In Vitro Toxicity Testing Sub-Group

Technical Report

Mouse Lymphoma Assay Inter-Laboratory Proficiency Study

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1. Summary

An inter-laboratory Mouse Lymphoma Assay (MLA) proficiency study was conducted by four member laboratories of the CORESTA Sub-Group *In vitro* Toxicity Testing (IVT). The objectives of the study were to evaluate the proficiency of each participating laboratory in conducting the MLA and to determine the discriminatory power of each laboratory's assay in relation to combustible tobacco products. For the study, total particulate matter (TPM) samples were generated from three cigarette test pieces (100 % Flue-cured, 100 % Burley and Kentucky Reference 3R4F) using the ISO smoking regime and collected on Cambridge filter pads before extraction in dimethylsulfoxide (DMSO). At least three replicates (defined as an independent smoking session carried out on a single day) of TPM per test piece were produced. Each participating laboratory used its own L5178Y tk+/- 3.7.2C cell line and standard operating procedures to conduct the assay on three replicates of TPM per test piece. All four laboratories assessed the mutagenicity of the TPM samples in the mandatory +S9 treatment condition, while only two laboratories carried out the optional -S9 treatment condition. Following completion of the experimental phase of the study, the raw data (compiled in worksheets) as well as non-statistical conclusions were submitted to the Study Coordinator by each laboratory. In addition, coded study data were transferred to the Study Statistician for statistical analysis. Solvent and positive control-derived data from three (A, B and C) of the four laboratories in the +S9 condition were OECD Test Guideline (TG) 490-compliant and generally in line with their historical control ranges. Related data from the fourth laboratory (D) were not compatible with acceptance criteria recommended in OECD TG 490 but they were consistent with its own historical control ranges. Data from solvent and positive controls produced by the two laboratories (A and C) participating in the -S9 condition were in line with OECD TG 490 acceptance criteria as well as their own historical control ranges. Each replicate TPM sample generated from the three test pieces induced concentration-dependent increases in cytotoxicity and mutagenicity in all four laboratories that carried out the +S9 condition. For laboratories A, B and C that generated OECD TG 490-compliant solvent control data, the mutagenic responses observed for each test piece were considered mutagenic according to OECD TG 490 evaluation criteria, i.e. they surpassed the global evaluation factor (GEF). However, the mutagenic responses induced by test piece LOT 2 in these laboratories were consistently the least potent among the three test pieces although not determined to be statistically significant. Mutagenic potency data from the other two test pieces (LOT 1 and LOT 3) were inconsistent between these laboratories and also not statistically significant, thus it was not possible to rank their mutagenic effects. It was also not possible to evaluate the mutagenic responses observed in laboratory D according to the same OECD TG 490-based criteria as its solvent control data were not in the acceptable range (50-170 mutants/10^6 viable cells) but test piece-induced mutation frequencies (MFs) were multi-fold higher than solvent control MFs. However, there were no statistically significant differences between the mutagenic responses observed by this laboratory. Each replicate TPM sample generated from the three test pieces induced concentration-dependent increases in cytotoxicity and mutagenicity in laboratories A and C that carried out the -S9 condition. All MF responses observed would be considered as mutagenic according to the evaluation criteria described in OECD TG 490 with the exception of test piece LOT 1 in laboratory C (did not surpass the GEF). It was not possible to rank their mutagenic potency in the -S9 condition due to a lack of statistically significant effects. Minimal detectable differences (MDD) for each laboratory's MLA (+S9) were calculated from the study data; for two test pieces evaluated across three replicates, the median MDD varied from 90-100 % between the four laboratories. In conclusion, although it was not possible to rank the mutagenic potency of the three test pieces due to a lack of statistical significance, in general OECD TG 490-compliant data were produced and mutagenic findings were reported by the four participating laboratories.

2. Introduction

One of the objectives of the *In vitro* Toxicity Testing Sub-Group is to organise and conduct proficiency testing of tobacco and tobacco related products using common experimental protocols and the Sub-Group's recommended test battery, in which MLA is included. At the 2016 fall meeting of the CORESTA *In vitro* Toxicity Testing Sub-Group, the Sub-Group confirmed its intention to conduct a MLA inter-laboratory proficiency study.

2.1 Objectives

To conduct an inter-laboratory MLA proficiency study using two test pieces and the Kentucky Reference (KR) 3R4F in the L5178Y tk+/- 3.7.2C mouse lymphoma cell line (generally called L5178Y) in order to evaluate the proficiency of each participating laboratory in conducting the MLA. In addition, the discriminatory power of the assay towards different tobacco products was also assessed.

Each participating laboratory used its preferred subline of tk+/- 3.7.2C L5178Y cells to evaluate the test items as well as its own protocol. Basic requirements in the Study Plan had to be followed. The Study Plan is based on OECD Test Guideline 490 which should be consulted for further details [1].

3. Test System

The MLA is an *in vitro* test that detects gene mutations induced at the tk genetic locus in L5178Y mouse lymphoma cells [1]. Exposure of heterozygous tk+/- L5178Y cells to mutagenic agents may result in mutation, and potentially inactivation, of their functional tk gene [2]. In this event, mutant tk cells acquire resistance to the pyrimidine analogue trifluorothymidine (TFT) and are able to proliferate in its presence, while parent tk+/- cells remain sensitive to TFT-induced cytostasis and death. Although typically used for evaluating single chemicals, published data have shown that the assay is also capable of assessing the mutagenic activity of tobacco smoke-derived aerosols [3-5].

4. Organisation

A list of the participating laboratories (in alphabetical order) and their respective contribution is provided in Table 1. The laboratories were coded LAB A to LAB D for this study. The codes do not correspond with the alphabetical order of the participating laboratories below.

Table 1: Participating laboratories

Organization	Study Contribution
Covance Laboratories, UK	Participant laboratory
Japan Tobacco Inc., Japan	Providing test pieces (LOT 1 and LOT 2)
JTI R&D, Oekolab, Austria	Participant laboratory Study coordination Statistical analysis
Philip Morris International, Switzerland	Participant laboratory Study Coordination Technical Report
Zhengzhou Tobacco Research Institution (ZTRI), China	Participant laboratory

5. Material & Methods

5.1 Test Pieces

LOT 1 (100 % single grade Flue-cured) and LOT 2 (100 % single grade Burley) were produced for the study and provided by Japan Tobacco Inc. As third test piece the reference cigarette Kentucky Reference 3R4F (LOT 3) was used. The test pieces were expected to generate different *in vitro* activities.

Table 2 summarizes the specifications of the test pieces.

Table 2: Specification of the test pieces

Lot	Type of tobacco	Tobacco weight (mg)	Size Length & Circumference (mm)	Cigarette Paper (CU)	Filter plug & Tow spec	Ventilation (%)
LOT 1	100 % single grade Flue-cured (FC, Zimbabwe)	646	Length: 57+27 Circumference: 24.6	35	Cellulose acetate 2.8Y35	34
LOT 2	100 % single grade Burley (BLY, Brazil)	614				37
LOT 3	Kentucky Reference 3R4F, Blend	775	Length: 57+27 Circumference: 24.9	24	Cellulose acetate 2.9Y41	29

The results of component analysis and smoke analysis according to ISO smoking regime are provided in the following table.

Table 3: Component analysis (% dry basis) and Smoke analysis (mg/cigarette)

LOT#	Component analysis (% dry basis)			Smoke analysis (mg/cigarette)				Puff count
	Volatile basic nitrogen	Sugars	Nicotine	TPM	Tar	Nicotine	CO	
LOT 1	0.41	22.4	2.25	13.1	10.9	1.07	11.0	8.4
LOT 2	1.39	0	5.00	14.1	10.8	2.19	10.5	7.3
LOT 3	n.a.	8.7	n.a.	11.0	9.4	0.73	12.0	9.0

5.2 Conditioning, smoking, extraction & sample preparation

Test pieces were conditioned for at least 48 hours at 22 ± 1 °C and 60 ± 3 % relative humidity according to International Standard ISO 3402 [6], and smoked following the smoking regime International Standard ISO 3308 and 4387, i.e. 35 ± 0.3 mL puff volume, 2 ± 0.02 sec. puff duration, 60 ± 0.5 sec. puff interval, no ventilation block [7-8].

TPM was collected on Cambridge filter pads and extracted with DMSO. Each lab followed its own procedure for TPM extraction, details of which were provided in the study data submitted to the Study Coordinator.

At least three replicates of TPM per lot were produced; for this study, a replicate was defined as an independent smoking session carried out on a single day. In order to assess the variability due to smoking and TPM extraction, each participating lab submitted at least 2 mL of 9 TPM extracts (3 replicates × 3 lots of test items) to the Study Coordinator who passed forward the coded samples to Japan Tobacco Inc. for analysis of nicotine content. In order to prevent decomposition of nicotine in transit, TPM extracts were shipped protected from light below - 70 °C. Table 4 below summarizes the results of TPM analysis performed by JT Inc.

Table 4: TPM analysis

	LOT #	TPM conc. (mg/mL DMSO)	Estimated (mg/mL DMSO)	Replicate 1 (mg/mL DMSO)	Replicate 2 (mg/mL DMSO)	Replicate 3 (mg/mL DMSO)	Replicate 4 (mg/mL DMSO)	Average (mg/mL DMSO)	TPM basis (% to TPM)	Actual/ Estimated
LAB 1	LOT 1	10	0.84	0.74	0.76	0.80	0.83	**0.77**	7.7	**0.91**
	LOT 2	10	1.56	1.57	1.46	1.33	1.50	**1.45**	14.5	**0.93**
	LOT 3	10	0.69	0.73	0.64	0.60	0.77	**0.66**	6.6	**0.95**
LAB 2	LOT 1	57.4, 55.7, 57.6	4.82, 4.67, 4.83	3.79	4.55	4.16	-	**4.17**	7.3	**0.90**
	LOT 2	65.3, 66.5, 68.6	10.18, 10.37, 10.70	7.91	7.29	7.98	-	**7.73**	11.6	**0.68**
	LOT 3	46.3, 48.5, 47.2	3.21, 3.36, 3.27	2.69	2.67 7	2.71	-	**2.69**	5.7	**0.69**
LAB 3	LOT 1	24	2.02	2.05	2.02	1.83	-	**1.97**	8.2	**0.98**
	LOT 2	24	3.74	3.91	4.05	3.85	-	**3.94**	16.4	**1.05**
	LOT 3	24	1.66	1.92	1.81	1.78	-	**1.84**	7.7	**1.11**
LAB 4	LOT 1	10	0.84	0.83	0.85	0.82	-	**0.84**	8.4	**0.99**
	LOT 2	10	1.56	1.56	1.60	1.56	-	**1.57**	15.7	**1.01**
	LOT 3	10	0.69	0.74	0.73	0.73	-	**0.74**	7.4	**1.06**

5.3 Negative/solvent-treated controls

All participant laboratories in this study used DMSO as their solvent control.

5.4 Positive controls

All participant laboratories in this study used OECD 490-recommended positive controls.

Table 5: Positive controls used by the participating laboratories in this study

	Positive control(s) (+S9)	Positive control(s) (-S9)
LAB A	Cyclophosphamide (CPA) Benzo[a]pyrene (BAP)	Methyl methanesulfonate (MMS) 4-Nitroquinoline-1-oxide (4-NQO)
LAB B	7,12-Dimethylbenz[a]anthracene (DMBA)	N/A
LAB C	BAP	MMS
LAB D	CPA	N/A

5.5 Cell lines

All participating laboratories in this study used the OECD 490-recommended cell line L5178Y tk+/- 3.7.2C. However, the supplier of this cell line varied from laboratory-to-laboratory as described in Table 6.

Table 6: The supplier and lot number of the L5178Y cell line for each participant laboratory

	L5178Y cell line supplier (lot number)
LAB A	Harlan Laboratories, Germany (24102011)
LAB B	Public Health England, UK (12H013)
LAB C	Dr Donald Clive, Burroughs Wellcome Co, USA (AZ1.11)
LAB D	Institute of Disease Control and Prevention, Academy of Military Medical Sciences, China (N/A)

5.6 Cytotoxicity and concentration setting

Cytotoxicity (as relative total growth; RTG) was measured concomitantly with mutation frequency (MF) by each participating laboratory. Each laboratory followed its own protocol for concentration setting. Concentrations that induced ≥ 90 % cytotoxicity, i.e. RTG ≤ 10 %, were omitted from data analysis [1].

5.7 Test design and description

Each participating laboratory used its own protocol. Basic requirements in the Study Plan based on OECD Test Guideline 490 had to be followed. Test design used by the participating laboratories is summarized in Table 7.

Table 7: Test design used by the participating laboratories

	Treatment condition(s)	Supplier and source of S9	Exposure time (h)	At least three TPM replicates assessed?	At least three non-zero concentrations assessed?	RTG 10-20 % limit of cytotoxicity reached?	Scoring method
LAB A	+S9 and S9	Trinova; Aroclor 1254-induced rat liver	3	Yes	Yes	Yes	By eye via incident light microscope
LAB B	+S9	Moltox; Aroclor 1254-induced rat liver	4	Yes	Yes	Yes	By eye via low power magnifi-ication
LAB C	+S9 and S9	Moltox; Aroclor 1254-induced rat liver	3	Yes	Yes	Yes	By eye
LAB D	+S9	PrimeTox; not specified	3	Yes	Yes	Yes	By eye

6. Statistical Analysis

Data were represented as concentration-response plots (MF and RTG).

A regression model was applied [3] to estimate the mutagenic potential of the items, e.g. non-linear regression: the concentration-response curve for MF is modelled by non-linear regression analysis with the power function $y = a + b \times x^c$. From the concentration-response curves, the equal mutagenic effect concentration for three times the spontaneous mutant frequency (C3B) was derived. Replicate C3B values were used for sample comparison (ANOVA) and to derive sample rank order in terms of mutagenicity.

The minimal detectable difference (MDD) was assessed.

7. Results

Laboratories provided experimental information, raw data, and summary data using the provided worksheets to the Study Coordinator. Raw data are available upon request.

7.1 Solvent control MF distributions (+S9)

The MFs of all solvent controls generated in this study by laboratories A, B and C were within the OECD recommended range of 50-170 mutants / 10^6 viable cells. However, for laboratories A and B, a number of solvent controls was outside their own historical control range. The MFs of solvent controls from Laboratory D were below the OECD recommended lower limit (50 mutants/10^6 viable cells) but were in a similar range to its own historical control range.

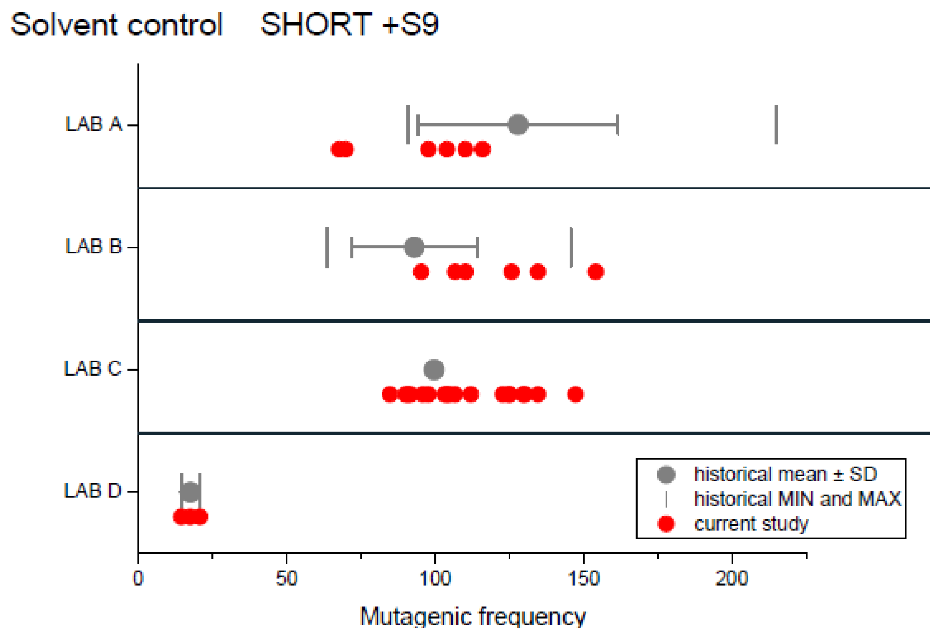


Figure 1: Solvent control MF distributions (+S9)

7.2 Positive control MF distributions (+S9)

All positive controls used in this study by laboratories A, B and C produced induced MFs above the OECD recommended value, i.e. an increase above background by at least 300 mutants/10^6 viable cells, and were generally in a similar range to their own historical control ranges. Although a clear induction in MF by the positive control was observed by Laboratory D, an increase above background by at least 300 mutants/10^6 viable cells was not achieved.

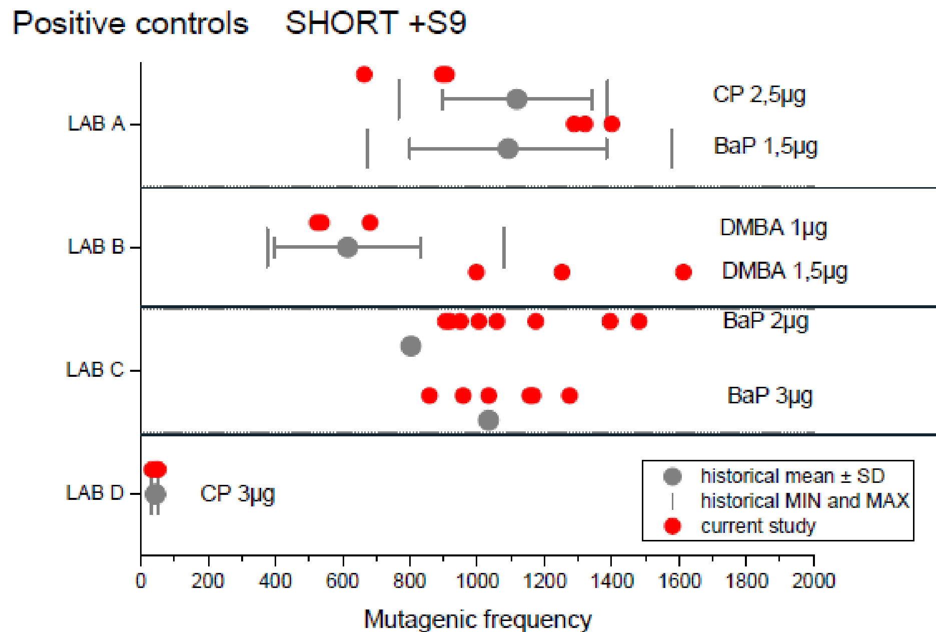


Figure 2: Positive control MF distributions (+S9)

7.3 Solvent control MF distributions (-S9)

For the two laboratories (A and C) that carried out the -S9 assay, the MFs of all solvent controls generated in this study were within the OECD recommended range of 50-170 mutants/10^6 viable cells and generally in line with their own historical control ranges.

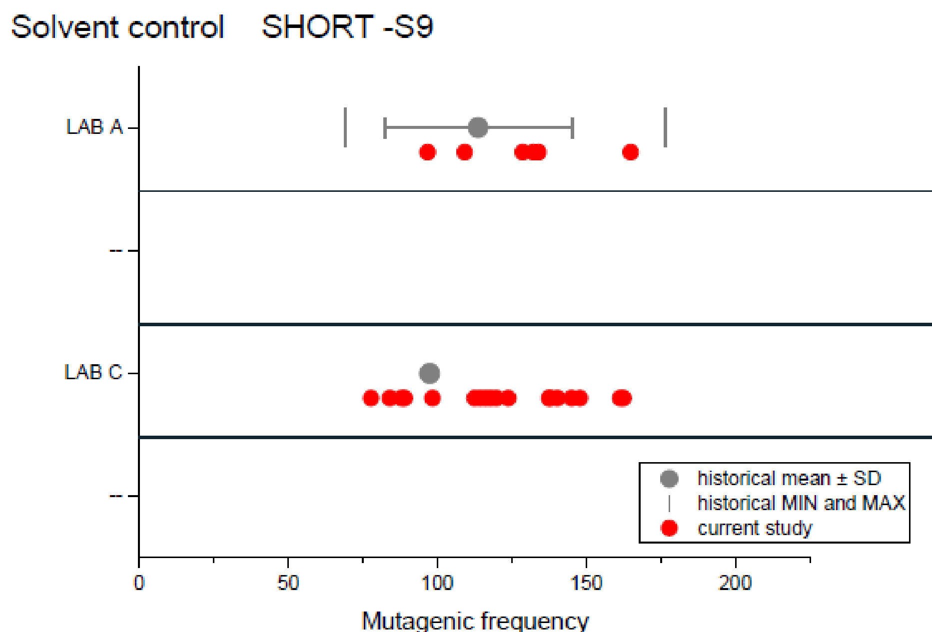


Figure 3: Solvent control MF distributions (-S9)

7.4 Positive control MF distributions (-S9)

For the two laboratories (A and C) that carried out the -S9 assay, their positive controls produced induced MFs above the OECD recommended value, i.e. an increase above background by at least 300 mutants/10^6 viable cells, and were generally in a similar range to their own historical control ranges.

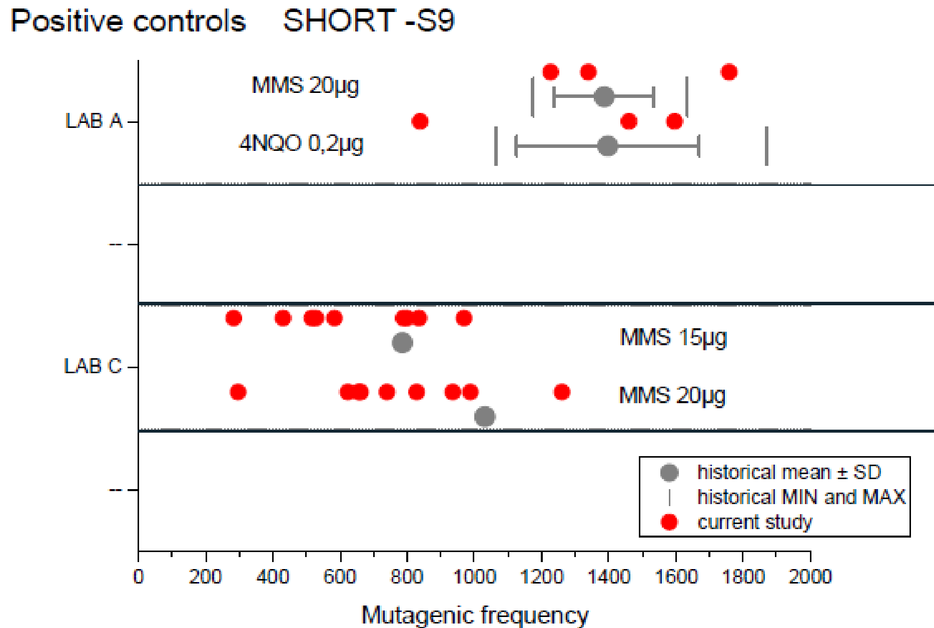
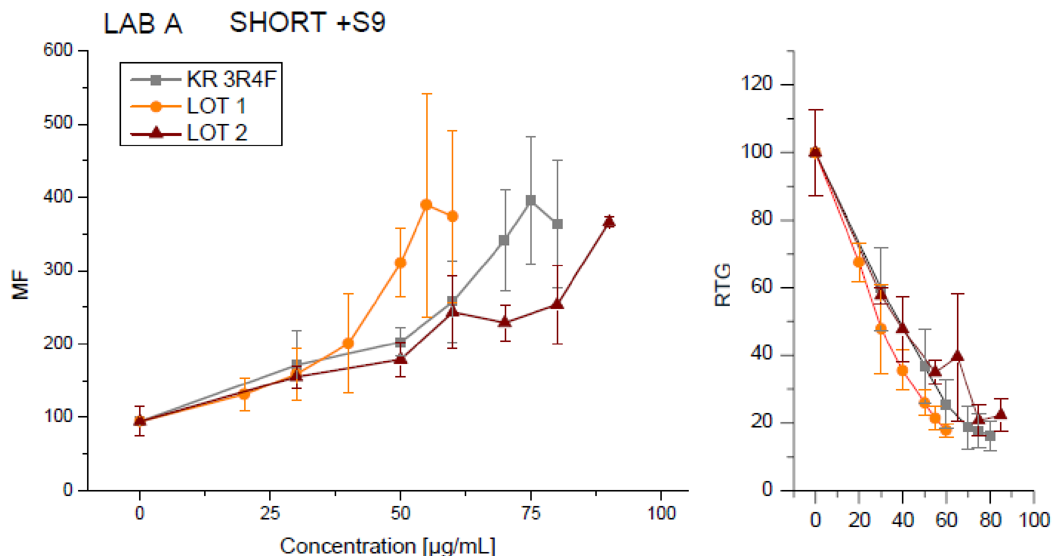


Figure 4: Positive control MF distributions (-S9)

7.5 Concentration-MF and RTG response relationships (+S9)

All laboratories observed reproducible, concentration-dependent increases in mutagenicity (increase in MF) and cytotoxicity (decrease in RTG) up to the cytotoxicity limit of the assay, i.e. RTG 10-20 %. In general, this limit was achieved at TPM concentrations between 60-150 µg/ml. The concentration-MF and RTG responses observed for all test pieces in laboratories A, B and C would be considered as mutagenic according to the evaluation criteria described in OECD 490, i.e. they produced an induced MF greater than 126 mutants/10^6 viable cells (the global evaluation factor).



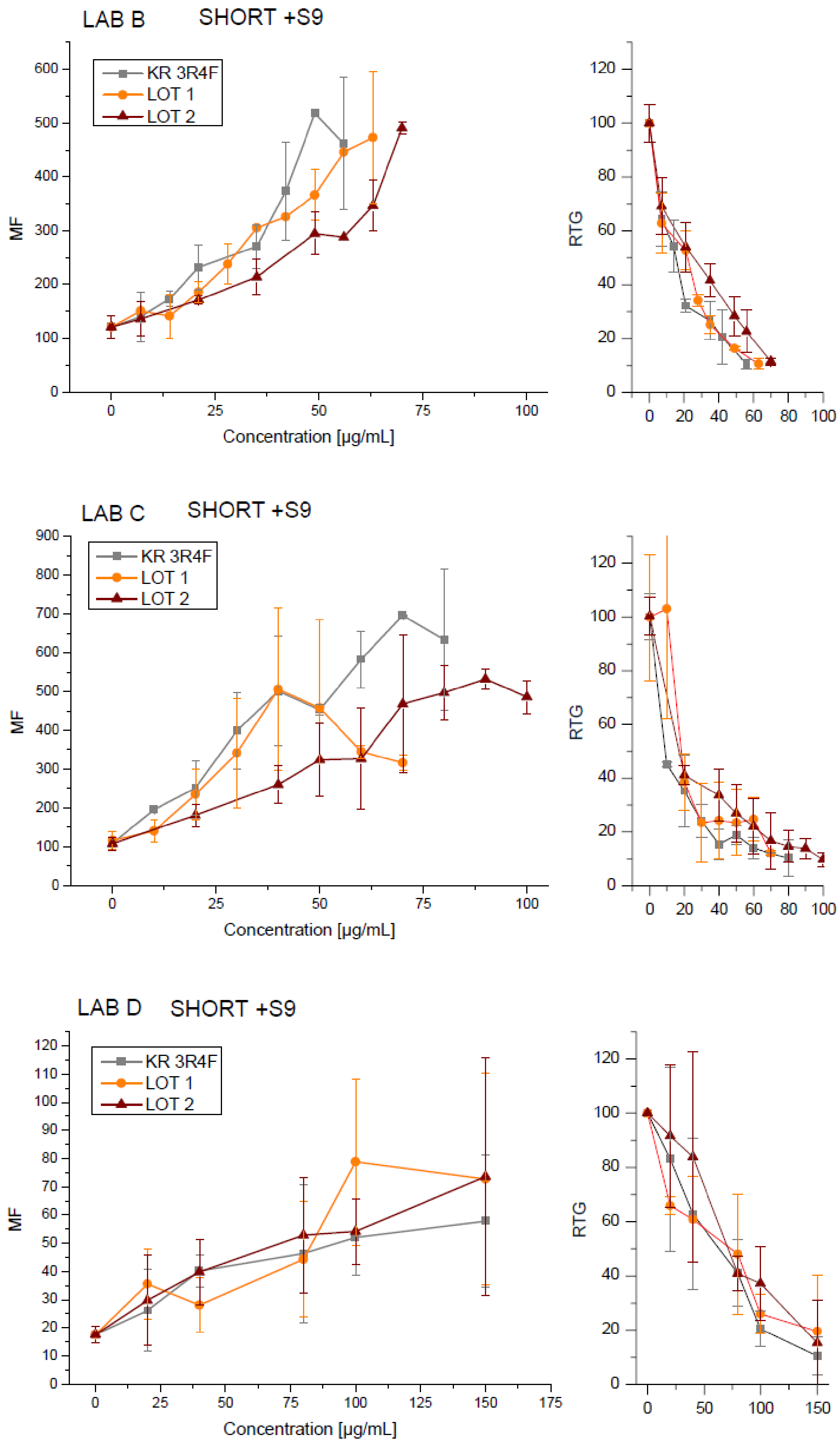


Figure 5: Concentration-MF and RTG response plots (+S9)

7.6 Concentration-MF and RTG response relationships (-S9)

The two laboratories (A and C) that carried out the -S9 assay observed reproducible, concentration-dependent increases in mutagenicity (increase in MF) and cytotoxicity (decrease in RTG) up to the cytotoxicity limit of the assay, i.e. RTG 10-20 %. In general, this limit was achieved at TPM concentrations between 60-80 µg/ml. The concentration-MF responses observed for all test pieces in laboratories A and C would be considered as mutagenic according to the evaluation criteria described in OECD 490 with the exception of LOT 1 in laboratory C.

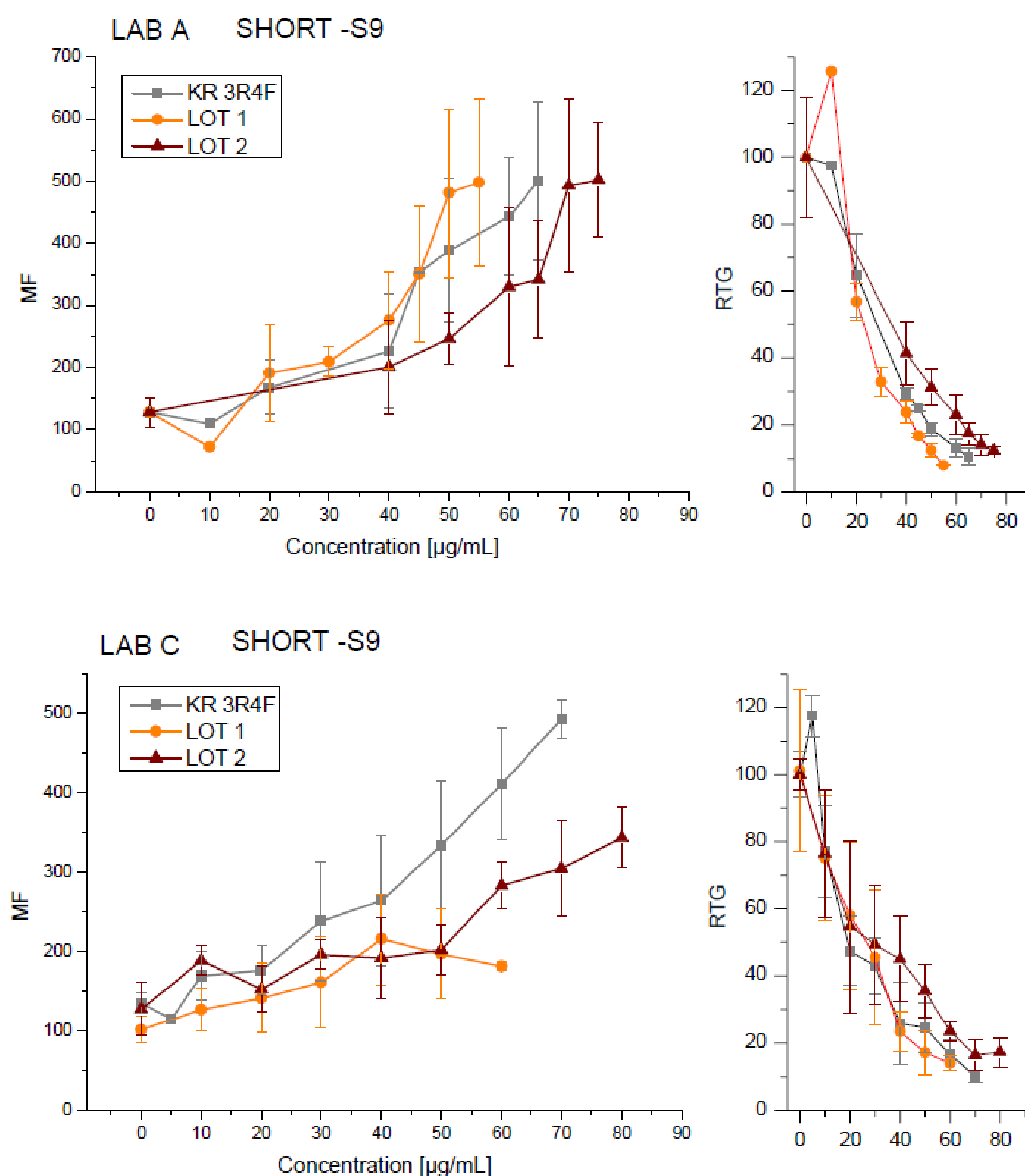


Figure 6: Concentration-MF and RTG response plots (-S9)

7.7 Ranking of mutagenic potencies (+S9)

The data produced in laboratories A, B and C revealed that the TPM derived from LOT 2 was consistently the least potent mutagen in the study, although this apparent difference was not determined to be statistically significant. It was not possible to rank the mutagenic potencies of LOT 1 and LOT 3 as a result of statistically non-significant findings. A different pattern of mutagenic potencies was found by Laboratory D but, again, any apparent differences were not statistically significant.

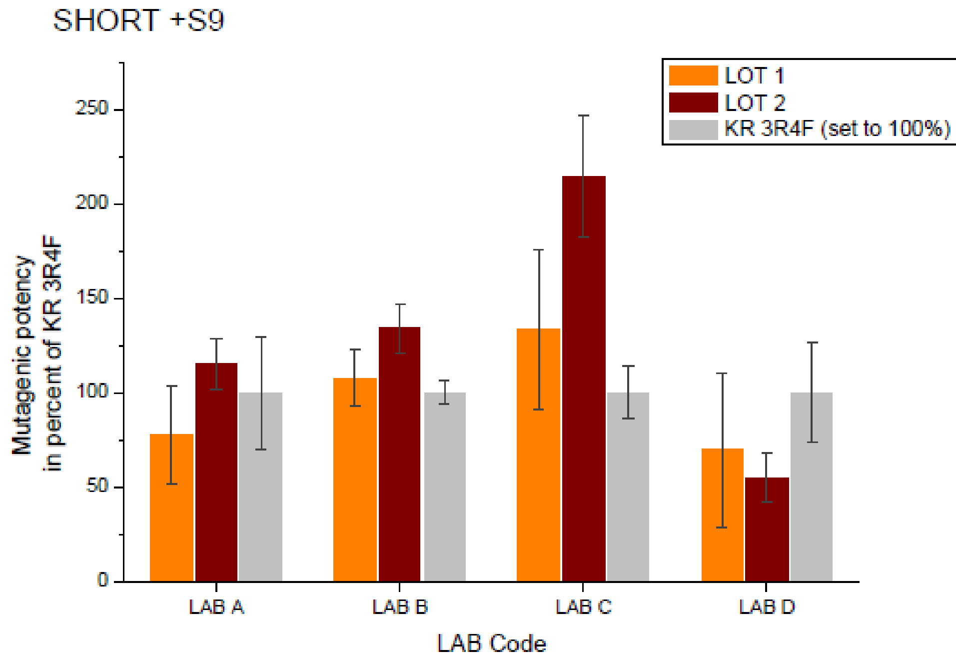


Figure 7: Ranking of mutagenic potency plots (+S9)

7.8 Ranking of mutagenic potencies (-S9)

The data produced in laboratories A and C was generally similar and revealed that the TPM derived from LOT 2 was consistently the least potent mutagen, although this apparent difference was not determined to be statistically significant. It was again not possible to rank the mutagenic potencies of LOT 1 and LOT 3 as a result of statistically non-significant findings.

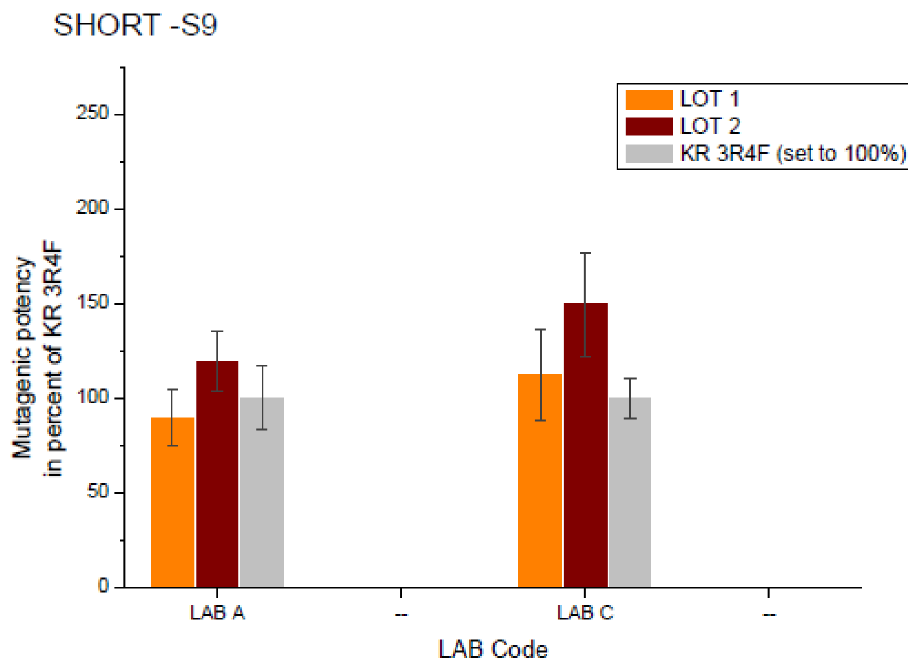


Figure 8: Ranking of mutagenic potency plots (-S9)

7.9 Summary of mutagenic potency ranking

Summary of mutagenic potency ranking is shown in Table 8.

Table 8: Summary of mutagenic potency ranking

		↑ high		mutagenicity		low ↓
		Rank 1		Rank 2		Rank 3
LAB A	SHORT +S9	LOT 1	=	KR 3R4F	=	LOT 2
	SHORT -S9	LOT 1	=	KR 3R4F	=	LOT 2
LAB B	SHORT +S9	KR 3R4F	=	LOT 1	=	LOT 2
	SHORT -S9	*not tested*				
LAB C	SHORT +S9	KR 3R4F	=	LOT 1	=	LOT 2
	SHORT -S9	KR 3R4F	=	LOT 1	=	LOT 2
LAB D	SHORT +S9	LOT 2	=	LOT 1	=	KR 3R4F
	SHORT -S9	*not tested*				

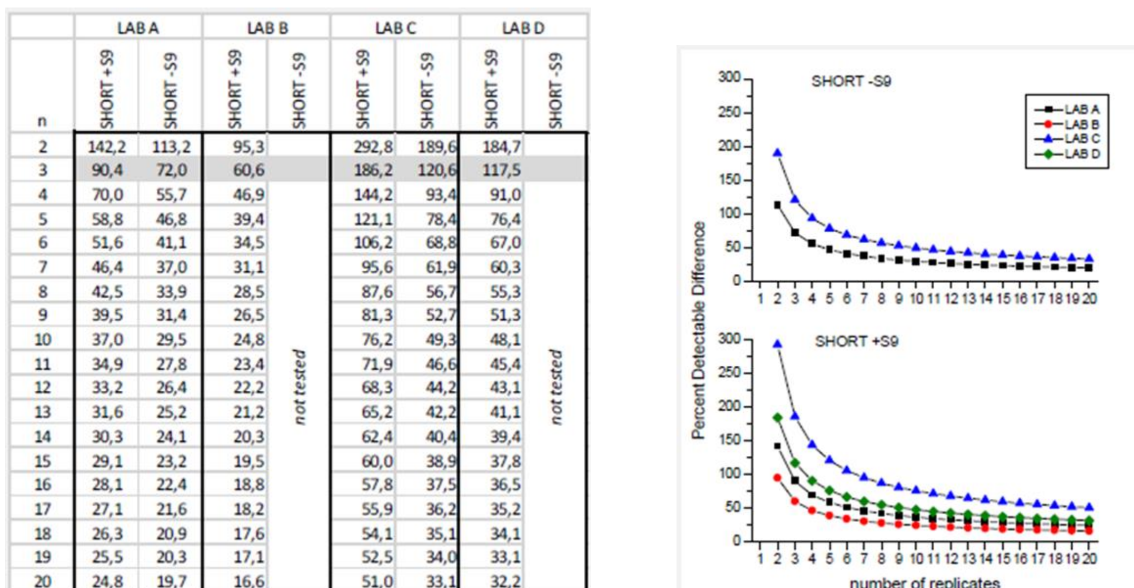
7.10 Statistical power – minimum detectable difference (MDD)

Using a two-tailed t-Test, the question can be answered how different the mean mutagenic potential values of two samples must be to detect a significant difference. The sample size is three replicates (n = 3), the significance level is set to α = 0.05 and we want to have a 90 % chance (power) to detect a difference, if one exits.

The following formula {F1} is used to calculate the MDD. The appropriate t parameters are derived from the t-distribution table and the variances (s^2) are pooled across samples.

$$\{F1\} \quad \delta \geq \sqrt{\frac{2s_p^2}{n}}\left(t_{\alpha,\upsilon} + t_{\beta(1),\upsilon}\right)$$

In the following, MDD plots are displayed for all treatments. In addition, the results are displayed in the Table.



	LAB A		LAB B		LAB C		LAB D	
n	SHORT +S9	SHORT -S9	SHORT +S9	SHORT -S9	SHORT +S9	SHORT -S9	SHORT +S9	SHORT -S9
2	142,2	113,2	95,3	*not tested*	292,8	189,6	184,7	*not tested*
3	90,4	72,0	60,6		186,2	120,6	117,5	
4	70,0	55,7	46,9		144,2	93,4	91,0	
5	58,8	46,8	39,4		121,1	78,4	76,4	
6	51,6	41,1	34,5		106,2	68,8	67,0	
7	46,4	37,0	31,1		95,6	61,9	60,3	
8	42,5	33,9	28,5		87,6	56,7	55,3	
9	39,5	31,4	26,5		81,3	52,7	51,3	
10	37,0	29,5	24,8		76,2	49,3	48,1	
11	34,9	27,8	23,4		71,9	46,6	45,4	
12	33,2	26,4	22,2		68,3	44,2	43,1	
13	31,6	25,2	21,2		65,2	42,2	41,1	
14	30,3	24,1	20,3		62,4	40,4	39,4	
15	29,1	23,2	19,5		60,0	38,9	37,8	
16	28,1	22,4	18,8		57,8	37,5	36,5	
17	27,1	21,6	18,2		55,9	36,2	35,2	
18	26,3	20,9	17,6		54,1	35,1	34,1	
19	25,5	20,3	17,1		52,5	34,0	33,1	
20	24,8	19,7	16,6		51,0	33,1	32,2	

Figure 9: Minimal detectable difference (tabular and graphical forms).

8. Conclusion

Three of the four participating laboratories (A, B and C) produced OECD TG 490-compliant solvent and positive control data and reported mutagenic findings for the TPM derived from the three test pieces in the +S9 condition. Although the control data from laboratory (D) were not congruent with the acceptance criteria recommended in OECD TG 490, they were in line with its own historical control ranges. However, laboratory D did report multi-fold increases in MF above background for the three test pieces. Both laboratories (A and C) that participated in the -S9 condition also produced OECD TG 490-compliant solvent and positive control data and reported mutagenic findings for the TPM derived from two test pieces (LOT 2 and LOT 3) but LOT 1 failed to meet the criteria for a mutagenic response, i.e. an induced MF more than the GEF (126 mutants/10^6 viable cells), in laboratory C. While LOT 2 was consistently the least potent mutagen across laboratories A, B and C in the +S9 condition, this effect as well as the mutagenic effects induced by the other two lots (LOT 1 and LOT 3) were not statistically significant. Similarly, the data generated by laboratory D (+S9) and laboratories A and C in the -S9 condition were also not determined to be statistically significantly different. The lack of statistically significant findings between the responses induced by the test pieces across this study (±S9 conditions) meant that the ranking of mutagenic potencies was not possible. The data however were used to calculate the MDD of each laboratory's MLA (+S9); for two test pieces evaluated across three replicates, the median MDD varied from 90-100 % between the four laboratories.

9. References

[1] OECD Guideline for the Testing of Chemicals, TG 490: *In Vitro* Mammalian Cell Gene Mutation Tests Using the Thymidine Kinase Gene. Adopted 29 July 2016.

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