkers (Srxn1 and Blvrb)

Reference MST showed weak activation of only one of the DNA damage biomarkers (Rtkn), in presence of S9

The MEC driving reporter activation ranged from 91–236 µg/mL (≥100-fold higher concentrations than combustibles)

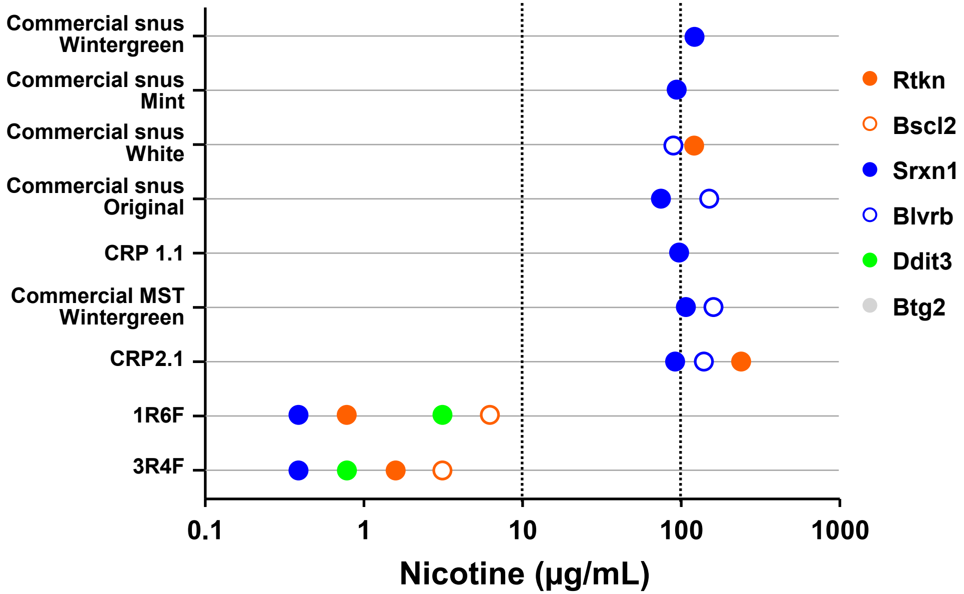
MST=Moist Smokeless Tobacco
MEC is the lowest nicotine concentration in the extract that shows ≥ 1.5-fold induction in any ToxTracker biomarker

Results: Oral Products: Snus

Category	Test Article	Metabolism	DNA Damage: Bscl2	DNA Damage: Rtkn	Protein Misfolding: Ddit3	Cell Stress: Btg2	Oxidative Stress: Srxn1	Oxidative Stress: Blvrb	Aneugenic Response
Snus	CRP 1.1	-S9	Negative	Negative	Negative	Negative	Positive	Equivocal	Negative
		+S9	Negative	Negative	Negative	Negative	Equivocal	Equivocal	Negative
	Commercial-Original	-S9	Negative	Negative	Negative	Negative	Equivocal	Equivocal	Negative
		+S9	Negative	Negative	Negative	Negative	Equivocal	Equivocal	Negative
	Commercial-White	-S9	Negative	Negative	Negative	Negative	Equivocal	Negative	Negative
		+S9	Negative	Equivocal	Negative	Negative	Equivocal	Equivocal	Negative
	Commercial-Mint	-S9	Negative	Negative	Negative	Negative	Positive	Equivocal	Negative
		+S9	Negative	Negative	Negative	Negative	Equivocal	Equivocal	Negative
	Commercial-Wintergreen	-S9	Negative	Negative	Negative	Negative	Negative	Negative	Negative
		+S9	Negative	Negative	Negative	Negative	Equivocal	Equivocal	Negative

Positive ≥2-fold Equivocal/Weak Positive 1.5–<2-fold Negative <1.5-fold

Minimum Effective Concentration



Most snus products showed weak activation of only the oxidative stress markers, under the study conditions

None of the DNA damage markers were activated in response to snus products

The MEC ranged from 74–148 µg/mL (≥100-fold higher concentrations than combustibles)

MST=Moist Smokeless Tobacco
MEC is the lowest nicotine concentration in the extract that shows ≥1.5-fold induction in any ToxTracker biomarker

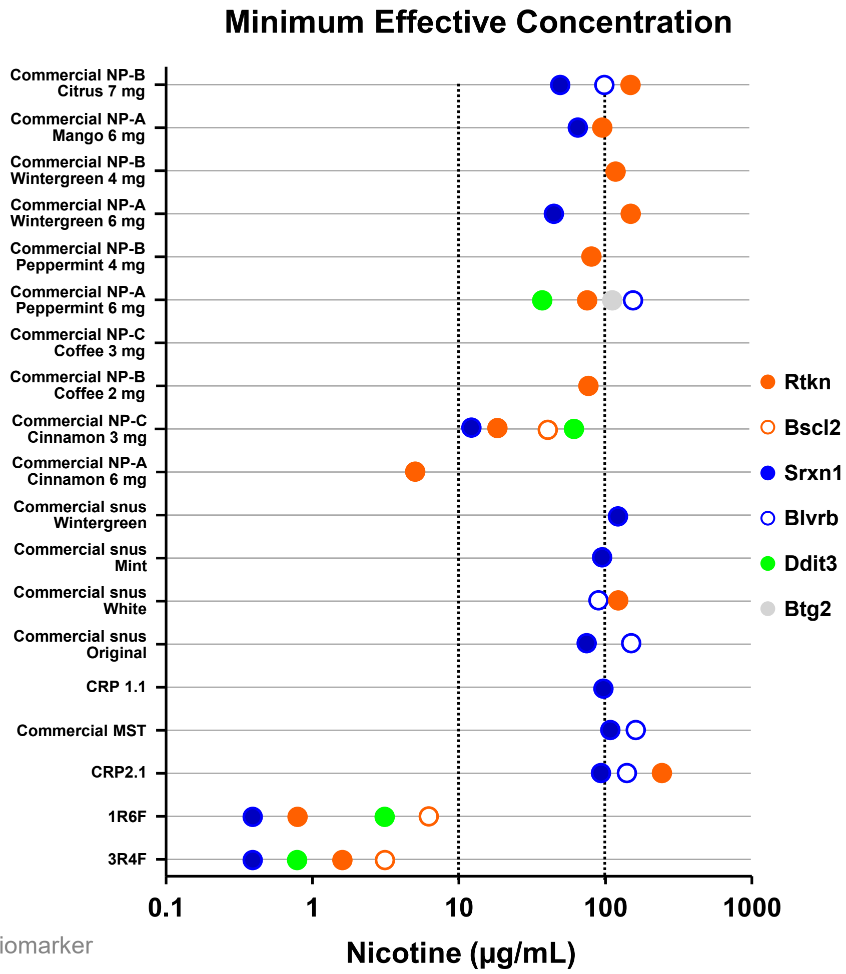
Results: Oral Products: Nicotine Pouches

Category	Test Article	Metabolism	DNA Damage: Bscl2	DNA Damage: Rtkn	Protein Misfolding: Ddit3	Cell Stress: Btg2	Oxidative Stress: Srxn1	Oxidative Stress: Blvrb	Aneugenic Response
Cinnamon Flavored NP	Brand A, 6 mg	-S9	Negative	Equivocal	Equivocal	Positive	Positive	Positive	Negative
		+S9	Negative	Positive	Negative	Positive	Positive	Positive	Negative
	Brand B, 7 mg	-S9	Negative	Negative	Negative	Negative	Negative	Negative	Negative
		+S9	Negative	Negative	Negative	Negative	Negative	Negative	Negative
	Brand C, 3 mg	-S9	Equivocal	Positive	Positive	Positive	Positive	Positive	Negative
		+S9	Negative	Positive	Negative	Positive	Positive	Positive	Negative
Coffee Flavored NP	Brand B, 2 mg	-S9	Negative	Negative	Negative	Negative	Negative	Negative	Negative
		+S9	Negative	Equivocal	Negative	Negative	Negative	Negative	Negative
	Brand C, 3 mg	-S9	Negative	Negative	Negative	Negative	Negative	Negative	Negative
		+S9	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Peppermint Flavored NP	Brand A, 6 mg	-S9	Negative	Negative	Equivocal	Equivocal	Positive	Negative	Negative
		+S9	Negative	Equivocal	Negative	Equivocal	Positive	Equivocal	Negative
	Brand B, 4 mg	-S9	Negative	Negative	Negative	Negative	Negative	Negative	Negative
		+S9	Negative	Positive	Negative	Negative	Negative	Negative	Negative
Wintergreen Flavored NP	Brand A, 6 mg	-S9	Negative	Negative	Negative	Equivocal	Positive	Negative	Negative
		+S9	Negative	Equivocal	Negative	Equivocal	Positive	Negative	Negative
	Brand B, 4 mg	-S9	Negative	Negative	Negative	Negative	Negative	Negative	Negative
		+S9	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Fruit Flavored NP	Brand A Mango, 6 mg	-S9	Negative	Negative	Negative	Equivocal	Positive	Negative	Negative
		+S9	Negative	Negative	Negative	Negative	Positive	Negative	Negative
	Brand B Citrus, 7 mg	-S9	Negative	Negative	Equivocal	Negative	Positive	Positive	Negative
		+S9	Negative	Negative	Equivocal	Negative	Positive	Positive	Negative
	Brand B Dragon fruit, 7 mg	-S9	Negative	Negative	Negative	Negative	Negative	Negative	Negative
		+S9	Negative	Negative	Negative	Negative	Negative	Negative	Negative

MST=Moist Smokeless Tobacco; NP=Nicotine Pouch

MEC is the lowest nicotine concentration in the extract that shows ≥ 1.5-fold induction in any ToxTracker biomarker

Positive ≥2-fold | Equivocal/Weak Positive 1.5–<2-fold | Negative <1.5-fold



Responses to market NP varied by the flavor type, with cinnamon flavored products being the most bioactive

- None of the NP products activated Bscl2 reporter or showed aneugenicity
  - The MEC ranged from 43–148 µg/mL (excluding cinnamon flavored products)
- For some NPs, only Rtkn was activated suggesting clastogenic or oxidative stress mediated effects
  - The effect of oxidative stress mediated effects can be evaluated using antioxidants (ToxTracker® AO assay) (CORESTA, 2025)

Conclusions

ToxTracker® revealed distinct differences in direct and indirect genotoxicity responses between combustible tobacco and oral tobacco/nicotine products

In contrast to combustible cigarettes, the tested oral tobacco/nicotine products did not exhibit direct DNA damage

---

Within oral nicotine pouch products, responses varied according to flavor category

For instance, certain cinnamon-flavored products (though not all) triggered activation of the indirect DNA damage marker Rtkn, accompanied by pronounced oxidative stress responses

Additional assays, such as ToxTracker® AO, can be employed to validate that indirect mechanisms (toxicity and/or oxidative stress) are the primary drivers of these responses (CORESTA, 2025)

Apart from cinnamon-flavored nicotine pouch products, most oral product-induced biomarker activations occurred at substantially higher concentrations (≥100-fold) compared to those seen with cigarette smoke

Overall, the ToxTracker® findings support a reduced toxicity and hazard potential for oral tobacco products, including nicotine pouches, relative to combustible tobacco products

References

- Brandsma, I., Moelijker, N., Derr, R., & Hendriks, G. (2020). Aneugen Versus Clastogen Evaluation and Oxidative Stress-Related Mode-of-Action Assessment of Genotoxic Compounds Using the ToxTracker Reporter Assay. *Toxicological Sciences*, 177(1), 202-213. doi:10.1093/toxsci/kfaa103
- Czekala, L., Chapman, F., Simms, L., Rudd, K., Trelles Sticken, E., Wieczorek, R., . . . Walele, T. (2021). The in vitro ToxTracker and Aneugen Clastogen Evaluation extension assay as a tool in the assessment of relative genotoxic potential of e-liquids and their aerosols. *Mutagenesis*. doi:10.1093/mutage/geaa033
- Doshi, U., Farcas, M., Biernacka, P., Gardner, W., Brandsma, I., Roberts, D., & Derr, R. Evaluation of genotoxicity of cinnamon flavored nicotine products using in vitro new approach methodologies. CORESTA Meeting, Smoke Science/Product Technology, Annecy, France 2025, STPOST52.
- Fisher, M., Tan-Torres, S., Gaworski, C., Black, R., Sarkar, M. Smokeless tobacco mortality risks: an analysis of two contemporary nationally representative longitudinal mortality studies. *Harm Reduct J*. 2019 Apr 11;16(1):27. doi: 10.1186/s12954-019-0294-6.
- Hashizume, T., Ishikawa, S., Matsumura, K., Ito, S., & Fukushima, T. (2023). Chemical and in vitro toxicological comparison of emissions from a heated tobacco product and the 1R6F reference cigarette. doi:10.1016/j.toxrep.2023.02.005
- Hendriks, G., Adriaens, E., Allemang, A., Clements, J., Cole, G., Derr, R., . . . Kirkland, D. (2024). Interlaboratory validation of the ToxTracker assay: An in vitro reporter assay for mechanistic genotoxicity assessment. *Environmental and molecular mutagenesis*, 65(1-2), 4-24. doi:10.1002/em.22592
- Kirkland, D., Aardema, M., Henderson, L., & Müller, L. (2005). Evaluation of the ability of a battery of three in vitro genotoxicity tests to discriminate rodent carcinogens and non-carcinogens I. Sensitivity, specificity and relative predictivity. *Mutation research*, 584(1-2), 1-256. doi:10.1016/j.mrgentox.2005.02.004
- Smart, D., Bozhilova, S., Miazzi, F., Haswell, L., Gaca, M., Thorne, D., & Breheny, D. (2022). Application of ToxTracker for the toxicological assessment of tobacco and nicotine delivery products. *Toxicology Letters*, 358, 59-68. doi:10.1016/j.toxlet.2022.01.005
- Yu, F., Bishop, E., Miazzi, F., Evans, R., Smart, D., Breheny, D., & Thorne, D. (2024). Multi-endpoint in vitro toxicological assessment of snus and tobacco-free nicotine pouch extracts. *Mutation Research, Genetic Toxicology and Environmental Mutagenesis*, 895, 503738. doi:10.1016/j.mrgentox.2024.503738

THANK YOU!