# Using the Mouse Lymphoma TK Assay to Compare the Mutagenicity of Mainstream Smoke Condensate from Research Cigarettes

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## Introduction

The mouse lymphoma TK assay (MLA) has been widely used by the chemical and pharmaceutical industry as an in vitro mammalian cell genotoxicity test to determine the genotoxic potential of new compounds. The assay measures the induction of forward mutations at the heterozygous thymidine kinase (*tk*) locus in mouse lymphoma cells, which results in the loss of thymidine kinase activity and, thus, the acquisition of trifluorothymidine resistance.

The MLA has now been established and optimized in our laboratory to quantitatively determine the in vitro genotoxicity of mainstream smoke condensate (MSC) in order to complement our existing testing battery.

The assay was validated with the positive control substances methyl methanesulfonate (MMS) and benzo(a)pyrene (B(a)P) and MSC from the Reference Cigarette 1R4F in the absence and in the presence of a metabolic activation system.

To determine whether the MLA can be used to discriminate different cigarette types, the mutagenicity of MSC from 5 research cigarettes containing different tobacco blends was compared.

## Materials and Methods

### Cigarettes

- University of Kentucky Reference Cigarette 1R4F
- 5 research cigarettes with different tobacco blends
  - Bright (100% Bright blend)
  - Burley (100% Burley blend)
  - Oriental (100% Oriental blend)
  - RS/Bright (50% Reconstituted Sheet/50% Bright)
  - Blend (35% Bright, 23% Burley, 15% Oriental, 27% Reconstituted Sheet)

### Mainstream Smoke Condensate

- Cigarettes smoked on 30-port smoking machine in basic conformity with ISO standards
- MSC collected in glass impaction traps, dissolved in dimethyl sulfoxide, and stored immediately after preparation at -75 °C
- For the research cigarettes, 2 batches of MSC, each prepared, assayed, and evaluated individually
- MSC yields
  - 10.4 mg per cigarette for the 1R4F
  - 22 to 35 mg per cigarette for the research cigarettes

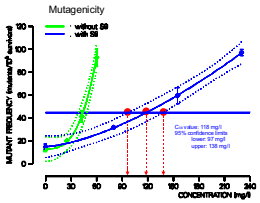
### Mouse Lymphoma TK Assay (MLA)

- Microtiter plate version of the MLA (Clive et al., 1972; Cole et al., 1986)
- Mouse lymphoma cell line L5178Y/$tk^{+/-}$-3.7.2C, growing in suspension
- Metabolic promutagen activation by S9 of livers from rats treated with Aroclor 1254, optimized S9 concentration of 5% during treatment
- 3 concentrations assayed per MSC batch, without and with metabolic activation; concentrations selected to cover a range from minimum to maximum cytotoxicity
- Cells exposed for 4 h, 1 cell culture per test substance concentration
- Cytotoxicity (e.g., relative total growth) of test substance determined after exposure
- After an expression period of 3 days, cells seeded for mutant frequency determination, mutant cells selected with trifluorothymidine

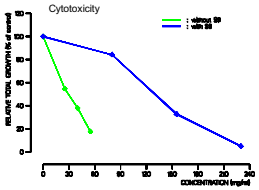
### Comparison of Mutagenicity

- Dose-response curve for the mutant frequency calculated by nonlinear regression analysis with the power function $y = a + bx^c$
- Calculation of the concentration for the effect level of 3 times the background (spontaneous) mutant frequency ($C_{3B}$) from the dose-response curves; confidence limits for the $C_{3B}$ values derived from the 95% confidence limits of the curves
  - the lower the $C_{3B}$ value the higher the mutagenic activity
- Comparison by testing whether the confidence limits of the $C_{3B}$ values overlap
- Mutagenicity of MSC from 2 cigarette types considered to be different if the confidence limits of the $C_{3B}$ values for each MSC batch do not overlap
- Inverse $C_{3B}$ values ($1/C_{3B}$) and confidence limits are given
  - the higher the $1/C_{3B}$ value the higher the mutagenic activity
  - inverse $C_{3B}$ values do not change the comparison results

Response to 1R4F MSC



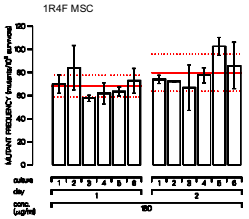

Remarks: mean ± SE, N = 3 microtiter plates
dashed lines: 95% confidence limits
horizontal line: 3 times background mutant frequency




## Results

### Reproducibility

**Intraday variation**: Reference Cigarette 1R4F and negative control

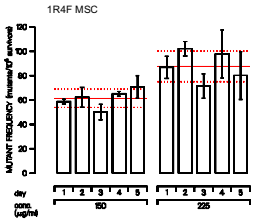



Remarks: mean ± SD, N = 3 microtiter plates
red lines: mean of 6 cell cultures ± SD

| Parameter | Mutant Frequency Mean (mut./$10^5$ surv.) | Coefficient of Variation (%) |
|---|---|---|
| solvent DMSO (1%), +S9 | | |
| day 1 | 19.0 | 10.3 |
| day 2 | 21.0 | 17.6 |
| test substance 1R4F MSC, +S9 | | |
| 180 mg/l: day 1 | 68.2 | 13.9 |
| day 2 | 79.8 | 16.1 |

- 2 experimental days
- 1 concentration for MSC and for negative control
- 6 cell cultures per concentration and day

**Interday variation**: Reference Cigarette 1R4F, negative and positive controls



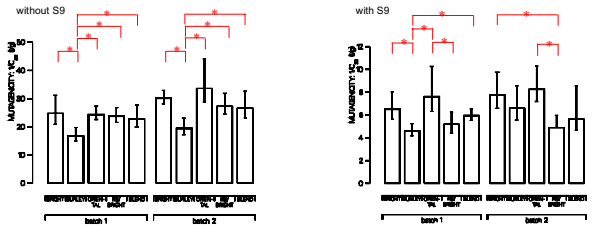

Remarks: mean ± SD, N = 3 microtiter plates
red lines: mean of 5 days ± SD

| Parameter | Mutant Frequency Mean (mut./$10^5$ surv.) | Coefficient of Variation (%) |
|---|---|---|
| solvent DMSO (1%), -S9 | 20.3 | 11.4 |
| +S9 | 23.1 | 6.2 |
| positive control MMS (20 mg/l), -S9 | 326 | 12.3 |
| B(a)P (2 mg/l), +S9 | 230 | 6.5 |
| test substance 1R4F MSC, +S9 | | |
| 150 mg/l | 61.4 | 12.4 |
| 225 mg/l | 87.7 | 14.3 |

- 5 experimental days
- 2 concentrations for MSC and 1 concentration for negative and positive controls
- 1 cell culture per concentration and day

- Reproducible responses for negative and positive control substances and for 1R4F MSC (coefficient of variation less than 20%)

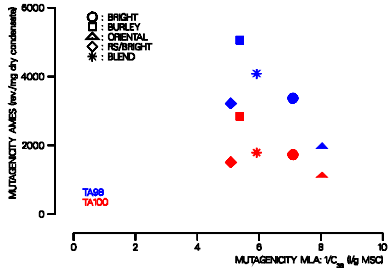
### Comparison of Mutagenicity of Research Cigarettes




Remarks: $1/C_{3B}$ value ± 95% confidence limits
*: confidence limits of $C_{3B}$ values do not overlap

- Without S9: Burley MSC different from other blends (up to 40% lower)
- With S9: RS/Bright MSC different from Oriental MSC (up to 40% lower)

### MLA Mutagenicity vs Ames Mutagenicity




Remarks: with S9
$1/C_{3B}$ values (MLA) and mean (Ames) from 2 batches of MSC for each cigarette type

- Ames Assay: mutagenic activity of Burley MSC higher than other blends in strains TA98 and TA100 with S9 (T.J. Meisgen, unpublished results)
- Ranking of mutagenic activity of single blends
  - MLA: Oriental > Bright > Burley
  - Ames: Burley > Bright > Oriental
- MLA: ranking of Bright and Burley in accordance with results of the mouse skin painting assay, where Bright condensate was more tumorigenic than Burley condensate (Wynder and Hoffmann, 1963).

## Conclusions

- The mouse lymphoma TK assay can be used to discriminate mainstream smoke condensate from cigarettes containing different tobacco blends.
- The converse results obtained in the 2 assays (MLA vs Ames) indicate the clear need for a genotoxicity testing battery of complementary assays for the comprehensive testing of cigarette smoke.

**References**
Clive, D., Flamm, W.G., Machesko, M.R., Bernheim, N.J., Mutat. Res. 16: 77-87 (1972)
Cole, J., Muriel, W.J., Bridges, B.A., Mutagenesis Vol. I (2): 157-167 (1986)
Wynder, E.L., Hoffmann, D., Dtsch. Med. Wochenschr. 88: 622-628 (1963)

SOT 2002, Nashville, TN, U.S.A.