CORESTA IN VITRO TOXICOLOGY TASK FORCE

Report on Interlaboratory Study of the *In Vitro* Toxicity of Particulate Matter from Four Cigarettes

(Ames, Neutral Red Cytotoxicity and Micronucleus Assays)

Study Design

The CORESTA *In Vitro* Toxicology Task Force planned an interlaboratory study using a common set of cigarettes. British American Tobacco GR&D Southampton agreed to provide three cigarettes; 2R4F, a Kentucky Reference cigarette, was obtained by each individual laboratory. The test cigarettes provided by British American Tobacco GR&D Southampton are described in Table 1:

Table 1. Description of Test Cigarettes

R309	100% single grade U.S. Burley (USB), cellulose acetate filter without ventilation
R310	100% single grade Brazilian flue-cured, cellulose acetate filter without ventilation
R311	1:1 mixture of Brazilian flue and American burley, cellulose acetate filter without ventilation

Laboratories conducted assays and submitted the data using provided templates. Each laboratory was assigned a code number. (Each laboratory was informed of their code number only).

Conditioning of Cigarettes and Collection of Particulate Matter

Cigarettes were conditioned prior to collection of particulate matter, with most laboratories following ISO 3402 methodology (73 ± 2 ºF [23 ºC]); 60% ± 2 RH (Table 2).

Table 2. Smoke Collection Methodology (as reported by each lab)

Lab	Cigarette conditioning prior to smoke collection	Smoke Method	Equipment
1	≥ 18 hrs.	FTC; 60 ± 2% RH; 75 ± 2F; exhaust hood target 200 mm/sec	Borgwaldt RM20/CS
2	48 hrs	ISO 3308	Rotative RM200
3	ISO 3402	ISO 3308	Borgwaldt RM20-CSR
4	ISO	ISO 3308	Filtrona SM350
5	≥ 48 hrs.	ISO 3308	Borgwaldt RM200
6	ISO 3402	ISO 3308	Cerulean ASM 516
7	≥ 48 hrs.	ISO 3308	Cerulean ASM 500
8	ISO 3402	ISO 3308	Borgwaldt RM200
9	ISO 3402	ISO 3308	20-port rotary RM20/CS
10	ISO 3402	ISO 3308	20-port Borgwaldt
11	ISO 48 hr	ISO 3308	Borgwaldt RM20
12	ISO 48 hr	ISO	Borgwaldt RM200
13	≥ 48 hrs.	ISO 3308	Cerulean ASM 500

Particulate matter was collected on Cambridge filter pads, primarily as specified by ISO 3308 (71.6 ± 3.6 ºF [22 ºC]; 60% ± 2 (RH) Smoke collection provided in Table 3.

Table 3. Smoke Chemistry Data[1]

Cigarette Description		mg TPM/cigt	mg nicotine/ cigt	mg water/ cigt	mg 'tar'/ cigt	Puffs/ cigt
R309	**100% burley**	24.89 ± 1.55	3.00 ± 1.03	3.63 ± 1.12	18.66 ± 1.52	8.75 ± 0.33
R310	**100% flue**	27.75 ± 1.64	2.82 ± 0.09	3.58 ± 1.26	21.40 ± 1.33	9.93 ± 0.39
R311	**1:1 mix**	26.28 ± 1.28	2.83 ±0.18	3.43 ± 1.06	20.10 ± 1.15	9.97 ± 0.41
2R4F	**Kentucky Reference**	10.78 ± 0.71	0.79 ± 0.05	1.08 ± 0.41	8.97 ± 0.56	8.76 ± 0.41

[1] Average of data provided by laboratories, ± S.D.

Preparation of cigarette smoke condensate

Cigarette smoke condensate was prepared according to procedures established in each laboratory, and are detailed in Table 4. In all cases the final solution was prepared in dimethylsulfoxide (DMSO).

Table 4. Preparation of Cigarette Smoke Condensate

Lab	CSC extraction method
1	44 mm pad extracted by adding DMSO at a final concentration of 20 mg/ml. Pad shaken for 30 minutes on wrist action shaker; extract filtered through 0.45 micron PTFE filter
2	Extracted at 20 mg TPM/ml by shaking in DMSO for 1 hour
3	DMSO
4	Extracted with 25 minute rotary shaker in DMSO; filtered through cheesecloth
5	Extracted with 30 minutes shaking in DMSO (1 ml DMSO/1 cig)
6	Extracted in DMSO with 3 hours wrist action shaker
7	44 mm pads extracted with DMSO added drop-wise to pad under vacuum
8	Shake filter with DMSO for 20 min; centrifuge 5 min. @ 1500 rpm using sterile mesh bag placed in conical centrifugation tube
9	Shaken for 25 min; filtered through cheesecloth
10	TPM from 2 filters extracted with 6 ml DMSO
11	Extracted at 20 mg TPM/mL by shaking in DMSO for 1 h.
12	Pad sectioned and ¾ pad extracted in DMSO under vacuum using glass funnel
13	DMSO added dropwise to pad under vacuum

AMES MUTAGENICITY ASSAYS

Thirteen laboratories participated in this study. Ten reported running more than ten Ames assays within the year prior to the study, and two reported running less than ten (this information not provided by 1 lab).

Ames assays were conducted in each laboratory following that lab's standard operating procedures. Laboratories were expected to follow guidelines described in the Task Force Report (OECD 471)[1]. Assays were conducted with TA98 and TA100 in the presence of S9 metabolic activation. Details of basic experimental parameters are described in Table 5.

Table 5. Ames Assay Experimental Conditions

Lab #	# Plates /concentration	# Concentrations tested	% S9 v/v
1	3	8	5
2	6	6	10
3	3	8	5
4	3	8	5
5	3	4	4
6	3	8	10
7	3	5	10
8	3	2	10
9	3	6	5
10	3	5	1 mg protein/plate
11	3	6	4
12	4	6	10
13	3	5	10

RESULTS OF AMES ASSAYS

Each laboratory followed internal procedures for calculating the response, typically expressed as revertants/mg (or µg) TPM. Data (revertants/µg TPM) are presented in Tables 6 and 7 and Figures 1 and 2.

Table 6. TA98 Revertants/µg TPM

Lab #	USB (R309)	Flue (R310)	Blend (R311)	2R4F
1	9.44	4.36	6.93	5.12
2	6.09	2.30	4.19	3.43
3	2.95	1.71	2.46	2.33
4	3.63	1.34	2.10	1.97
5	4.96	2.67	4.08	3.20
6	2.06	0.76	1.50	1.13
7	3.68	1.64	2.56	1.95
8	5.61	2.36	4.18	3.43
9	5.26	1.98	3.71	3.27
10	3.62	1.64	2.98	2.25
11	6.04	2.47	5.72	2.05
12	3.70	1.40	2.59	2.03
13	3.27	1.41	2.28	2.07
Average ± S.D.	**4.64 ±1.91**	**2.00 ± 0.89**	**3.48 ± 1.54**	**2.63 ± 1.02**

Table 7. TA100 Revertants/µg TPM

Lab #	USB (R309)	Flue (R310)	Blend (R311)	2R4F
1	2.80	1.95	1.88	1.75
2	1.77	0.98	1.43	1.12
3	0.65	0.56	0.66	0.56
4	1.0	0.70	0.94	0.92
5	5.02	4.40	4.45	4.62
6	0.88	0.50	0.79	0.62
7	1.52	0.93	1.10	0.90
8	1.01	0.58	0.88	0.79
9	0.59	0.37	0.54	0.57
10	1.72	0.91	1.19	1.13
11	0.78	0.49	0.63	0.78
12	0.41	0.26	0.36	0.33
13	1.14	0.68	1.02	0.81
Average ± S.D.	**1.48 ± 1.24**	**1.02 ± 1.10**	**1.22 ± 1.05**	**1.15 ± 1.10**

Figure 1. Revertants/μg TPM with TA98 with S9 metabolic activation

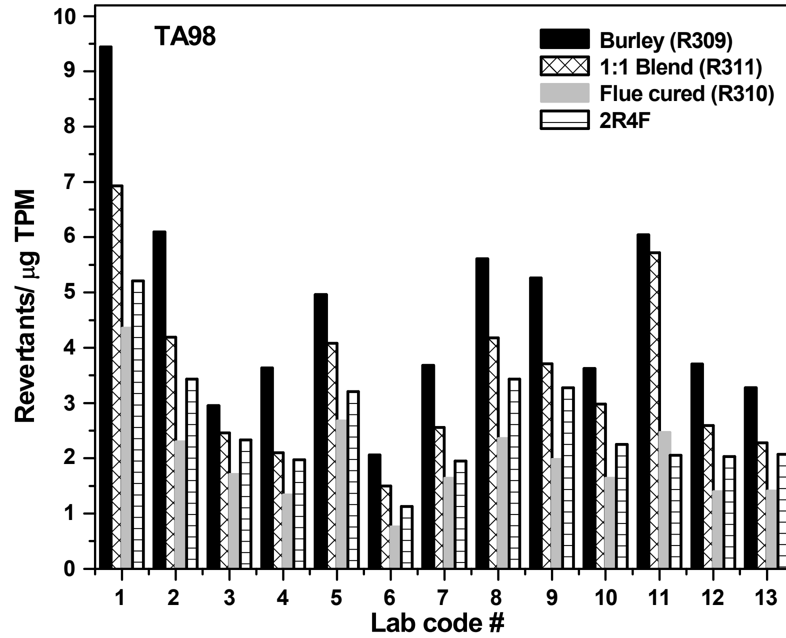
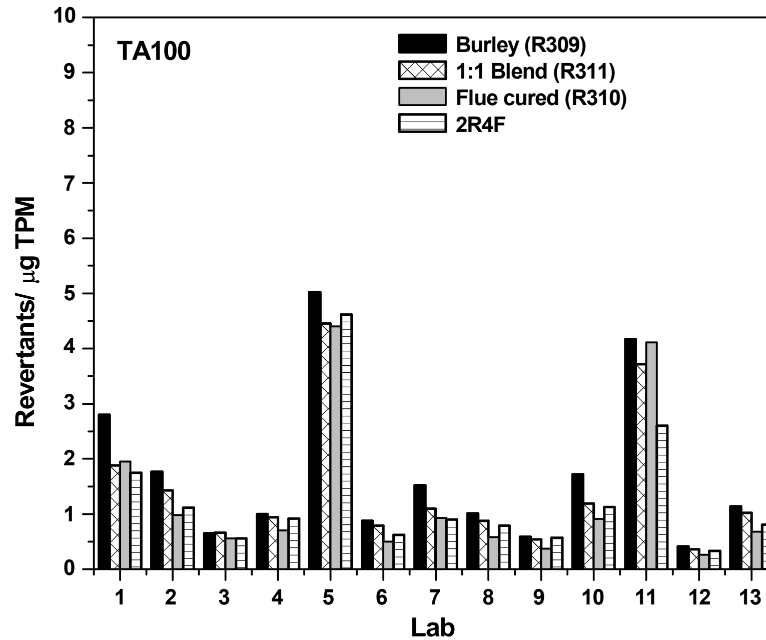


Figure 2. Revertants/μg TPM with TA100 with S9 metabolic activation



Responses of the three test cigarettes were also compared to that of the 2R4F reference cigarette (Figures 3 and 4).

Figure 3. Percent response compared to 2R4F, TA98 with S9

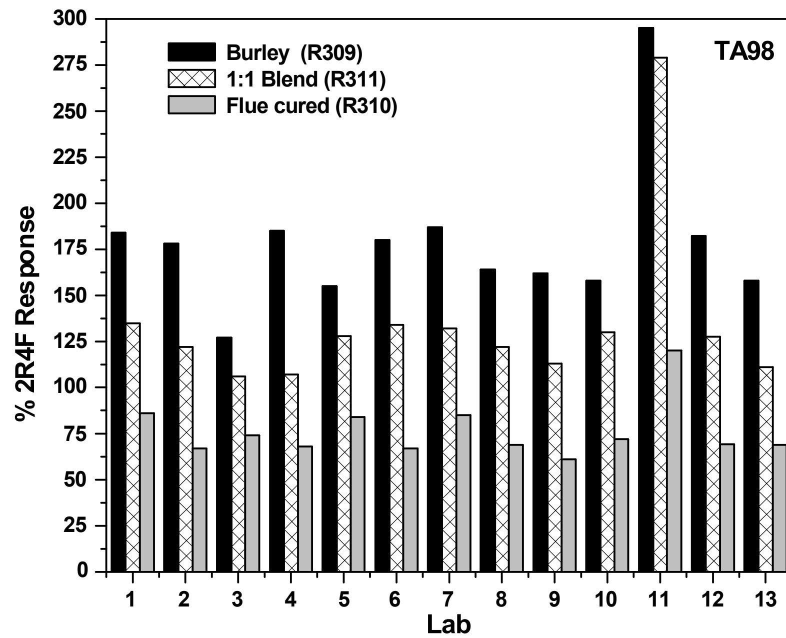
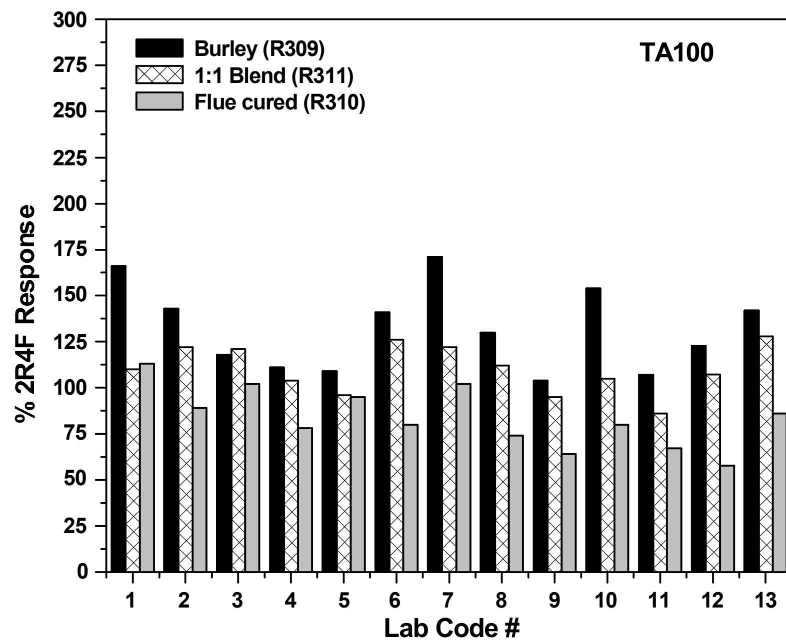


Figure 4. Percent response compared to 2R4F, TA100 with S9



Mutagenicity comparisons were conducted and rankings were reported by each individual laboratory, and are summarized in Table 8.

Table 8. Mutagenicity ranking

Lab	TA 98 Summary	TA 100 Summary
1	**Burley** > Flue, Blend, 2R4F; Blend > Flue	**Burley** > 2R4F & Blend
2	**Burley** > Blend > 2R4F > Flue	**Burley** > Blend > Flue & 2R4F
3	**Burley** > Flue, Blend, 2R4F Blend & 2R4F > Flue	No significant differences
4	**Burley** > Flue, Blend, 2R4F; Blend & 2R4F > Flue	No significant differences
5	**Burley** > Blend > 2R4F > Flue	No significant differences
6	**Burley** > Flue, 2R4F; Blend > Flue	**Burley** > Flue; Blend > Flue
7	**Burley** > Flue, Blend, 2R4F	**Burley** > Flue & Blend
8	**Burley** > Blend > Flue (statistical method still under consideration)	(statistical method still under consideration)
9	**Burley** > Blend; 2R4F > Flue	**Burley** > Flue; **Burley** ≥ Blend; 2R4F ≥ Flue
10	**Burley** > Blend, Flue, 2R4F; Blend > 2R4F > Flue	**Burley** > Flue, Blend, 2R4F; Blend, 2R4F > Flue
11	**Burley** & Blend > Flue	No significant differences
12	**Burley** > Blend > 2R4F > Flue	**Burley** > Flue
13	*None provided*	*None provided*

CONCLUSIONS FROM AMES ASSAYS

All thirteen labs reported greater mutagenicity with particulate matter from burley cigarettes than with that from flue-cured cigarettes when using TA98. Fewer labs were able to discriminate between any of the cigarettes with TA100.

Possible sources of variation were differences in preparation of cigarette smoke condensate, assay methodology (i.e., preincubation vs. plate incorporation, S9 source and concentration) and data/statistical analysis.

NEUTRAL RED CYTOTOXICITY ASSAYS

Twelve labs reported data for assays of particulate matter (lab # 13 submitted data after the meeting which is not included in all the tables). One lab submitted data for gas vapor phase (GVP) as well as particulate matter (PM) + GVP; one lab submitted data on a puff-basis as well as TPM basis. Several different cell lines were used and experimental design varied from lab to lab (Table 9).

Table 9. NRU methodology

Lab #	Cells	# replicates	# plates/assay	# concentrations
1	CHO	4	3	8
2	CHO	3	4	6
3	CHO	3	4	8
4	CHO	2	2	8
5	HepG2	2	3	8
6	Swiss 3T3	4	1	4
7	V79	2	1	8
8	CHO	3	3	10
9	BALB/c 3T3	2	3	8
10	BALB/c 3T3	3	4	8
11	HepG2	2	2	8
12	V79	3	4	8

Each participating laboratory submitted results expressed as IC_{50}, i.e., the concentration of particulate matter resulting in 50% reduction in cell viability as measured by the NRU assay. Summary results are presented in Table 10 and in Figure 5. Laboratories also provided information concerning the response of each Test cigarette compared to that of 2R4F; that data is presented in Table 11 and Figure 6.

Table 10. Neutral Red Cytotoxicity, IC_{50}

Lab #	USB (R309)	Flue (R310)	Blend (R311)	2R4F
1	46.9	35.6	40.1	44.9
2	70.0	75.0	72.0	62.0
3	100.0	97.4	105.0	94.5
4	66.8	45.2	53.1	49.5
5	23.7	29.7	25.8	27.7
6	121.0	81.0	146.0	79.0
7	35.3	25.6	30.7	33.5
8	95.8	63.0	75.2	69.9
9	120.3	97.6	111.0	103.9
10	104.8	92.7	94.0	97.8
11	8.5	14.1	14.6	15.0
12	27.3	29.1	30.3	31.1

Figure 5. Cytotoxicity Assay Results, IC_{50}

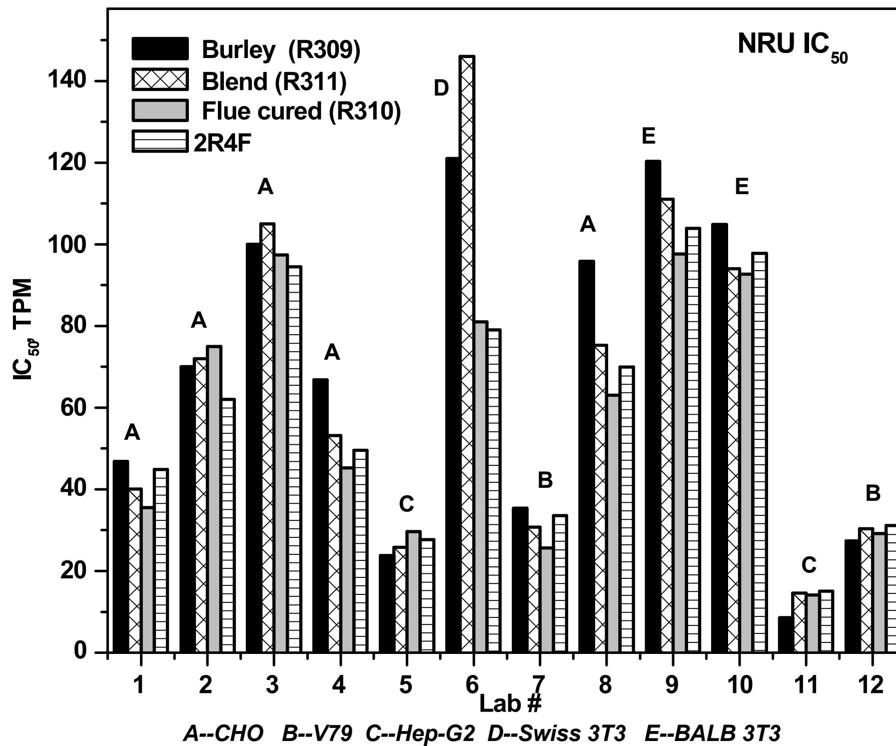
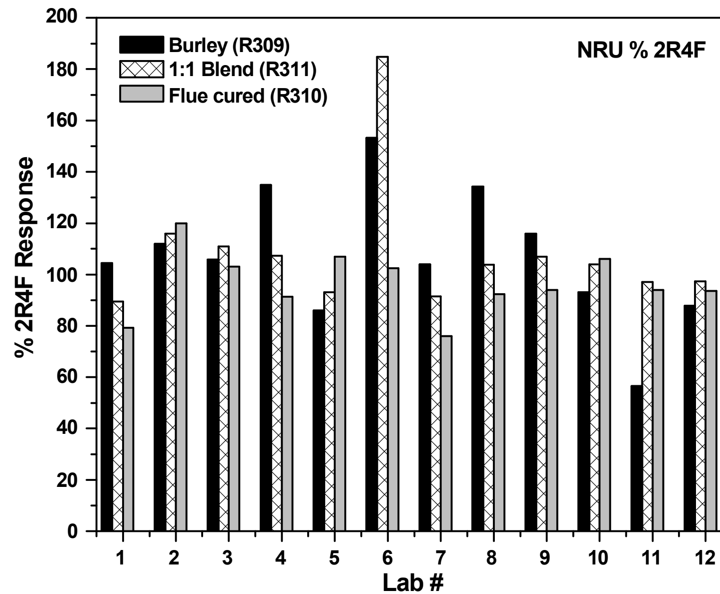


Table 11. Percent response compared to 2R4F

Lab #	USB (R309)	Flue (R310)	Blend (R311)
1	104.5	79.3	90.0
2	112.0	120.0	116.0
3	105.8	103.1	111.1
4	134.9	91.3	107.3
5	86.0	107.0	93
6	153.2	102.5	184.8
7	104.0	76.0	91.5
8	137.2	90.4	107.5
9	116.0	94.0	107.0
10	93.0	104.0	106.0
11	56.6	94.0	97.0
12	87.8	93.6	94.4

Figure 6. Percent response compared to 2R4F



The variability of responses was greater in the neutral red assay than observed in the Ames assay data. In order to gain a better understanding of what may have contributed to this variability, Wendy Wright (Labstat) agreed to take a closer look at the NRU data, using the same methodology to calculate IC_{50} responses. Table 13 summarizes conclusions reported by each laboratory and conclusions based on reanalysis of the data using the same methods (ANOVA and S-N-K).

Table 13. Neutral Red Cytotoxicity Results

Lab #	Reported Conclusions (TPM Basis)	ANOVA and S-N-K Conclusions (TPM basis)
1	Flue > {2R4F, Burley}	Flue > {Burley, Blend, 2R4F} (ANOVA: p<0.01)
8	Trend: Flue > Blend > Burley	{Flue, Blend, 2R4F} > Burley (ANOVA: p<0.02)
3	No significant difference	No significant difference (ANOVA: p=0.63)
6*	No significant difference	No significant difference (ANOVA: p=0.51)
7	No significant difference	No significant difference (ANOVA: p=0.75)
12	No significant difference	No significant difference (ANOVA: p=0.83)
13^	Lab unable to rank responses	No significant difference (ANOVA: p=0.77)
5	Burley > Flue, Blend in between	{Burley, Blend} > {Flue, 2R4F} (ANOVA: p<0.03)
11* ^	Burley > {Flue, Blend}	Burley > {Flue, Blend, 2R4F} (ANOVA: p<0.02)
2^	2R4F > Burley ≥ Blend ≥ Flue	No significant difference (ANOVA: p=0.20)
4	Flue > {Burley, Blend}; Blend > Burley	No significant difference (ANOVA: p=0.30)
9	Flue > 2R4F > Blend > Burley	No significant difference (ANOVA: p=0.38)
10	Trend: {Flue, Blend} > Burley	No significant difference (ANOVA: p=0.35)

* assay data contained no maximum response plateau, logistic model assumes max = 100
^ raw data submission was incomplete or no raw data was reported

A consensus among participating laboratories regarding the cytotoxicity rank-order could not be made with this data set. Differences are due to several factors: variability in experimental design (plate design, # repeat assay plates per replicate, and # replicates) and in analytical methodology.

The NRU presentation concluded with the following recommendations:

Given the impact of Experimental Design on our ability to detect differences in cytotoxicity among brands, future experiments should consider:

- ***Assay plate design***
  - *Number of wells dedicated to CSC treatments*
  - *CSC concentrations to produce sigmoid dose-response*
- ***Number of "repeat" assay plates per "replicate"***
- ***Number of "replicate" assays for each brand***
  - *Sources of variation associated with defining a "replicate"*
- ***Methodology for data analysis***
  - *Blank-correction and relative absorbance calculations*
  - *Methodology for determining cytotoxicity estimate (i.e. IC_{50})*
  - *Methodology for comparing cytotoxicity estimates among brands*

IN VITRO MICRONUCLEUS ASSAY

The third assay used in the CORESTA Interlaboratory Study was the *in vitro* micronucleus assay. The Task Force recommended following OECD guidelines (still in draft as of April 2007; revisions in these guidelines have occurred over the time period of the Interlaboratory Study). Nine laboratories submitted data for at least one experimental condition. Six labs reported conducting more than 10 micronucleus assays within the past year. Experimental methodology and design are detailed in Tables 14 and 15; conclusions reported by each individual lab are presented in Table 16.

Table 14. Micronucleus Assay Experimental Design

Lab	Assay conditions
1	1 smoking; 4 replicates of each dose
2	3 smokings; CSC pooled & tested in 2 independent experiments
3	Duplicate assays
4	Duplicate assays
5	Duplicate assays
7	Duplicate assays
8	Duplicate assays
11	Duplicate assays
12	Duplicate assays

Table 15. Micronucleus Assay Methodology

Lab	Cell Line	CYB*	Cytotox method	Solvent/ level	Hrs. of short treatment	Hrs. of overnight treatment	# Non-zero doses	Dose range µg/ml		
								short + S9	Short -S9	Overnight -S9
1	CHO	Yes	CBPI**	DMSO	4	24	Report 3 of 7	3.125 - 200	3.125 - 200	3.125 - 200
2	CHO	No	NRU	DMSO	--	24	4	--	--	15 - 60
3	CHO	No	Cell count	DMSO	3	30	4	75 - 200	75 - 200	25 - 100
4	CHO-WBL	Yes	CBPI**	DMSO 1%	4	24	6	80 - 200	40 - 160	40 - 140
5	V79	No	NRU	DMSO 0.75-2%	1h + 3h	24	4	52-130	50 – 200	30 - 75
7	V79	Yes	CBPI**	DMSO 1%	3	20	4	80 - 140	50 - 110	10 – 40
8	CHL/ IU	No	Cell count		3		6	50 - 300	50 - 300	--
11	CHO	No	Cell count	2%	3	24	6	50 – 200	40 – 125	10 - 60
12	V79	Yes	CBPI	DMSO	3		6	70 – 120	50 - 100	

* CYB = cytochalasin B (a cytokinesis inhibitor)
** CBPI = cytokinesis block proliferation index

Table 16. Micronucleus Assay Conclusions

Lab	Plus S9 short	No S9 short	No S9 overnight
1	No significant differences	Blend > Flue & **Burley**	No significant differences
2	---------	---------	Global ANOVA: No significant differences GLM: different, but not same for each dose
3	No significant differences	Flue > Burley, Blend & 2R4F	No significant differences
4	Rep 1: Flue > **Burley** & Blend. Rep 2: Flue & 2R4F > **Burley** Combined: Flue > **Burley** & Blend; 2R4F > **Burley**	Rep. 1: Flue, Blend, 2R4F > **Burley** Rep. 2: No significant differences Combined: No significant differences	Rep 1: Flue & Blend > **Burley** Rep 2: No significant differences Combined: Flue > **Burley**
5	Flue, Blend, 2R4F > **Burle**y; 2R4F = Flue, Blend	Blend > **Burley** at 1 dose; Flue = 2R4F	No significant differences
7	Flue > **Burley** & Blend at 3 doses	Flue > **Burley** & Blend at 3 doses; Blend > Burley at 1 dose	Flue > **Burley** & Blend at 2 doses; Blend > **Burley** at 1 dose
8	Data submitted; no ranking assessed	Data submitted; no ranking assessed	---------
11	*1-way + Tukey:* Rep 1: 2R4F > Flue, Blend Rep 2: Flue > Blend; 2R4F > Blend	*1-way + Tukey*: Rep 1: 2R4F > **Burley** , Flue & Blend Rep 2: Flue > 2R4F	
	2-way: Rep 1: 2R4F > Blend & Flue @ ≥ 2 conc. Rep 2: 2R4F > Blend & **Burley** @ ≥ 2 conc.	*2-way:* Rep 1 & Rep 2: 2R4F > Blend & Flue & **Burley** @ ≥ 2 conc.	*2-way:* Rep 1: 2R4F > Blend @ ≥ 2 conc. Rep 2: 2R4F > Blend & **Burley** @ ≥ 1 conc.
12	Rep. 1: Flue , Blend & 2R4F > **Burley**; Flue > Blend; 2R4F > Blend Rep. 2: Flue & 2R4F > **Burley**; Flue > Blend; 2R4F > Flue & Blend	Rep. 1: Flue , Blend & 2R4F > **Burley**; Flue > Blend Rep. 2: 2R4F > **Burley** & Blend Flue > Blend	---------

Based on the data and conclusions submitted, there is no complete consensus about the rank ordering of the cigarettes in the micronucleus assay, although overall there is a trend for flue to give a greater response than burley. Differences in final conclusions may be due to differences in extraction methods, cell line, concentration of S9, experimental design (# concentrations tested, length of time, etc) and statistical methods.

CONCLUDING OBSERVATIONS

This interlaboratory study was an ambitious undertaking in a relatively short period of time, with a large number of variables from assay to assay and from laboratory to laboratory, and a large amount of data. Not surprisingly, data from the assay with the greatest historical use, the Ames assay, showed the most concordance among the laboratories, while data from both the Neutral red cytotoxicity and the *In Vitro* Micronucleus assays exhibited much greater variability.

Members of the Task Force have expressed interest in proficiency testing. If such testing is to take place, adequate discussions and attention to experimental design and detail must be given to assure greater concordance.

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[1] OECD Guideline for testing of chemicals. Bacterial Reverse Mutation Test, 471.