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Bacterial Reverse Mutation Assay for Mainstream Tobacco Smoke

Canada

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Page: 1 of 19

1 SCOPE OF APPLICATIONS

- 1.1 This document sets out how to test the total particulate matter (TPM) from mainstream tobacco smoke in the Bacterial Reverse Mutation Assay (Ames Assay) with the pre-incubation method.

2 NORMATIVE REFERENCES

- 2.1 Organization for Economic Co-operation and Development. Guideline for testing chemicals OECD No. 471: Bacterial Reverse Mutation Assay. July 21, 1997.
- 2.2 Health Canada Official Method T-115. Determination of Tar, Water, Nicotine and Carbon Monoxide in Mainstream Tobacco Smoke. Second Edition. 2003. (Parts applicable to manufactured cigarettes only)
- 2.3 Health Canada Official Method T-304. Determination of Humectants in Whole Tobacco. Second Edition. 2003.
- 2.4 International Organization for Standardization. Standard ISO 4387. Cigarettes - Determination of total and nicotine-free dry matter using a routine analytical smoking machine. Third Edition. 2000.
- 2.5 Maron, D.M. and Ames, B.N. Revised Methods for the *Salmonella* Mutagenicity Test. *Mutation Research*, 113:173-215, 1983.
- 2.6 Mortelmans, K. and Zeiger, E. The Ames *Salmonella*/Microsome Mutagenicity Assay. *Mutation Research*, 455: 29-60, 2000.

3 DEFINITIONS AND ABBREVIATIONS

- 3.1 **Ames Assay:** A bacterial mutagenicity assay, also known as the "Bacterial Reverse Mutation Assay", "*Salmonella* Mutagenicity Assay", "*Salmonella* Mammalian Microsome Mutagenicity Assay" or "*Salmonella typhimurium* Reverse Mutation Assay". The *Salmonella typhimurium* histidine (his) reversion system is a microbial assay that detects mutation in a gene of a histidine auxotroph to produce a wild type via base-pair and/or reading-frame changes in the DNA sequence of the *Salmonella typhimurium* bacterium.
- 3.2 **Aseptic technique:** Procedures used to keep cultures, instruments, media and samples free of microbial contamination.
- 3.3 **Base-pair substitution:** A substitution of one pair of nitrogenous bases for another in a DNA sequence.
- 3.4 **Cofactor:** A non-protein organic substance that binds to a protein in order to form an active enzyme.
- 3.5 **DMSO:** Dimethylsulphoxide
- 3.6 **Glass fibre filter disc (pad):** A pad used to collect particulate matter from tobacco smoke.
- 3.7 **Histidine (his):** An essential amino acid required, in the case of this bioassay, for the growth of *Salmonella* bacteria.

- 3.8 Mutagen:** A substance that has been shown to cause permanent changes in the coding sequence of DNA and/or permanent changes in the chromosomal structure.
- 3.9 Mutant:** An organism differing from the parental strain as a result of mutation.
- 3.10 Mutation:** The process producing a gene or a chromosome differing from the wild type.
- 3.11 Pre-incubation assay:** This is the modified Ames assay procedure, in which the mixture of bacterial tester strains, the test compound and the S9 metabolic activation mix or phosphate buffer is incubated at 37°C for 20 minutes before being added to the top agar and over-layered on the minimal glucose agar plates.
- 3.12 R-factor:** A plasmid containing the damage-inducible genes MucA/B, as well as a gene for antibiotic resistance. The MucA/B genes increase the susceptibility of *Salmonella* to DNA – damaging agents.
- 3.13 Replicate:** A TPM preparation generated by an independent smoking of cigarettes taken from the same cigarette sample.
- 3.14 Revertant:** A wild type bacterium that was formerly an auxotroph mutant.
- 3.15 rfa mutation:** A mutation that results in increased permeability of the bacterial cell wall to large molecules.
- 3.16 *Salmonella typhimurium* tester strain:** The genetically altered *Salmonella typhimurium* bacteria used in this assay.
- 3.17 S9 rat liver fraction:** The supernatant of liver homogenates prepared from male rats exposed to an enzyme-activity-inducing substance (such as Aroclor-1254, phenobarbitone or β -naphthoflavone). The fraction permits in vitro simulations of the metabolic activation reactions that are ordinarily restricted to mammalian liver.
- 3.18 S9 mix:** The combined mixture of S9 rat liver fraction and other co-factors, which provides a metabolic activation system in this assay.
- 3.19 Spontaneous mutation:** A mutation not caused by addition of the test or control substance.
- 3.20 Total particulate matter:** That portion of the mainstream smoke which is trapped on the glass fibre filter disc (pad).
- 3.21 uvrB mutation:** A mutation resulting in bacteria that are abnormally sensitive to a variety of physical and chemical DNA-damaging agents, including ultraviolet light.

4 METHOD SUMMARY

- 4.1** Mainstream smoke of cigarettes (e.g. 20 cigarettes), smoked on a rotary smoking machine under modified (intense) ISO conditions, is trapped onto either a 44 mm or 92 mm diameter glass fibre filter disc (pad), as per Appendix 1. (See note in Appendix regarding the size of the filter pad.) The number of replicates is as per the applicable regulations.
- 4.2** The total particulate matter trapped on a pad is extracted with dimethylsulphoxide (DMSO) to achieve a target concentration of 10 mg TPM/mL of DMSO, as per Appendix 2.
- 4.3** Samples of TPM are further characterized (water, nicotine, tar, glycerol, menthol and propylene glycol, where applicable), as per Appendix 3.

- 4.4 In the pre-incubation method, developed by Yahagi et al (1975), a histidine-dependent (his⁻) mutant strain of *Salmonella typhimurium* is mixed with a testing chemical (which in this case is the TPM mixture) in the presence or absence of rat liver enzymes (S-9 mix). Molten soft agar (top agar) is then added and the mixture is over-layered on top of minimal glucose agar. After the agar solidifies, the plate is incubated at 37 ± 1°C for 48 - 72 hours.

Note: The pre-incubation modification of Ames Assay shows increased sensitivity for some chemicals over the plate incorporation method.

- 4.5 The colonies, which are histidine independent (his⁺) wild type revertants, are counted.
- 4.6 The number of replicates is as per the applicable regulations.

Warning: The testing and evaluation of certain products against this test method may require the use of materials and or equipment that are potentially hazardous and this document does not purport to address all the safety aspects associated with its use. Anyone using this test method has the responsibility to consult with the appropriate authorities and to establish health and safety practices in conjunction with all existing applicable regulatory requirements prior to its use.

5 APPARATUS AND EQUIPMENT

- 5.1 Equipment needed for collection of TPM as per Appendix 1.
- 5.2 Equipment needed for preparation of TPM as per Appendix 2.
- 5.3 Equipment needed for further characterization of TPM as per Appendix 3.
- 5.4 All appropriate apparatus and equipment needed to perform the Ames Assay. Refer to Maron and Ames (1983) and Mortelmans and Zeiger (2000) for details.

6 REAGENTS AND SUPPLIES

Note: All reagents shall be free of mutagens or, at least, should not increase the rate of spontaneous mutation. Use analytical grade chemicals whenever possible.

- 6.1 For the requirement of chemicals, refer to Maron and Ames (1983) and Mortelmans and Zeiger (2000).
- 6.2 *Salmonella typhimurium* tester strains TA98, TA100, TA102, TA1535, TA1537
- 6.3 Reagents and Supplies needed for collection of TPM as per Appendix 1
- 6.4 Reagents and Supplies needed for preparation of TPM as per Appendix 2.
- 6.5 Reagents and Supplies needed for further characterization of TPM as per Appendix 3.

7 PREPARATION OF GLASSWARE AND PLASTIC WARE

- 7.1 Glassware and plastic ware should be cleaned and sterilized.
- 7.2 Cleaning required for collection of TPM as per Appendix 1
- 7.3 Cleaning required for preparation of TPM as per Appendix 2.
- 7.4 Cleaning required for further characterization of TPM as per Appendix 3.

8 PREPARATION OF SOLUTIONS AND MEDIA

- 8.1 Potential carcinogens should be prepared in a fume hood with caution appropriate for this type of hazardous material.
- 8.2 Refer to Maron and Ames (1983) for the preparation of solutions and media required for the assay.
- 8.3 Prepare positive control solutions required for the analysis. For example, refer to Mortelmans and Zeiger (2000).
- 8.4 For solutions needed for the further characterization of TPM, refer to Appendix 3.

9 TESTER STRAINS

9.1 Bacterial Tester Strain Cultures

- 9.1.1 Obtain *Salmonella typhimurium* tester strains TA98, TA100, TA102, TA1535, TA1537.

9.2 Preparation of Tester Strain Cultures

- 9.2.1 Refer to Mortelmans and Zeiger (2000) for culture preparation, storage, testing, purification and quality control procedures for tester strains.
- 9.2.2 Refer to Maron and Ames (1983) for the methodology to confirm the genotypes of the tester strains with respect to the following:
- Histidine Requirement
 - *rfa* Mutation
 - *uvrB* Mutation
 - Presence of R-factor (Ampicillin and Ampicillin-Tetracycline Resistance)

10 COLLECTION OF TPM

- 10.1 Refer to Appendix 1: Collection of TPM on glass fibre filter disc.

11 PROCESSING OF TPM

- 11.1 Refer to Appendix 2: Preparation of TPM from glass fibre filter disc.
- 11.2 Samples of TPM are further characterized as per Appendix 3.

12 MUTAGENICITY TESTING

- 12.1 Follow standard aseptic procedures for microbiological investigations.
- 12.2 Preparation of Fresh Overnight Bacterial Culture for Mutagenicity Assay
- 12.2.1 Prepare tester strain culture to a density of $1-2 \times 10^9$ bacteria per mL as described by Maron and Ames (1983).
- 12.2.2 The bacterial density of the culture used should be estimated by measuring the optical density at 650 nm and by bacterial viability count.

12.2.3 Confirm the tester strain culture density by bacterial viability count as follows:

- Perform serial dilutions by mixing appropriate quantity of bacterial culture (e.g. 100 µL of bacterial culture) and nutrient broth (e.g. 900 µL of nutrient broth) and plate the appropriate dilution on nutrient agar plates.
- Incubate the plates at $37 \pm 1^\circ\text{C}$ and, after 24 hours, count the number of colonies either manually or using an appropriate automated counting device.

12.2.4 Maintain the culture(s) at room temperature (i.e. $22 \pm 2^\circ\text{C}$) during the assay.

12.3 Assay (Pre-incubation Method):

12.3.1 To prepare for the assay, perform the following on the day of the assay:

- Prepare 5% S9 mixture as follows, adding the ingredients in the order indicated:

Ingredients	(per 50 mL)
Sterile deionized water	19.25 mL
0.2M Phosphate Buffer, pH 7.4	25.00 mL
0.1M Nicotine Adenine Dinucleotide Phosphate (NADP) solution	2.00 mL
1M Glucose-6-Phosphate solution	0.25 mL
0.4M Magnesium Chloride (MgCl_2)+1.65M Potassium Chloride (KCl) solution	1.00 mL
S9 rat liver fraction	2.50 mL

- Prepare Top Agar as per Maron and Ames (1983).
- Remove samples from the cryofreezer (-70°C or below) and thaw at room temperature.
- Remove required controls from storage (freezer/refrigerator) and equilibrate to room temperature.
- Label all tubes and plates needed for samples and controls for the assay.

12.3.2 Prepare reaction mixture in a sterile pre-labelled reaction tube by adding appropriate amount of phosphate buffer or S9 Mix, appropriate amount of either solvent or TPM sample or positive control solution and fresh overnight bacterial culture. Refer to OECD No. 471 (1997) for details.

Note: The dosing protocol must include at least one concentration which is in the toxicity range. At least seven (7) non-zero concentrations are to be employed. The actual concentrations cannot be specified in advance, as they are dependent on the nature of the sample under investigation.

Note: It has been observed that for TPM mixture prepared from “typical” commercially available Canadian flue-cured tobacco cigarettes, concentrations of 0, 25, 50, 75, 100, 125, 250, 500 µg per plate will generally give a satisfactory response. For atypical cigarettes, a range finding experiment will be necessary.

12.3.3 Incubate the tubes in the shaker incubator (100-120 rpm) for 20 ± 2 min. at $37 \pm 1^\circ\text{C}$.

12.3.4 Record the presence of precipitates in the reaction mixture, if any.

12.3.5 After incubation, add 2 mL of top agar to each reaction mixture tube and vortex to mix.

12.3.6 Overlay the mixture of top agar and reaction mixture on top of minimal glucose agar.

Note: Complete the above two steps within 20 seconds.

12.3.7 Prepare three plates for each concentration.

12.3.8 After the overlay has solidified, invert the plates and incubate at $37 \pm 1^\circ\text{C}$ for 48 – 72 hrs.

12.4 Procedure for Counting Revertant Colonies:

Note: Normal practice is to consider the colony counts obtained from three plates at each concentration when samples are examined for evidence of mutagenicity.

12.4.1 Examine the plate after incubation for background lawn and count the number of revertant colonies.

12.4.2 Record the number of revertant colonies for individual plates.

12.4.3 Record any evidence of thinning or scarcity of the bacterial lawn.

12.4.4 Record the presence/absence of any precipitation found on assay plate.

13 QUALITY CONTROL AND DOCUMENTATION

13.1 Chemicals and Media

13.1.1 Verify and record the sterility of media, reagents and solutions as per good laboratory practice for microbiology laboratories. Verify the performance characteristics of the control solutions.

13.2 Genotypes of the Tester Strains

13.2.1 All the genotypic characteristics of a strain as indicated in this Official Method must be present. Please refer to 9.2.2.

13.3 Laboratory Controls

13.3.1 To assess the overall performance of the analysis, a Kentucky Reference 2R4F control cigarette must be included in the sample. (The results of the control cigarette may be compared, using appropriate statistical techniques, to “expected values” generated by the laboratory or, if none exist, to values found in literature. This will provide information to the laboratory on test accuracy and precision.)

13.3.2 Each analysis should include positive and solvent controls in triplicate for each tester strain used.

13.3.3 Triplicate plates treated with solvent control solution, with and/or without metabolic activation, are used to define background counts (i.e. negative controls) as per OECD No. 471 (1997).

Note: For example, where DMSO is used the concentration of DMSO should not exceed 4% v/v.

13.3.4 When the S9 mix is used, a control plate without the S9 mix must also be included.

13.4 Evaluation of Negative controls

13.4.1 The tester strain is mixed with the top agar and seeded on a minimal glucose agar in triplicate at the same time that the assay is performed. The number of spontaneous revertant colonies is recorded. This number should be checked frequently.

Note: The average colony count for the triplicate plates must fall within a pre-defined range determined in an appropriate manner (see table 1 below for an example).

Note: It is incumbent upon each laboratory to determine and to communicate acceptability ranges for each bacterial culture using appropriate statistical methodologies. One possibility would be to determine the 5th and 95th percentiles from control data accumulated over several months of testing. These would then become the lower (5th percentile) and the upper (95th percentile) bounds for acceptability.

Table 1: Typical Ranges for Background Colony Counts

<i>Salmonella typhimurium</i> Strain	Typical Colony Count Range for Negative (solvent) Control
TA98	20 - 50
TA100	70 - 200
TA102	150 - 400
TA1535	5 - 20
TA1537	5 - 20

13.5 Evaluation of Positive Controls

13.5.1 Triplicate culture plates are also to be prepared and treated with a specific concentration of a chemical known to give a demonstrable response as per OECD No. 471 (1997) when evaluated at the same time as test samples (see Table 2 below). The average colony count for positive control replicate plates must exhibit at least a three-fold increase over that of the respective average negative control for each bacterial strain. Table 2 also lists some ranges for typical revertant counts per plate that would be seen under normal practice of the method using the selected strain and chemical control combinations.

Table 2: Typical Response Ranges for Positive Chemical Controls

Bacteria Strain	S9 Activation	Positive Control Substance	Concentration (µg/plate)	Typical Range (revertants/plate)
TA98	+S9	2-aminoanthracene	2	1250 - 2100
TA100	+S9	2-aminoanthracene	2	1250 - 2100
TA1535	+S9	2-aminoanthracene	4	300 - 600
TA1537	+S9	2-aminoanthracene	4	300 - 600
TA102	+S9	2-aminoanthracene	7.5	1450 - 2100
TA98	-S9	2-nitrofluorene	4	500 - 1750
TA100	-S9	sodium azide	1	400 - 600
TA1535	-S9	sodium azide	1	400 - 600
TA1537	-S9	9-aminoacridine	100	400 - 750
TA102	-S9	Mitomycin C	0.5	1150 - 1600

13.6 Bacterial Growth

13.6.1 The density of the bacterial culture used for the assay should be a minimum of 1×10^9 viable bacteria per mL of nutrient broth.

13.6.2 The bacterial background lawn should be routinely examined using a microscope under 'low power' as an aid to the identification of toxicity. Scarcity of the bacterial lawn usually indicates that the testing concentration is beyond an acceptable range.

14 STATISTICAL ANALYSIS AND INTERPRETATION OF RESULTS

14.1 Evaluate Plate-to-Plate Variation within an Assay

14.1.1 The criteria used for this test is based on the properties of the chi-square distribution:

$$(\chi^2), \text{ where } (n-1) \frac{s^2}{\sigma^2} \sim \chi_{n-1}^2$$

14.1.2 In the above equation, $n = 3$ represents the triplicate plate results at each concentration, s^2 is the observed variance of the triplicate plates at each concentration and σ^2 is the expected variance at each concentration.

14.1.3 Table 3 lists some typical expected variance statistic (σ^2) estimates for some typical TPM concentration/bacterial strain/S9 activation combinations based on normal practice of the method.

Table 3: Typical Variance Estimates for Triplicate Plate Revertant Counts

Strain and S9 Activation	Estimates of σ^2 for triplicate plates at TPM concentrations							
	0	0.025	0.050	0.075	0.100	0.125	0.250	0.500
TA98 (+S9)	17	71	103	152	220	296	529	619
TA98 (-S9)	5	6	10	7	10	10	10	12
TA100 (+S9)	36	34	43	48	64	74	108	212
TA100 (-S9)	57	55	50	58	51	71	125	100
TA1535 (+S9)	2	2	3	3	3	5	4	7
TA1535 (-S9)	2	4	4	5	4	4	5	8
TA1537 (+S9)	4	7	10	11	16	18	22	32
TA1537 (-S9)	4	5	6	6	6	6	8	3
TA102 (+S9)	143	108	144	129	154	210	240	236
TA102 (-S9)	115	124	120	151	162	95	143	160

15 REPORTING OF ASSAY RESULTS

15.1 Reports of mutagenicity data must include the following elements, as per Appendix 4:

- Sample ID (for reference to cigarette brand)
- Smoking data (smoking machine identity, smoking date, puff count, number of test samples smoked, amount of total particulate matter)
- Control data
- Chemical data (including water, nicotine, propylene glycol and glycerol contents, where applicable)
- Assay observations: sample ID, strain, concentration, with/without S9, count (for each replicate), mean, standard deviation.

16 REFERENCES

- 16.1 Ames, B.N., McCann, J., Yamasaki, E., Methods for Detecting Carcinogens and Mutagens with the Salmonella/Mammalian-microsome Mutagenicity Test. *Mutation Research* 31:347-364, 1975.
- 16.2 Bernstein, L., Kaldor, J., McCann, J., Pike, M.C., An Empirical Approach to the Statistical Analysis of Mutagenesis Data from the Salmonella Test. *Mutation Research*, 97:267-281, 1982.
- 16.3 Edler, L., Statistical Methods for Short-term Tests in Genetic Toxicology: The First Fifteen Years. *Mutation Research*, 277:11-33, 1992.
- 16.4 Sokal, R.R., and Rohlf, F.J., *Biometry*. 3rd edition. W.H. Freeman and Co, San Francisco. pp. 493-521, 1995.
- 16.5 Yahagi, T., Degawa, M., Seino Y., Mat Sishima, T., Nagoa, M., Sugimura, T. and Hashimoto, Y., Mutagenicity of Carcinogenic Azo Dyes and Their Derivatives. *Cancer Letter* 1, 91-96, 1975.

APPENDIX 1

COLLECTION OF TOTAL PARTICULATE MATTER (TPM) ON GLASS FIBRE FILTER DISC

1 SUMMARY

- 1.1** Mainstream smoke of cigarettes (e.g. 20 cigarettes), smoked on a rotary smoking machine under modified (intense) ISO conditions, is trapped onto either a 44-mm or 92-mm diameter glass fibre filter disc.

Note: Smoke a sufficient amount of cigarettes such that breakthrough of TPM does not occur, and the limits of TPM, defined in ISO 4387, are not exceeded. The number of test samples may also need to be adjusted to provide a minimum of 180 mg of TPM per 92-mm collection pad, or 100 mg of TPM per 44-mm collection pad.

2 APPARATUS AND EQUIPMENT

- 2.1** Equipment needed to perform conditioning as specified in Health Canada Official Method T-115.
- 2.2** Equipment needed to perform marking for butt length as specified in Health Canada Official Method T-115.
- 2.3** Equipment needed to perform smoking of cigarette as specified in Health Canada Official Method T-115.

3 REAGENTS AND SUPPLIES

Note: Wherever possible, reagents are identified by their Chemical Abstract Service [CAS] registry numbers in square brackets. All reagents shall be at least analytical reagent grade.

- 3.1** Ethanol [67-17-5] 70% (v/v)
- 3.2** Reagents and supplies as specified in Health Canada official Method T-115.

4 PREPARATION OF GLASSWARE

- 4.1** Clean and dry glassware in a manner to ensure that contamination from residues on glassware does not occur.
- 4.2** Sterilize all lab ware by autoclaving at 121°C for 30 minutes at 15 pounds per square inch (psi).

5 SAMPLING

- 5.1** The sampling of tobacco products for the purpose of testing shall be as specified in Health Canada Official Method T-115.

6 CIGARETTE PREPARATION

- 6.1** Mark cigarettes for butt length as specified in Health Canada Official Method T-115.
- 6.2** Prepare cigarettes to be smoked as specified in Health Canada Official Method T-115.
- 6.3** Condition cigarettes as specified in Health Canada Official T-115.

7 SMOKING MACHINE PREPARATION

- 7.1** The ambient conditions for smoking shall be as specified in Health Canada Official Method T-115.
- 7.2** Use only non-UV lighting in the rooms in which the sample generation and sample analyses are conducted.
- 7.3** The machine conditions for a rotary machine shall be as specified in Health Canada Official Method T-115, noting the following:
- To reduce bacterial contamination, all neoprene washers and labyrinth seals are to be cleaned with 70% ethanol.
- 7.4** To reduce bacterial contamination, various smoking machine parts are cleaned with a 70% ethanol solution prior to smoking. These parts are as follows:
- Ash plate
 - Ports
 - Pad holders
 - All work surfaces, including the exterior of extraction flasks and stoppers
 - Any other items, such as gloves, that may come in contact with the sample or cleaned work surfaces

8 TPM GENERATION

- 8.1** Smoke the cigarettes and collect the TPM as specified in Health Canada Official Method T-115 with the following modifications:
- The smoking conditions are modified in the following manner:
 - puff volume is increased from 35 mL to 55 mL,
 - puff interval is decreased from 60 s to 30 s, and
 - all ventilation holes are blocked by placing over them a strip of Mylar adhesive tape, Scotch Brand product no. 600 Transparent Tape, and the tape must be cut so that it covers the circumference and is tightly secured from the end of the filter to the tipping over-wrap seam, or by another method of equivalent efficiency.
 - After smoking the required number of cigarettes, perform three clearing puffs and remove the pad holder from the smoking machine.
- 8.2** The number of replicates to be generated is as per applicable regulations. All replicates must be analyzed on the same day.
- 8.3** Determine the TPM as specified in Health Canada Official Method T-115.
- 8.4** Upon completion of TPM determination, the pad is to be removed from the pad holder, folded into quarters (TPM side in), and the pad holder wiped with the folded pad.
- 8.5** Perform sample extraction as per Appendix 2.

APPENDIX 2

PREPARATION OF TOTAL PARTICULATE MATTER (TPM) FROM GLASS FIBRE FILTER DISC

1 SUMMARY

- 1.1 Total particulate matter smoke trapped on a glass fibre filter disc is extracted with DMSO to achieve a target concentration of 10 mg TPM/mL of DMSO.

2 APPARATUS AND EQUIPMENT

- 2.1 Centrifuge
- 2.2 Pipettors and sterile tips (various sizes)
- 2.3 Freezer, -20°C to -86°C
- 2.4 Fume hood, class II, type B
- 2.5 Rotary shaker at 200 rpm
- 2.6 Wrist action shaker
- 2.7 Thermometer
- 2.8 Timers
- 2.9 Incubator

3 REAGENTS AND SUPPLIES

Note: Wherever possible, reagents are identified by their Chemical Abstract Service [CAS] registry numbers in square brackets. All reagents shall be at least analytical reagent grade.

- 3.1 Glass fibre filter disc and holder
- 3.2 Polymethylpentene (PMP) Erlenmeyer flask (125 mL) or equivalent
- 3.3 Dimethylsulphoxide (DMSO) [67-68-5]
- 3.4 Sterile 25 mL amber bottles
- 3.5 Sterile amber vials
- 3.6 Sterile cheesecloth
- 3.7 Sterile disposable graduated pipettes (5 mL and 10 mL)
- 3.8 Sterile disposable plastic conical tubes (50 mL capacity)
- 3.9 Sterile forceps
- 3.10 Sterile funnels
- 3.11 Sterile graduated centrifuge tubes (15 mL and 50 mL)
- 3.12 Sterile nylon mesh (e.g. Tissue specimen bags from Thermo Shandon)

4 PREPARATION OF GLASSWARE

- 4.1 Sterilize all lab ware to be used by autoclaving at 121°C at 15 psi until sterility is achieved.

5 SAMPLE PREPARATION

Note: All procedures are to be performed such that background contamination is minimized by sterilizing/disinfecting equipment and work surfaces.

Note: If the glass fibre filter disc prepared as in Appendix 1 is not extracted immediately, it can be stored in an airtight flask at -70°C or below. The pads must be allowed to come to room temperature before extraction with DMSO.

- 5.1 Transfer the glass fibre filter disc prepared as in Appendix 1 to a sterile 125 mL PMP Erlenmeyer flask.

- 5.2** Pipette the appropriate amount of DMSO to the flask to achieve a target concentration of 10 mg TPM/mL DMSO. The volume of DMSO required to prepare a 10mg/mL solution of TPM is determined by the following calculation:

$$\text{Volume (mL)} = \text{Total Weight TPM}/10$$

Note: The volume of DMSO to be added is to be determined to two decimal places.

- 5.3** Shake the PMP Erlenmeyer flask for 20 minutes on a wrist action shaker.
- 5.4** Filter the DMSO extract into a 50 mL sterile centrifuge tube through sterile cheesecloth to remove the filter disc material.

Note: If TPM was collected on a 44mm glass fibre filter disc, removal of the filter disc material may also be performed as follows:

- Shake the filter disc with the appropriate amount of DMSO in a 25 mL amber bottle on a rotary shaker.
 - Centrifuge at 1500 rpm for 5 minutes using a sterilized mesh bag placed in a conical centrifugation tube.
- 5.5** Dispense an aliquot/aliquots of DMSO extract into appropriately pre-labelled sterile amber vial(s). This extract is the TPM stock sample solution.
- 5.6** Check the sterility of the TPM stock sample solution by plating onto and incubating a nutrient agar plate at 37°C for 48 hours.
- 5.7** Store all solutions at -70°C or below until used.

APPENDIX 3

DETERMINATION OF GLYCEROL, MENTHOL, NICOTINE, PROPYLENE GLYCOL, WATER AND TAR IN CONJUNCTION WITH THE ANALYSIS OF TOTAL PARTICULATE MATTER (TPM)

1 METHOD SUMMARY

- 1.1 Total particulate matter (TPM) from either parallel smoking runs (alternating nicotine/water smoking with Ames smoking), or a sub-sample of the TPM used for the Ames assay, is extracted following Health Canada Official Method T-115.

Note: When using parallel runs, the same number of cigarettes will be smoked as used for the generation of TPM of the Ames assay.
- 1.2 An aliquot of the extract is analyzed for nicotine and water as per Health Canada Official Method T-115.
- 1.3 A second aliquot is used to determine glycerol, menthol and propylene glycol as per Health Canada Official Method T-304. Nicotine data is also available from this analysis.
- 1.4 Two blank pads will be analyzed for water with each sample for chemical analysis in order to perform the proper sample blank determination and correction for water.
- 1.5 Quantification is achieved using an internal standard calibration by comparing the FID or TCD response of the analytes in the samples against a multi-point calibration of the corresponding standards.

2 APPARATUS AND EQUIPMENT

- 2.1 Equipment as described in Health Canada Official Method T-115.
- 2.2 Equipment as described in Health Canada Official Method T-304.

3 REAGENTS AND SUPPLIES

Note: Wherever possible, reagents are identified by their Chemical Abstract Service [CAS] registry numbers in square brackets. All reagents shall be at least analytical reagent grade.

- 3.1 Reagents as described in Health Canada Official Method T-115.
- 3.2 Reagents as described in Health Canada Official Method T-304.
- 3.3 Menthol [89-78-1] Distilled-in-Glass

4 PREPARATION OF SOLUTIONS

- 4.1 Prepare solutions as described in Health Canada Official Method T-115.

5 PREPARATION OF STANDARDS

- 5.1 Prepare standards as described in Health Canada Official Methods T-115 and T-304 using trans-anethole as the internal standard for T-304.

5.2 Preparation of the Menthol Standard

- 5.2.1** Menthol Primary Stock – Dissolve 1 g of menthol in a 100 mL volumetric flask with extraction solution.
- 5.2.2** Secondary Stock - Dilute to 100 mL in a volumetric flask with extraction solution 5 mL of menthol primary stock along with 5 mL each of glycerol, propylene glycol and nicotine primary stock solutions.
- 5.2.3** Low Standard - Dilute 1 mL of secondary stock solution to 50 mL with extraction solution (menthol 0.01 mg/mL).
- 5.2.4** Medium Standard - Dilute 10 mL of secondary stock solution to 50 mL with extraction solution (menthol 0.1 mg/mL).
- 5.2.5** High Standard - Dilute 25 mL of secondary stock solution to 50 mL with extraction solution (menthol 0.5 mg/mL).

6 PREPARATION OF GLASSWARE

- 6.1** Clean and dry glassware in a manner to ensure that contamination from residues on glassware does not occur.

7 SAMPLE PREPARATION

- 7.1** Collect TPM as per Appendix 1.

Note: Two separate collections of TPM may be required; one used for the Ames assay, the second to be analyzed for chemical compounds. When using parallel runs, the same number of test samples will be smoked as used for the generation of TPM of the Ames assay.

- 7.2** Preparation of the TPM for chemistry analysis is as described in Health Canada Official Method T-115 with the following modifications:

- 7.2.1** Cut the pad into quarters instead of folding.
- 7.2.2** Use 125 mL Erlenmeyer flasks.
- 7.2.3** Prepare two laboratory reagent blanks with each smoked sample.
- 7.2.4** Add 80 mL of extraction solution to the flasks.
- 7.2.5** Wrap the flasks in foil after the addition of extraction solution.
- 7.2.6** Shake the sample and blanks for 30 minutes using a wrist-action shaker.

8 SAMPLE ANALYSIS

- 8.1** Determine the tar, water content and nicotine as per Health Canada Official Method T-115.

8.2 Determination of Glycerol, Propylene Glycol and Menthol

- 8.2.1** Samples are analyzed for glycerol, nicotine, propylene glycol, and menthol as per Health Canada Official Method T-304 with the following modifications:
- Extraction solution used is as per Health Canada Official Method T-115
 - The internal standard is trans-anethole as per Health Canada Official Method T-115
 - Two blanks are analyzed per set of samples
 - Prepare the menthol standard as per 5.2

- Quantification of menthol and nicotine is achieved in the same manner as the humectants described in T-304 with trans-anethole as the internal standard.

9 CALCULATIONS

9.1 Calculations are done as described in Health Canada Official Methods T-115 and T-304 (using the appropriate internal standard).

9.2 Combine the calculated contents from the independent runs.

10 QUALITY CONTROL

10.1 For quality control measures, refer to Health Canada Official Methods T-115 and T-304.

APPENDIX 4

SAMPLE REPORTING FORMATS FOR AMES REVERSE MUTATION ASSAY DATA

1 SAMPLE ID

Laboratory Sample ID	Sample Description
030001	Kentucky Reference 2R4F
030002	Brand X regular full flavour
030003	Brand Y King size Medium
030004	Brand Z 100-mm Gold

2 SMOKING DATA

Set Number	Run Number	Sample ID	Replicate Number	Smoking Date	Cigarettes Smoked	Puff Count (per cig)	Weight of MS CSC (mg)*	Smoking Machine
1	3	030001	1	14-Jul-03	20	6.9	269	rotary
1	4	030001	2	14-Jul-03	20	6.9	275	rotary
1	6	030001	3	14-Jul-03	20	6.9	246	rotary
1	5	030002	1	14-Jul-03	20	7.4	271	rotary
1	7	030002	2	14-Jul-03	20	7.5	274	rotary
1	8	030002	3	14-Jul-03	20	7.4	276	rotary
1	2	030003	1	14-Jul-03	20	8.2	257	rotary
1	9	030003	2	14-Jul-03	20	8.2	261	rotary
1	10	030003	3	14-Jul-03	20	8.2	257	rotary
1	1	030004	1	14-Jul-03	20	8.6	227	rotary
1	11	030004	2	14-Jul-03	20	8.5	222	rotary
1	12	030004	3	14-Jul-03	20	8.7	223	rotary

* Samples extracted in DMSO to give a final concentration of 10.0 mg/ml.

* MS CSC = Mainstream total particulate matter

3 CONTROL DATA

Control Substance	Assay Date	Concentration (µg/plate)	TA98 (+S9)			TA100 (+S9)			TA1535 (+S9)			TA1537 (+S9)			TA102 (+S9)		
			P1	P2	P3	P1	P2	P3	P1	P2	P3	P1	P2	P3	P1	P2	P3
Negative Control (-)	July 16, 2003		25	24	38												
	July 17, 2003					147	129	135									
	July 21, 2003								16	15	18						
	July 25, 2003														262	258	253
	August 6, 2003											12	14	14			
	August 7, 2003					135	124	138	13	13	14						
Positive Control (+)																	
2-aminoanthracene	July 16, 2003	2	1571	1651	1716												
	July 17, 2003	2				1795	1742	1856									
	August 7, 2003	2				1740	1686	1746									
2-aminoanthracene	July 21, 2003	4							319	320	303						
	August 6, 2003	4										541	562	582			
	August 7, 2003	4							354	348	389						
2-aminoanthracene	July 25, 2003	7.5													1522	1633	1575

4 CHEMICAL DATA

Set Number	Run Number	Sample ID	Replicate Number	Weight (mg/cig)	Puff Count (per cig)	MS TPM (mg/cig)	Water (mg/cig)	Nicotine (mg/cig)	Tar (mg/cig)	Propylene Glycol (mg/cig)	Glycerol (mg/cig)
1	11	030001	1	983	7.0	12.7	1.61	0.912	10.2	< 0.008 but > 0.002	1.10
2	23	030001	2	996	7.3	13.2	2.54	0.896	9.76	< 0.008 but > 0.002	1.12
3	23	030001	3	990	7.2	13.3	2.04	0.907	10.4	< 0.008 but > 0.002	1.14
4	5	030001	4	978	7.1	12.7	1.80	0.902	10.0	< 0.008 but > 0.002	1.05
5	6	030001	5	999	7.2	13.2	2.34	0.873	10.0	< 0.008 but > 0.002	1.11
Average				989	7.2	13.0	2.06	0.898	10.1	NQ	1.10
Std. Dev.				9	0.1	0.3	0.38	0.015	0.2	NQ	0.03
Coeff. Var.				0.9	1.7	2.2	18.4	1.7	2.3	N/A	3.1

NQ: Below the Limit of Quantification

N/A: Not Applicable

5 MEAN RESPONSE

Sample ID	Replicate Number	Dose (µg/plate)	TA98 (+S9)		TA100 (+S9)		TA1535 (+S9)		TA537 (+S9)		TA102 (+S9)	
			Mean	St Dev	Mean	St Dev	Mean	St Dev	Mean	St Dev	Mean	St Dev
030001	1	0	26	5	141	3	12	1	14	1	255	11
030001	1	25	61	8	154	7	13	1	22	2	261	3
030001	1	50	118	8	161	6	13	1	29	2	279	5
030001	1	75	139	5	176	10	16	1	43	2	292	10
030001	1	100	237	19	201	3	17	0	52	7	303	9
030001	1	125	271	16	231	6	18	1	67	5	325	7
030001	1	250	404	7	322	17	20	1	78	3	329	14
030001	1	500	470	18	333	17	21	1	61	3	320	5
030001	2	0	25	5	143	3	11	1	13	1	235	7
030001	2	25	55	6	160	5	12	1	24	1	253	3
030001	2	50	126	6	174	1	13	1	31	4	266	4
030001	2	75	142	12	182	4	15	1	44	2	279	7
030001	2	100	225	18	204	9	16	1	50	4	294	5
030001	2	125	257	35	243	6	18	2	66	6	312	2
030001	2	250	435	23	307	19	20	3	77	6	319	3
030001	2	500	483	58	349	4	20	2	68	8	315	7
030001	3	0	26	2	148	10	12	2	14	1	244	6
030001	3	25	58	2	158	5	13	1	24	2	260	6
030001	3	50	122	2	174	16	13	2	34	2	279	8
030001	3	75	147	5	187	2	15	1	43	4	286	5
030001	3	100	230	10	222	18	16	1	52	5	300	1
030001	3	125	271	6	259	2	17	1	66	3	323	9
030001	3	250	395	6	306	9	19	1	72	5	329	4
030001	3	500	462	20	332	26	20	1	65	2	312	3

6 OBSERVATIONS (EXAMPLE WITH S9)

Sample ID	Replicate Number	Dose (µg/plate)	TA98 (+S9)			TA100 (+S9)			TA1535 (+S9)			TA1537 (+S9)			TA102 (+S9)		
			P1	P2	P3	P1	P2	P3	P1	P2	P3	P1	P2	P3	P1	P2	P3
030001	1	0	31	25	22	139	144	140	11	13	12	14	14	13	242	259	263
030001	1	25	69	61	53	151	162	149	13	12	13	22	20	24	260	265	259
030001	1	50	109	123	122	158	168	158	14	14	12	28	27	31	274	284	279
030001	1	75	143	139	134	165	179	185	15	16	16	41	45	43	291	303	283
030001	1	100	222	231	258	198	202	204	17	17	17	48	48	60	301	295	312
030001	1	125	289	265	259	224	235	234	18	18	19	62	68	71	320	321	333
030001	1	250	398	412	403	320	339	306	20	21	20	80	79	75	339	334	313
030001	1	500	453	469	489	352	329	318	21	21	20	61	59	64	320	325	316
030001	2	0	20	24	30	141	143	146	10	12	12	12	13	14	230	232	243
030001	2	25	56	60	49	156	165	159	13	12	12	23	25	24	250	253	256
030001	2	50	133	123	121	174	175	174	12	14	14	29	35	28	261	268	268
030001	2	75	129	146	152	179	186	180	14	16	15	44	46	42	271	284	283
030001	2	100	221	209	245	214	201	198	17	15	17	50	46	54	291	292	300
030001	2	125	239	235	297	247	237	246	16	18	19	65	72	61	310	314	313
030001	2	250	414	433	459	294	299	329	23	18	18	81	79	70	320	321	315
030001	2	500	470	433	546	349	345	353	19	20	22	70	75	60	322	309	315
030001	3	0	25	28	26	158	148	138	12	13	10	15	13	13	241	251	240
030001	3	25	56	59	59	161	160	152	12	13	13	26	23	23	265	261	254
030001	3	50	121	124	120	159	172	190	15	12	13	32	34	36	270	281	286
030001	3	75	142	151	149	185	186	189	14	15	15	39	47	43	291	281	287
030001	3	100	229	240	221	209	NA	235	16	17	16	48	51	58	300	301	299
030001	3	125	271	276	265	258	261	259	17	18	17	69	67	63	316	321	333
030001	3	250	398	388	399	298	305	315	18	19	19	78	70	69	326	329	333
030001	3	500	445	456	484	314	320	361	20	20	21	64	65	67	315	309	311



Health
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Official Method T – 502

Second Edition
2004-11-01

Neutral Red Uptake Assay for Mainstream Tobacco Smoke

Canada

No: T-502, Second Edition
Date: November 1, 2004
Page: 1 of 18

1 SCOPE OF APPLICATIONS

- 1.1** Applicable to the assessment of the cytotoxic potential of cigarette smoke. The method includes the preparation of Chinese Hamster Ovary (CHO) cells for assay, treatment with different fractions of cigarette smoke, uptake of neutral red dye by untreated and treated CHO cells and analysis of results.
- 1.2** Applicable to the assessment of:
- 1.2.1** Particulate phase (PP) of cigarette smoke
 - 1.2.2** Gas-vapour phase (GVP) of cigarette smoke
 - 1.2.3** Combination of particulate phase and gas-vapour phase (PP+GVP).

2 NORMATIVE REFERENCES

- 2.1** Health Canada Official Method T-115. Determination of Tar, Water, Nicotine and Carbon Monoxide in Mainstream Tobacco Smoke. Second Edition. 2003. (Parts applicable to manufactured cigarettes only)
- 2.2** International Organization for Standardization method ISO 3308. Cigarettes – Routine Analytical Cigarette-Smoking Machine - Definitions and Standard Conditions. 2000.
- 2.3** International Organization for Standardization ISO 4387. Cigarettes – Determination of total and nicotine-free dry matter using a routine analytical smoking machine. 2000.
- 2.4** US National Institutes of Health. NIH Publication No: 01-4500: Guidance Document on Using *In Vitro* Data to Estimate *In Vivo* Starting Doses for Acute Toxicity. 2001. (Appendix C only)

3 DEFINITIONS AND ABBREVIATIONS

- 3.1 CHO cells:** Chinese hamster ovary cells.
- 3.2 CMF-PBS:** Phosphate-buffered saline without calcium chloride and magnesium chloride.
- 3.3 Cytotoxicity:** Cell injury, shedding of cells, death and/or impaired growth of a cell population caused by toxic substances.
- 3.4 DMSO:** Dimethylsulphoxide
- 3.5 Glass fibre filter disc (pad):** A pad used to collect particulate matter from tobacco smoke.
- 3.6 GVP:** Gas-vapour phase of the smoke.
- 3.7 GVP+PP:** A 1:1 (v/v) combination of gas-vapour phase and particulate phase.

- 3.8 Hemocytometer (hemacytometer):** A specialized microscopic grid used to count the number of cells in a suspension.
- 3.9 IC₅₀:** Concentration that causes 50% reduction in cell activity as measured by absorbance of Neutral Red dye.
- 3.10 Neutral Red:** A dye that diffuses into viable cells and accumulates in lysosomes, where dye binds to anionic sites on the lysosomal matrix.
- 3.11 PP:** Particulate phase of the smoke.
- 3.12 Replicate:** A TPM preparation generated by an independent smoking of cigarettes taken from the same cigarette sample.

4 METHOD SUMMARY

- 4.1** The cigarettes (e.g. 20 cigarettes) are smoked under modified (intense) ISO conditions using a 20-port rotary smoking machine.
- 4.2** The mainstream smoke is passed through a 92-mm glass fibre filter disc for PP collection, and into the cooled impinger containing phosphate-buffered saline (CMF-PBS) for GVP collection.
- 4.3** Both fractions, either in DMSO (PP) or phosphate-buffered saline (GVP), are prepared to approximately 10 mg PP/mL solution (or 10 mg PP equivalent/mL solution in the case of GVP).
- 4.4** Chinese hamster ovary (CHO) cells are treated with the test sample in a tissue culture plate within 1 hour of smoking, due to the instability of the GVP extract. Following treatment, the test sample is removed and replaced with a neutral red solution.
- Note:** Blank, negative (solvent) and positive controls are assayed concurrently.
- 4.5** Cells are washed and fixed, and the neutral red dye is then extracted using a glacial acetic acid/ethanol extraction solution.
- 4.6** Absorbance is measured at 540 nm for each culture.
- 4.7** Absorbance averages and standard deviations are calculated from 8 wells of the microtitre plate for each concentration of test sample. Cytotoxicity is indicated by a decreased ability of the injured cell to take up the neutral red dye into the lysosome. It is calculated by dividing the absorbance of the treated cells with the absorbance of the negative control cells multiplied by 100.
- 4.8** The number of replicates is as per the applicable regulations.

Warning: The testing and evaluation of certain products against this test method may require the use of materials and or equipment that are potentially hazardous and this document does not purport to address all the safety aspects associated with its use. Anyone using this test method has the responsibility to consult with the appropriate authorities and to establish health and safety practices in conjunction with all existing applicable regulatory requirements prior to its use.

5 APPARATUS AND EQUIPMENT

- 5.1 Equipment needed for collection of PP and GVP as per Appendix 1.
- 5.2 All appropriate apparatus and equipment needed to perform the Neutral Red Uptake Assay. Refer to NIH Publication No:01-4500 (2001).
- 5.3 Inverted microscope (or equivalent)
- 5.4 Microplate reader (wavelength: 540nm) [e.g. Bio-Tek Instruments Inc. Universal Microplate Reader, model ELx800 or equivalent]

6 REAGENTS AND SUPPLIES

Note: Wherever possible, reagents are identified by their Chemical Abstract Service [CAS] registry numbers in square brackets. Use analytical grade chemicals whenever possible.

- 6.1 Reagents and supplies shall be as mentioned in NIH Publication No: 01-4500 (2001), except as follows:
 - 6.1.1 CHO cells in growth media
 - 6.1.2 Neutral Red dye
 - 6.1.3 Fetal bovine serum
 - 6.1.4 Nutrient mixture (F-12 Ham with L-glutamine and NaHCO_3)
 - 6.1.5 Calcium-magnesium free phosphate buffered saline (CMF-PBS)
 - 6.1.6 Penicillin-streptomycin solution
 - 6.1.7 Trypan blue solution (0.4%)
 - 6.1.8 Trypsin [9002-07-7 or equivalent]
 - 6.1.9 Formalin
 - 6.1.10 Sodium lauryl sulphate (SLS) [151-21-3]
 - 6.1.11 Ethanol (denatured, 2A) [64-17-5]
 - 6.1.12 0.2 μM sterile filter
 - 6.1.13 Sterile deionised water
 - 6.1.14 Reagents and supplies needed for collection and preparation of test samples as per Appendix 1

7 PREPARATION OF GLASSWARE AND PLASTIC WARE

- 7.1 Glassware and plastic ware should be sterile, clean and, when necessary, disposable.
- 7.2 Cleaning required for collection and preparation of test samples as per Appendix 1.

8 PREPARATION OF SOLUTIONS AND MEDIA

Note: All reagent and media solutions preparation should follow standard aseptic procedures and whenever applicable follow the manufacturer instructions for the preparation of solutions.

Note: Potential carcinogen(s)/toxin(s) should be prepared in a fume hood with caution appropriate for this type of hazardous material.

- 8.1 Prepare the complete growth media by mixing 90% nutrient mixture with 10% fetal bovine serum, 100 units/mL penicillin and 100 $\mu\text{g/mL}$ streptomycin.

Note: Growth media is used as the diluent and the assay blank in this assay.

- 8.2 Prepare CMF-PBS following manufacturer's instructions.
- 8.3 Prepare 0.25% (w/v) trypsin solution in CMF-PBS one day prior to performing the assay.

- 8.4** Prepare a 50 µg/mL neutral red dye solution (crystal-free) in nutrient mixture the day before required. Place the solution, loosely capped, in a humidified CO₂ incubator overnight at 37 ± 1°C and 5% CO₂ atmosphere. The next day remove the crystals from the solution by filtration through sterile disposable 0.2 µm porosity filters.

Note: Alternatively, prepare 50 µg/mL neutral red dye solution (crystal-free) in nutrient mixture F-12 Ham with L-glutamine as per NIH Publication No: 01-4500 (2001).

- 8.5** Prepare 1% (v/v) formalin solution in sterile deionised water on day of use.
- 8.6** Prepare a solution for extracting the neutral red dye containing: 50% (v/v) ethanol solution, 1% (v/v) glacial acetic acid, and 49% (v/v) sterile deionised water on day of use.

8.7 Preparation of Negative Control Solutions

- 8.7.1** Prepare a 2% (v/v) solution of each of the following solvents in growth media as the negative controls:

- DMSO
- CMF-PBS
- DMSO/CMF-PBS (1:1)

8.8 Preparation of Positive Control Solutions

- 8.8.1** Prepare a stock concentration of SLS solution in sterile deionised water (e.g. 20 mg SLS/mL).

- 8.8.2** At the start of an assay, prepare two appropriate concentrations of SLS solution in growth media.

Note: One solution is estimated to be lethal to 50% of the cells (e.g. 110 µg/mL). One solution is estimated to be lethal to all cells (e.g. 200 µg/mL).

9 PREPARATION OF CHO CELL CULTURE SUSPENSION

- 9.1** Calculate the amount of CHO cell suspension to harvest in order to conduct the assay. Twenty mL of a 50,000 viable cell/mL culture is required per 96 well assay plate. Set up and use four 96 well plates for each sample (8 wells per concentration per plate).

- 9.2** CHO cells are routinely grown as a monolayer in tissue culture grade flasks at 37 ± 1°C in a humidified atmosphere of 5% CO₂. When cells approach confluence they can be harvested or sub-cultured by trypsinization as follows:

- 9.2.1** Remove the growth medium.

- 9.2.2** Add CMF-PBS to rinse.

- 9.2.3** Discard the washing solution.

- 9.2.4** Add CMF-PBS for a second time to rinse.

- 9.2.5** Discard the washing solution.

- 9.2.6** Add 0.25% trypsin solution to the monolayer for desired period of time, and then remove solution.

9.2.7 Add growth medium to the flask and mix well to make a single cell suspension.

9.3 Counting the Number of CHO Cells in Single Cell Suspension

9.3.1 Mix 0.5 mL of cell suspension with 0.5 mL of 0.4% Trypan Blue solution. Care should be exercised when working with Trypan Blue, as it is toxic and may be carcinogenic.

9.3.2 Allow this mixture to stand for at least 5 minutes but no more than 15 minutes.

9.3.3 Make sure that hemocytometer is clean and dry prior to use. Keep the cover glass in place on the hemocytometer.

9.3.4 Transfer the cell/trypan blue suspension to the chamber of hemocytometer with a capillary tube or other suitable device. Do not overfill or underfill the chambers.

9.3.5 Count the number of viable and non-viable cells from 4 corner squares and 1 central square. Non-viable cells will stain blue.

9.3.6 Calculate average viable cell count per square.

9.3.7 Calculate the number of viable cells per mL of cell suspension as follows:

Viable cells /mL = (Average cell count/square) x (Dilution factor) x 10^4 (Chamber conversion factor).

Example: (125 cells) x (2) x 10^4 = 2.5×10^6 cells/mL.

9.3.8 After determination of cell number, the cell suspension can be diluted with growth media and seeded into 96-well tissue culture plates at a density of 5×10^4 cells/mL or re-cultured into another flask.

10 COLLECTION OF PARTICULATE PHASE AND GAS-VAPOUR PHASE OF THE SMOKE

10.1 Refer to Appendix 1: Collection and preparation of test samples.

11 PREPARATION OF TEST SAMPLE (PP; GVP; PP+GVP)

11.1 Refer to Appendix 1: Collection and preparation of test samples.

12 THE CYTOTOXICITY TEST

12.1 Preparation of 96-Well Tissue Culture Plate

12.1.1 Leave the first column of wells free of CHO cells as the assay blank.

12.1.2 Dispense 200 μ L of the cell suspension into each of the remaining wells of 96-well tissue culture plate.

12.1.3 Confirm presence or absence of CHO cells using an inverted microscope.

12.1.4 Incubate the plate at $37 \pm 1^\circ\text{C}$ in a humidified, 5% CO_2 atmosphere for 24 ± 3 hours.

12.2 Preparing Desired Concentrations of Sample and Controls for the Test

12.2.1 Prepare the desired concentrations of the negative, cigarette smoke sample (PP or GVP or PP+GVP) or positive control solution by mixing with the appropriate amount of growth medium.

Note: A range finder experiment as per NIH Publication No: 01-4500 (2001) may be required to ensure these concentrations produce approximately 10% to 90% inhibition of Neutral Red Uptake.

Note: It has been observed that for PP, GVP, and PP+GVP prepared from “typical” Canadian flue-cured cigarettes, concentrations of 0, 10, 50, 75, 100, 120, 140, 160, and 200 µg/mL will generally give a satisfactory response.

Note: Solvent used to prepare dilutions shall correspond to solvent used in preparation of test sample (e.g. DMSO, CMF-PBS, DMSO/CMF-PBS).

12.3 Exposing CHO Cells to Smoke Fractions

12.3.1 Remove the culture medium from each well.

12.3.2 Treat all 8 wells of each column (e.g. rows A-H) with either assay blank, negative control, positive control or each different concentration of the tobacco smoke fraction.

12.3.3 Add 200 µL of growth medium containing negative control/positive control/differing concentrations of tobacco smoke fraction.

12.3.4 Incubate the prepared 96-well plate at $37 \pm 1^\circ\text{C}$ in a humidified, 5% CO_2 atmosphere for 24 hours.

12.4 Treating CHO Cells with Neutral Red Dye for Cellular Uptake

12.4.1 Remove the medium from the wells after 24 hours incubation.

12.4.2 Add 200 µL pre-warmed ($37 \pm 1^\circ\text{C}$) CMF-PBS.

12.4.3 Remove CMF-PBS.

12.4.4 Add 200 µL of freshly prepared filtered neutral red dye solution to each well.

12.4.5 Incubate the cells at $37 \pm 1^\circ\text{C}$ for 3 hours in a humidified, 5% CO_2 atmosphere.

12.5 Fixing CHO Cells After Incubation with 1% Formalin

12.5.1 Remove the neutral red solution from the plate.

12.5.2 Add 200 µL of freshly prepared 1% formalin solution to each well.

12.5.3 Remove the formalin solution after 1 minute but within 2 minutes of addition.

12.6 Extraction of Neutral Red Dye from the Fixed CHO Cells

12.6.1 After removal of 1% Formalin solution from the wells, add 200 µL of freshly prepared 50% ethanol solution containing 1% acetic acid to each well.

12.6.2 Shake the plate on a microtitre plate shaker for 5 - 10 minutes.

12.7 Determination of Absorbance of Neutral Red Dye Extracted from CHO Cells

12.7.1 Read the absorbance from the wells containing the extracted neutral red solution on a microplate reader at a wavelength of 540 nm.

13 CALCULATION OF RELATIVE ABSORBANCE

Note: In order to compare results between assays, the raw absorbance results from each assay plate are blank-corrected and normalized to the negative control prior to data analysis.

13.1 The average absorbance of all assay blank wells is subtracted from each negative control, positive control and treatment well uncorrected absorbance value to produce blank-corrected absorbance values.

13.2 To calculate relative absorbance, divide each blank-corrected positive control and treatment well absorbance value by the average blank-corrected negative control absorbance value for the plate. Express each absorbance fraction as a percent of the negative control to obtain the relative absorbance (%).

14 QUALITY CONTROL AND DOCUMENTATION**14.1 Chemicals and Media**

14.1.1 Verify and record the sterility of media, reagents and solutions as per good laboratory practice for tissue culture laboratories. Verify the performance characteristics of the control solutions.

14.2 Cell Culture Maintenance

14.2.1 The cell culture should be examined daily under an inverted microscope and any changes in morphology or adhesive properties noted.

14.2.2 Cells should be checked regularly for the absence of mycoplasma contamination and only used if demonstrated to be mycoplasma-free (e.g. using Sigma mycoplasma staining kit).

14.3 Laboratory Controls

14.3.1 To assess the overall performance of the analysis, a Kentucky Reference 2R4F control cigarette must be included in the sample. (The results of the control cigarette may be compared, using appropriate statistical techniques, to "expected values" generated by the laboratory or, if none exist, to values found in literature. This will provide information to the laboratory on test accuracy and precision.)

Note: The IC₅₀ result from each reference cigarette smoke fraction assay should be evaluated using appropriate control-charting techniques. For examples see *Introduction to Statistical Quality Control*, John Wiley & Sons, Inc., ISBN 0-471-51988-X.

- 14.3.2** Each 96-well tissue culture plate should contain a blank column that contains only media (to control for plate material absorbance).
- 14.3.3** Each tissue culture plate should have one control column (8 rows) that contains solvent only (negative control).
- 14.3.4** Each tissue culture plate should contain two 8-row columns containing differing concentrations of SLS, which function as positive controls.

14.4 Evaluation of Negative Controls

- 14.4.1** The average uncorrected absorbance for the negative control of each assay plate should be greater than the pre-defined minimum absorbance (see Table 1 below), which is dependent on the solvent medium.

Table 1: Typical Values for Absorbance Minimums

Smoke Fraction	Solvent Control	Uncorrected Absorbance Minimum (540 nm)
PP	DMSO	0.3
GVP	PBS	0.2
PP + GVP	DMSO + PBS	0.3

14.5 Evaluation of Positive Controls

- 14.5.1** The average relative absorbance of the positive controls must pass appropriate acceptance criteria defined by the individual laboratory.

15 REPORTING OF ASSAY RESULTS

- 15.1** Reports of cytotoxicity data must include the following elements, as per Appendix 2:

- Sample ID (for reference to cigarette brand)
- Smoking data (smoking machine identity, smoking date, puff count, number of test samples smoked, total particulate matter, lag time)
- Uncorrected absorbance values for all assay blank, negative control, treatment and positive control wells of each assay plate (include average, standard deviation and coefficient of variation statistics for each assay plate column)
- Blank-corrected absorbance values for all negative control, treatment and positive control wells of each assay plate (include average, standard deviation and coefficient of variation statistics for each assay plate column)
- Relative absorbance values for all treatment and positive control wells of each assay plate (include average, standard deviation and coefficient of variation statistics for each assay plate column)
- Data for PP alone, GVP alone, and combination PP+GVP

16 REFERENCES

- 16.1** Babich, H. and Borenfreund, E. (1990) Cytotoxic effects of food additives and pharmaceuticals on cells in culture as determined with the Neutral Red assay. *Journal of Pharmaceutical Sciences* 79: 592-594.
- 16.2** Bombick, B.R., Murli, H., Avalos, J.T., Bombick, D.W., Morgan, W.T., Putnam, K.P., and Doolittle, D.J. (1997) Chemical and biological studies of a new cigarette that primarily heats tobacco. Part 2. In vitro toxicology of mainstream smoke condensate. *Food and Chemical Toxicology* 36: 183-190.
- 16.3** Bombick, D.W., Ayres, P.H., Putnam, K., Bombick, B.R., and Doolittle, D.J. (1998) Chemical and biological studies of a new cigarette that primarily heats tobacco. Part 3. In vitro toxicity of whole smoke. *Food and Chemical Toxicology* 36: 191-197.
- 16.4** Borenfreund, E. and Puerner, J.A. (1985) Toxicity determined in vitro by morphological alterations and neutral red absorption. *Toxicology Letters* 24: 119-124.

APPENDIX 1

PREPARATION AND COLLECTION OF TEST SAMPLES FOR NEUTRAL RED CYTOTOXICITY ASSAY

1 SUMMARY

- 1.1 The cigarettes (e.g. 20 cigarettes) are smoked under modified (intense) ISO conditions using a 20-port rotary smoking machine.
- 1.2 Mainstream smoke is passed through a 92-mm glass fibre filter disc for PP collection, and into the cooled impinger containing phosphate-buffered saline (CMF-PBS) for GVP collection.
- 1.3 Both fractions, either in DMSO (PP) or phosphate-buffered saline (GVP), are prepared to approximately 10 mg PP/mL solution (or 10 mg PP equivalent/mL solution in the case of GVP).

Note: Smoke a sufficient amount of cigarettes such that breakthrough of particulate matter does not occur, and the limits of total particulate matter, defined in ISO 4387, are not exceeded. The number of cigarettes will also need to be adjusted to provide a minimum of 180 mg total particulate matter per 92-mm disc collection.

2 APPARATUS AND EQUIPMENT

- 2.1 Equipment needed to perform conditioning as specified in Health Canada Official Method T-115.
- 2.2 Equipment needed to perform marking for butt length as specified in Health Canada Official Method T-115.
- 2.3 Equipment needed to perform smoking of tobacco product as specified in Health Canada Official Method T-115.

3 REAGENTS AND SUPPLIES

Note: Wherever possible, reagents are identified by their Chemical Abstract Service [CAS] registry numbers in square brackets. All reagents shall be at least analytical grade.

- 3.1 Dimethylsulphoxide (DMSO) [67-68-5]
- 3.2 Sterile phosphate buffered Saline (without CaCl_2 and MgCl_2) (CMF-PBS)
- 3.3 Ethanol [67-17-5]
- 3.4 Sterile serological pipettes (5 mL and 10 mL)
- 3.5 Impinger (approx. volume 70 mL) with extra coarse (EC) frit (Kimble 170-200 μm pore diameter) and Teflon sleeve
- 3.6 Glass fibre filter disc (e.g. Cambridge filter pad or equivalent) and holder
- 3.7 Ice water bath and thermometer
- 3.8 Eppendorf (or equivalent) pipettes and sterile tips (various sizes)
- 3.9 Sterile conical tubes (15 mL and 50 mL)
- 3.10 Sterile cheese cloth
- 3.11 Aluminum foil
- 3.12 Tubing and connectors (Nalgene food grade or equivalent)
- 3.13 Polymethylpentene (PMP) Erlenmeyer flask (125 mL) or equivalent

4 PREPARATION OF GLASSWARE

- 4.1 Clean and dry glassware in such a manner to ensure that contamination does not occur.

- 4.2 All lab ware to be used should be sterilized by autoclaving at 121°C for 30 minutes at 15 pounds per square inch (psi).

5 SAMPLING

- 5.1 The sampling of cigarettes for the purpose of testing shall be as specified in Health Canada Official Method T-115.

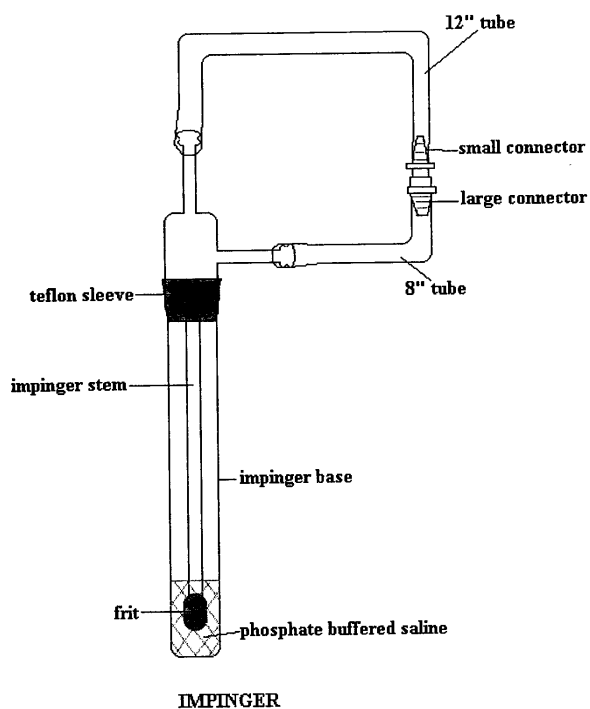
6 CIGARETTE PREPARATION

- 6.1 Mark test samples for butt length as specified in Health Canada Official Method T-115.
- 6.2 Prepare cigarettes to be smoked as specified in Health Canada Official Method T-115.
- 6.3 Condition cigarettes as specified in Health Canada Official Method T-115.

7 PREPARATION OF IMPINGERS

Note: All procedures are to be performed such that background contamination is minimized by sterilizing/disinfecting equipment and work surfaces.

- 7.1 Wrap the following separately in aluminum foil and autoclave: impinger base, regular impinger stem with EC frit, top and side connectors.
- 7.2 Cut the plastic tubing into 30 cm (12 inch) and 20 cm (8 inch) lengths.
- 7.3 Rinse the inside of the tubing as well as the plastic tube connectors with ethanol in order to disinfect and wrap in aluminum foil.
- 7.4 Assemble the tubing and plastic connectors on the impinger stem with the EC frit. See Figure 1 for proper assembly.

Figure 1: Preparation of Impingers for Gas-vapour Phase Collection

Note: Do not remove the aluminum foil wrapping from around the regular stem EC frit and sleeve. Make sure the Teflon sleeve has no creases or holes.

- 7.5 Add 15mL of ice cold CMF-PBS to impinger base using serological pipette.
- 7.6 Gently remove the aluminum foil wrapped around the regular stem with EC frit and sleeve, immerse the stem into the CMF-PBS.
- 7.7 Temporarily remove foil wrapping around base to ensure that the EC frit is immersed in the CMF-PBS. Replace the foil.
- 7.8 Place the closed impinger unit into ice bath containing ice, water and thermometer ensuring that the temperature does not exceed 1°C.

8 SMOKING MACHINE PREPARATION

- 8.1 The ambient conditions for smoking shall be as specified in Health Canada Official Method T-115.
- 8.2 Use only non-UV lighting in the rooms in which the sample generation and sample analyses are conducted.
- 8.3 The machine conditions for a rotary machine shall be as specified in Health Canada Official Method T-115 noting the following:

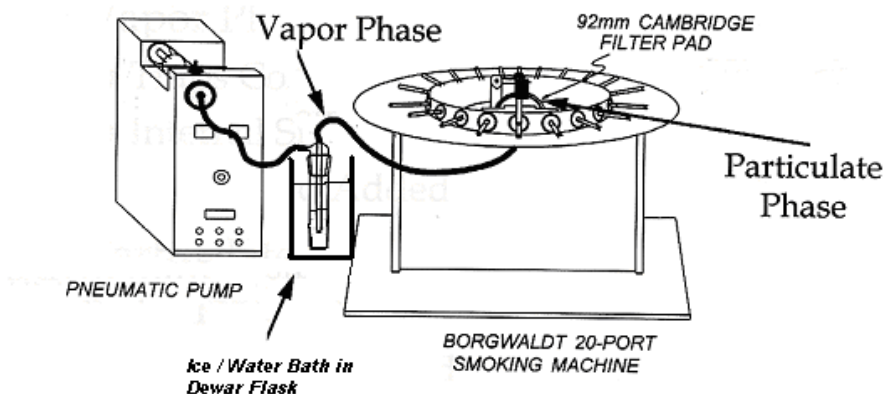
8.3.1 To reduce bacterial contamination, all neoprene washers and labyrinth seals are to be cleaned with 70% ethanol.

8.3.2 To reduce bacterial contamination, various smoking machine parts are cleaned with a 70% ethanol solution prior to smoking. These parts are as follows:

- Ash plate
- Ports
- Pad holders
- Smoke train
- All work surfaces, including the exterior of extraction flasks and stoppers
- Any other items, such as gloves worn by the technician that may come in contact with the sample or cleaned work surfaces

8.4 Assemble the smoke train as illustrated in Figure 2.

Figure 2: Sample Collection of Particulate and Gas Phase Samples for NRU Assay



9 SAMPLE GENERATION

9.1 Smoke the cigarettes using the above smoke train and collect the total particulate matter as specified in Health Canada Official method T-115 with the following modifications:

9.1.1 The smoking conditions are modified in the following manner:

- puff volume is increased from 35 mL to 55 mL,
- puff interval is decreased from 60 s to 30 s, and
- all ventilation holes are blocked by placing over them a strip of Mylar adhesive tape, Scotch Brand product no. 600 Transparent Tape, and the tape must be cut so that it covers the circumference and is tightly secured from the end of the filter to the tipping over-wrap seam, or by another method of equivalent efficiency.

9.1.2 After smoking the required number of test samples, perform three clearing puffs.

- 9.1.3 Record the time at which smoking is complete as well as sample preparation/processing time.
- 9.1.4 Record the temperature of the ice bath in which impinger is kept.
- 9.1.5 Disassemble smoke train by removing the pad holder from the smoking machine and close the impinger by connecting the two ends together.
- 9.2 The number of replicates to be generated is as per applicable regulations. All replicates must be analyzed on the same day.
- 9.3 Determine the total particulate matter as specified in Health Canada Official Method T-115.

10 SAMPLE PREPARATION

10.1 Particulate Phase

- 10.1.1 Upon completion of total particulate matter determination, the pad is to be removed from the pad holder, folded into quarters (total particulate matter side in), and the pad holder wiped with the folded pad.
- 10.1.2 Transfer the collection pad to a sterile 125 mL PMP Erlenmeyer flask.
- 10.1.3 Pipette the appropriate amount of DMSO to the flask such that the final concentration of total particulate matter is 10 mg/mL.
- 10.1.4 Record the amount of DMSO added to the flask.
- 10.1.5 Extract the pad in the PMP Erlenmeyer flask for 20 minutes on the wrist action shaker
- 10.1.6 This solution is then filtered through sterile cheesecloth and administered to the cells within approximately 1 hour after the completion of sample generation.

10.2 Gas-Vapour Phase

- 10.2.1 Upon completion of total particulate matter determination, transfer additional cold CMF-PBS to the impinger, such that the total volume of CMF-PBS is equal to the amount of DMSO used to extract the particulate phase.
- 10.2.2 Record the total amount of CMF-PBS added to the flask such that the GVP concentration is representative of a 10 mg PP equivalent/mL CMF-PBS.
- 10.2.3 Ensure that CMF-PBS in the impinger is mixed properly.
- 10.2.4 Transfer enough impinger solution to carry out testing to a 50 mL sterile conical tube.
- 10.2.5 Cover the tubes with clean aluminum foil.
- 10.2.6 The CMF-PBS sample (as per Section 10.2.4) solution is to be administered to the cells within 1 hour of the completion of sample generation.

10.3 Particulate Phase and Gas-Vapour Phase – Combined

- 10.3.1** Pipette the appropriate amount of impinger solution (e.g. 1.5 mL) to a 15 mL sterile conical tube.
- 10.3.2** Pipette an equal amount (e.g. 1.5 mL) of the particulate phase extract to the same 15 mL sterile conical tube that contains the CMF-PBS aliquot, such that the combined solution contains equal amounts of total particulate matter (or PP equivalent) from the particulate phase and gas-vapour phase.
- 10.3.3** Ensure the sample is adequately mixed before being administered to the cells.

APPENDIX 2

SAMPLE REPORTING FORMATS FOR NRU ASSAY DATA

1 SAMPLE ID

Laboratory Sample ID	Sample Description
030001	Kentucky Reference 2R4F
030002	Brand X regular full flavour
030003	Brand Y King size Medium

2 SMOKING DATA

Set Number	Run Number	Sample ID	Smoke Fraction	Replicate Number	Smoking Date	Cigarettes Smoked	Puff Count (per cig.)	Weight of MSTPM (mg) ¹	Smoking Machine	Lag Time ² (hours)
1	1	030001	A	1	February 10, 2004	20	9.6	218	Borgwaldt rotary	00:59
2	1	030001	B	1	February 18, 2004	20	9.2	243	Borgwaldt rotary	00:19
2	1	030001	C	1	February 18, 2004	20	9.2	243	Borgwaldt rotary	00:46
1	3	030002	A	1	February 10, 2004	100	7.1	203	Borgwaldt rotary	00:51
1	3	030002	B	1	February 10, 2004	100	7.1	203	Borgwaldt rotary	00:19
1	3	030002	C	1	February 10, 2004	100	7.1	203	Borgwaldt rotary	00:39
2	2	030003	A	1	February 18, 2004	11	7.5	219	Borgwaldt rotary	00:54
1	2	030003	B	1	February 10, 2004	11	7.6	215	Borgwaldt rotary	00:17
2	2	030003	C	1	February 18, 2004	11	7.5	219	Borgwaldt rotary	00:47

Note: The following codes are used to indicate the applied fraction of mainstream smoke:

A - Particulate (DMSO), **B** - Gas/Vapour (PBS), **C** - A + B (DMSO + PBS)

1. Samples extracted in appropriate solvent control to give a final concentration of 10.0 mg/mL

2. Time lapsed between completion of smoking and test initiation (in hours)

3 UNCORRECTED ABSORBANCE DATA

Neutral Red Cytotoxicity Assay Results
(Uncorrected Absorbance Data)

Set-Run Number	Sample ID	Smoke Fraction	Replicate Number	Plate Number	Well Number	Raw Assay Plate Absorbance Readings													
						Assay Blank	Control * Reading	Cigarette Smoke Condensate (µg/mL)										SLS (µg/mL)	
								10	50	75	100	120	140	160	200	110	200		
1-1	030001	A	1	1	1	0.082	0.479	0.495	0.424	0.316	0.204	0.136	0.118	0.101	0.09	0.139	0.085		
1-1	030001	A	1	1	2	0.085	0.505	0.523	0.402	0.353	0.207	0.147	0.123	0.105	0.093	0.116	0.086		
1-1	030001	A	1	1	3	0.084	0.508	0.488	0.401	0.32	0.23	0.116	0.113	0.096	0.086	0.109	0.083		
1-1	030001	A	1	1	4	0.082	0.471	0.473	0.418	0.24	0.211	0.114	0.104	0.096	0.086	0.104	0.084		
1-1	030001	A	1	1	5	0.083	0.507	0.538	0.404	0.348	0.218	0.119	0.107	0.094	0.088	0.125	0.085		
1-1	030001	A	1	1	6	0.084	0.499	0.48	0.396	0.28	0.193	0.115	0.1	0.094	0.089	0.116	0.103		
1-1	030001	A	1	1	7	0.082	0.505	0.514	0.403	0.322	0.199	0.116	0.102	0.09	0.085	0.118	0.09		
1-1	030001	A	1	1	8	0.08	0.477	0.474	0.387	0.277	0.183	0.118	0.098	0.097	0.084	0.129	0.086		
Average						0.083	0.494	0.498	0.404	0.307	0.206	0.123	0.108	0.097	0.088	0.120	0.088		
Std. Dev.						0.002	0.015	0.024	0.012	0.039	0.015	0.012	0.009	0.005	0.003	0.011	0.006		
Coeff. Var.						1.9	3.1	4.9	2.9	12.5	7.1	9.9	8.3	4.8	3.4	9.4	7.4		
1-1	030001	A	1	2	1	0.08	0.482	0.47	0.389	0.314	0.227	0.13	0.117	0.102	0.089	0.103	0.085		
1-1	030001	A	1	2	2	0.08	0.498	0.506	0.394	0.303	0.227	0.13	0.113	0.104	0.096	0.103	0.085		
1-1	030001	A	1	2	3	0.082	0.494	0.527	0.41	0.31	0.191	0.123	0.106	0.096	0.088	0.11	0.084		
1-1	030001	A	1	2	4	0.081	0.513	0.512	0.415	0.311	0.205	0.118	0.097	0.096	0.087	0.107	0.084		
1-1	030001	A	1	2	5	0.081	0.505	0.503	0.396	0.306	0.192	0.101	0.099	0.086	0.085	0.107	0.085		
1-1	030001	A	1	2	6	0.081	0.487	0.538	0.438	0.363	0.189	0.103	0.1	0.095	0.087	0.107	0.083		
1-1	030001	A	1	2	7	0.082	0.494	0.516	0.405	0.351	0.196	0.115	0.094	0.091	0.085	0.107	0.084		
1-1	030001	A	1	2	8	0.079	0.506	0.51	0.379	0.326	0.187	0.12	0.098	0.097	0.086	0.099	0.084		
Average						0.081	0.497	0.510	0.403	0.323	0.202	0.118	0.103	0.096	0.088	0.105	0.084		
Std. Dev.						0.001	0.010	0.020	0.018	0.022	0.017	0.011	0.008	0.006	0.004	0.003	0.001		
Coeff. Var.						1.3	2.1	3.9	4.5	6.9	8.2	9.3	8.0	5.9	4.1	3.3	0.8		

Note: The following codes are used to indicate the applied fraction of mainstream smoke:

A - Particulate (DMSO), B - Gas/Vapour (PBS), C - A + B (DMSO + PBS)

4 BLANK-CORRECTED ABSORBANCE DATA

Neutral Red Cytotoxicity Assay Results
(Blank-Corrected Absorbance Data)

Run-Port Number	Sample ID	Smoke Fraction	Replicate Number	Plate Number	Well Number	Blank-Corrected Assay Plate Absorbance Readings												
						Control * Reading	Cigarette Smoke Condensate (µg/mL)										SLS (µg/mL)	
							10	50	75	100	120	140	160	200	110	200		
1-1	030001	A	1	1	1	0.396	0.412	0.341	0.233	0.121	0.053	0.035	0.018	0.007	0.056	0.002		
1-1	030001	A	1	1	2	0.422	0.440	0.319	0.270	0.124	0.064	0.040	0.022	0.010	0.033	0.003		
1-1	030001	A	1	1	3	0.425	0.405	0.318	0.237	0.147	0.033	0.030	0.013	0.003	0.026	0.000		
1-1	030001	A	1	1	4	0.388	0.390	0.335	0.157	0.128	0.031	0.021	0.013	0.003	0.021	0.001		
1-1	030001	A	1	1	5	0.424	0.455	0.321	0.265	0.135	0.036	0.024	0.011	0.005	0.042	0.002		
1-1	030001	A	1	1	6	0.416	0.397	0.313	0.197	0.110	0.032	0.017	0.011	0.006	0.033	0.020		
1-1	030001	A	1	1	7	0.422	0.431	0.320	0.239	0.116	0.033	0.019	0.007	0.002	0.035	0.007		
1-1	030001	A	1	1	8	0.394	0.391	0.304	0.194	0.100	0.035	0.015	0.014	0.001	0.046	0.003		
	Average					0.411	0.415	0.322	0.224	0.123	0.040	0.025	0.014	0.005	0.037	0.005		
	Std. Dev.					0.015	0.024	0.012	0.039	0.015	0.012	0.009	0.005	0.003	0.011	0.006		
	Coeff. Var.					3.8	5.8	3.6	17.2	11.9	30.4	35.5	33.1	61.0	30.5	129.9		
1-1	030001	A	1	2	1	0.401	0.389	0.308	0.233	0.146	0.049	0.036	0.021	0.008	0.022	0.004		
1-1	030001	A	1	2	2	0.417	0.425	0.313	0.222	0.146	0.049	0.032	0.023	0.015	0.022	0.004		
1-1	030001	A	1	2	3	0.413	0.446	0.329	0.229	0.110	0.042	0.025	0.015	0.007	0.029	0.003		
1-1	030001	A	1	2	4	0.432	0.431	0.334	0.230	0.124	0.037	0.016	0.015	0.006	0.026	0.003		
1-1	030001	A	1	2	5	0.424	0.422	0.315	0.225	0.111	0.020	0.018	0.005	0.004	0.026	0.004		
1-1	030001	A	1	2	6	0.406	0.457	0.357	0.282	0.108	0.022	0.019	0.014	0.006	0.026	0.002		
1-1	030001	A	1	2	7	0.413	0.435	0.324	0.270	0.115	0.034	0.013	0.010	0.004	0.026	0.003		
1-1	030001	A	1	2	8	0.425	0.429	0.298	0.245	0.106	0.039	0.017	0.016	0.005	0.018	0.003		
	Average					0.417	0.430	0.323	0.242	0.121	0.037	0.022	0.015	0.007	0.025	0.004		
	Std. Dev.					0.010	0.020	0.018	0.022	0.017	0.011	0.008	0.006	0.004	0.003	0.001		
	Coeff. Var.					2.5	4.6	5.6	9.2	13.6	29.8	36.9	37.6	50.0	14.1	20.2		

Note: The following codes are used to indicate the applied fraction of mainstream smoke:

A - Particulate (DMSO), B - Gas/Vapour (PBS), C - A + B (DMSO + PBS)

5 RELATIVE ABSORBANCE DATA

Neutral Red Cytotoxicity Assay Results
(Relative Absorbance Data)

Run-Port Number	Sample ID	Smoke Fraction	Replicate Number	Plate Number	Well Number	Relative Assay Plate Absorbance Readings									
						Cigarette Smoke Condensate (µg/mL)								SLS (µg/mL)	
						10	50	75	100	120	140	160	200	110	200
1-1	030001	A	1	1	1	100	83.0	56.7	29.5	13.0	8.57	4.44	1.76	13.7	0.547
1-1	030001	A	1	1	2	107	77.7	65.7	30.2	15.6	9.79	5.41	2.49	8.09	0.791
1-1	030001	A	1	1	3	98.6	77.4	57.7	35.8	8.09	7.36	3.22	0.791	6.38	0.061
1-1	030001	A	1	1	4	94.9	81.5	38.2	31.2	7.60	5.17	3.22	0.791	5.17	0.304
1-1	030001	A	1	1	5	111	78.1	64.5	32.9	8.82	5.90	2.74	1.28	10.3	0.547
1-1	030001	A	1	1	6	96.6	76.2	48.0	26.8	7.84	4.20	2.74	1.52	8.09	4.93
1-1	030001	A	1	1	7	105	77.9	58.2	28.3	8.09	4.68	1.76	0.547	8.57	1.76
1-1	030001	A	1	1	8	95.2	74.0	47.2	24.4	8.57	3.71	3.47	0.304	11.2	0.791
	Average					101	78.2	54.5	29.9	9.70	6.17	3.37	1.19	8.94	1.22
	Std. Dev.					6	2.9	9.4	3.6	2.95	2.19	1.12	0.72	2.72	1.58
	Coeff. Var.					5.8	3.6	17.2	11.9	30.4	35.5	33.1	61.0	30.5	129.9
1-1	030001	A	1	2	1	93.4	74.0	56.0	35.1	11.8	8.70	5.10	1.98	5.34	1.02
1-1	030001	A	1	2	2	102	75.2	53.3	35.1	11.8	7.74	5.58	3.66	5.34	1.02
1-1	030001	A	1	2	3	107	79.0	55.0	26.5	10.1	6.06	3.66	1.74	7.02	0.780
1-1	030001	A	1	2	4	104	80.2	55.3	29.8	8.94	3.90	3.66	1.50	6.30	0.780
1-1	030001	A	1	2	5	101	75.7	54.1	26.7	4.86	4.38	1.26	1.02	6.30	1.02
1-1	030001	A	1	2	6	110	85.7	67.7	26.0	5.34	4.62	3.42	1.50	6.30	0.540
1-1	030001	A	1	2	7	104	77.8	64.9	27.7	8.22	3.18	2.46	1.02	6.30	0.780
1-1	030001	A	1	2	8	103	71.6	58.9	25.5	9.42	4.14	3.90	1.26	4.38	0.780
	Average					103	77.4	58.1	29.0	8.82	5.34	3.63	1.71	5.91	0.840
	Std. Dev.					5	4.4	5.3	4.0	2.63	1.97	1.37	0.86	0.83	0.170
	Coeff. Var.					4.6	5.6	9.2	13.6	29.8	36.9	37.6	50.0	14.1	20.2

Note: The following codes are used to indicate the applied fraction of mainstream smoke:

A - Particulate (DMSO), **B** - Gas/Vapour (PBS), **C** - A + B (DMSO + PBS)



Health
Canada

Santé
Canada

Official Method T – 503

Second Edition
2004-11-01

In Vitro Micronucleus Assay for Mainstream Tobacco Smoke

Canada

No: T-503, Second Edition
Date: November 1, 2004
Page: 1 of 16

1 SCOPE OF APPLICATIONS

- 1.1 This document sets out how to test the total particulate matter from mainstream tobacco smoke on Chinese Hamster Ovary cells using the *in vitro* Micronucleus Assay.

2 NORMATIVE REFERENCES

- 2.1 Health Canada Official Method T-115. Determination of Tar, Water, Nicotine and Carbon Monoxide in Mainstream Tobacco Smoke. Second Edition. 2003. (Parts applicable to manufactured cigarettes only)
- 2.2 International Organization for Standardization. Standard ISO 4387: Cigarettes – Determination of total and nicotine-free dry particulate matter using a routine analytical smoking machine. Third Edition. 2000.

3 DEFINITIONS AND ABBREVIATIONS

- 3.1 **CHO cells:** Chinese hamster ovary cells.
- 3.2 **CMF-PBS:** Phosphate buffered saline without calcium chloride and magnesium chloride.
- 3.3 **CP:** Cyclophosphamide.
- 3.4 **DMSO:** Dimethylsulphoxide.
- 3.5 **Glass fibre filter disc (pad):** A pad used to collect particulate matter from tobacco smoke.
- 3.6 **Hemocytometer (hemacytometer):** A specialized microscopic grid used to count the number of cells in a suspension.
- 3.7 **Micronucleus (MN):** A small extra-nuclear body formed at mitosis from acentric chromosome fragments or whole chromosomes that are not incorporated into either daughter nucleus.
- 3.8 **MMC:** Mitomycin C.
- 3.9 **NADP:** Nicotinamide adenine dinucleotide phosphate.
- 3.10 **S9 rat liver fraction:** The supernatant of liver homogenates prepared from male rats exposed to an enzyme-activity-inducing substance (such as Aroclor-1254, phenobarbitone or β -naphthoflavone). The fraction permits *in vitro* simulations of the metabolic activation reactions that are ordinarily restricted to mammalian liver.
- 3.11 **TPM:** That portion of the mainstream smoke that is trapped on the glass fibre filter disc (pad).

4 METHOD SUMMARY

- 4.1 Cigarettes (e.g. 20 cigarettes) are smoked under modified (intense) ISO conditions using a 20-port rotary smoking machine.

- 4.2 Mainstream smoke is passed through a 44-mm or a 92-mm glass fibre filter disc for TPM collection.
- 4.3 A solution of TPM is prepared to a concentration of approximately 10 mg of TPM/mL DMSO.
- 4.4 Chinese hamster ovary (CHO) cells are treated with different doses of the TPM solution in a tissue culture vessel (in duplicate). It is incumbent on each laboratory to determine cell cycle time and adjust treatment and recovery schedules accordingly. An example treatment schedule is (i) short-term exposure (e.g. 3 hour) without metabolic activation followed by recovery (e.g. 27 hour), (ii) short-term exposure (e.g. 3 hour) with metabolic activation followed by recovery (e.g. 27 hour), and (iii) long-term continuous exposure (e.g. 30 hour) without metabolic activation.

Note 1: Negative (solvent) and positive controls are assayed concurrently.

Note 2: All treatment schedules may be run concurrently, or schedules (i) and (ii) may be performed first, using schedule (iii) as a confirmatory test following negative or equivocal results in both schedules (i) and (ii).

- 4.5 The *in vitro* micronucleus assay is performed without cytochalasin-B.
- 4.6 After treatment, cells are harvested by trypsinization followed by fixation.
- 4.7 Slides are prepared using a cytospin centrifuge. Alternatively, the cells may be dropped, spread or smeared on clean slides.
- 4.8 Staining of cells is performed with Acridine Orange to show micronucleus (MN).
- 4.9 A minimum of 1000 randomly selected interphase cells are scored from each flask for the presence of micronuclei.

Warning: The testing and evaluation of certain products against this test method may require the use of materials and or equipment that are potentially hazardous and this document does not purport to address all the safety aspects associated with its use. Anyone using this test method has the responsibility to consult with the appropriate authorities and to establish health and safety practices in conjunction with all existing applicable regulatory requirements prior to its use.

5 APPARATUS AND EQUIPMENT

- 5.1 Equipment needed for collection of TPM and sample preparation as per Appendices 1 and 2.
- 5.2 Inverted microscope (or equivalent)
- 5.3 Laminar flow biological containment cabinet – Class II, type B
- 5.4 CO₂ incubator
- 5.5 Automatic pipettes
- 5.6 Cytospin centrifuge
- 5.7 Slide dryer
- 5.8 Fluorescence microscope equipped with relevant filters for Acridine Orange stain
- 5.9 Key counter
- 5.10 Tissue culture flask
- 5.11 10 mL sterile disposable pipettes
- 5.12 5 mL sterile disposable pipettes
- 5.13 Hemocytometer
- 5.14 15 mL sterile disposable tubes
- 5.15 Water bath
- 5.16 Glass slides and cover-slips
- 5.17 Balance
- 5.18 pH meter

6 REAGENTS AND SUPPLIES

Note: Wherever possible, reagents are identified by their Chemical Abstract Service [CAS] registry numbers in square brackets. Use analytical grade chemicals whenever possible.

- 6.1 Fetal Bovine Serum
- 6.2 CHO cells
- 6.3 Nutrient mixture (F-12 Ham with L-glutamine and NaHCO₃)
- 6.4 Calcium-magnesium free phosphate buffered saline (CMF-PBS)
- 6.5 Penicillin-streptomycin (e.g. in solution or powder form)
- 6.6 Trypan blue solution (0.4%) [72-57-1]
- 6.7 Trypsin [9002-07-7] or equivalent
- 6.8 Dimethyl sulphoxide [67-68-9]
- 6.9 Mitomycin C [50-07-7]
- 6.10 Colchicine [64-86-8]
- 6.11 Cyclophosphamide [6055-19-2]
- 6.12 S9 rat liver fraction
- 6.13 NADP [1184-16-3]
- 6.14 Isocitric acid [1637-73-6]
- 6.15 Methanol [67-56-1]
- 6.16 Glacial acetic acid [64-19-7]
- 6.17 Phosphate buffer (e. g. Sorenson)
- 6.18 Acridine Orange
- 6.19 Mounting media
- 6.20 Sterile, deionised water
- 6.21 0.2 µm sterile filter
- 6.22 Reagents and supplies needed for collection and preparation of test samples as per Appendices 1 and 2.

7 PREPARATION OF GLASSWARE AND PLASTIC WARE

- 7.1 Glassware and plastic ware should be sterile, clean and, when necessary, disposable.
- 7.2 Cleaning required for collection and preparation of test samples as per Appendices 1 and 2.

8 PREPARATION OF SOLUTIONS AND MEDIA

Note 1: All reagent and media solutions preparation should follow standard aseptic procedures and, whenever applicable, follow the manufacturer's instructions for the preparation of solutions.

Note 2: Potential carcinogen(s)/toxin(s) should be prepared in a laminar flow biological containment cabinet with caution appropriate for this type of hazardous material.

- 8.1** Prepare the complete growth media by mixing 90% nutrient mixture with 10% fetal bovine serum, 100 units/mL penicillin and 100 µg/mL streptomycin.

Note: Growth media is also used as the diluent in this assay in absence of metabolic activation.

- 8.2** Prepare CMF-PBS following manufacturer's instructions.

- 8.3** Prepare 0.25% (w/v) trypsin solution in CMF-PBS prior to performing the assay.

- 8.4** Prepare fixative solution by mixing glacial acetic acid with methanol (1:3 v/v).

Note: Other fixative solutions may be used, as applicable.

- 8.5** Prepare sufficient quantity of S9 mixture on the day of the assay [section 12.3.2] by mixing the following:

Ingredients	(for 0.275 mL of S9 mixture)
DL-Isocitric acid trisodium salt solution (13.5 %, w/v, FW 258.1)	0.100 mL
NADP solution (75 mg/mL, FW 765.4)	0.100 mL
S9 rat liver fraction	0.075 mL

8.6 Preparation of Negative Control Solutions

- 8.6.1** Use DMSO in growth media as the negative control. Use the same volume of DMSO as that used in the maximum dose level of test sample.

8.7 Preparation of Positive Control Solutions

8.7.1 Mitomycin C (MMC) solution

- 8.7.1.1** Prepare a stock solution of MMC (e.g. 2 mg MMC/4 mL sterile deionised water).

- 8.7.1.2** At the start of an assay, prepare appropriate working concentrations of MMC solution (e.g. in absence of metabolic activation system: 2.0 µg/mL of growth media for short-term treatment or 0.5 µg/mL in growth media for long-term continuous treatment)

8.7.2 Colchicine solution

- 8.7.2.1** Prepare a stock solution of Colchicine (e.g. 10 mg Colchicine/10mL sterile deionised water).

- 8.7.2.2** At the start of an assay, prepare appropriate working concentrations of Colchicine solution (e.g. in absence of metabolic activation system: 2.0 µg/mL)

of growth media for short-term treatment or 0.5 µg/mL in growth media for long-term continuous treatment)

8.7.3 Cyclophosphamide (CP) solution

8.7.3.1 Prepare a stock solution of cyclophosphamide (e.g. 75 mg CP/10 mL sterile, deionised water).

8.7.3.2 At the start of an assay, prepare an appropriate working concentration of CP solution (e.g. in presence of metabolic activation system: 7.5 µg/mL in growth media for short-term treatment)

8.8 Preparation of staining solution

8.8.1 Prepare a stock solution of Acridine Orange (e.g. 10 mg Acridine Orange/4 mL Phosphate buffer)

8.8.2 Prepare an appropriate working concentration of Acridine Orange (e.g. 20 µL stock Acridine Orange solution in 50 mL phosphate buffer)

9 PREPARATION OF CHO CELL SUSPENSION

9.1 Calculate the amount of CHO cell suspension to harvest in order to conduct the assay. Five mL of a 100,000 viable cell/mL culture is required per 25 cm² (50 mL) culture flask.

Note: Set up and use 2 flasks for each dose of the sample, negative (solvent) control and positive control.

9.2 CHO cells are grown as a monolayer in tissue culture grade flasks at 37 ± 1°C in a humidified atmosphere of 5% CO₂. When cells approach confluence they can be harvested or sub-cultured by trypsinization as follows:

9.2.1 Remove the growth medium.

9.2.2 Add CMF-PBS to rinse.

9.2.3 Discard the washing solution.

9.2.4 Add CMF-PBS for a second time to rinse.

9.2.5 Discard the washing solution.

9.2.6 Add 0.25% trypsin solution to the monolayer for an appropriate amount of time, and then remove solution. The amount will vary depending on the age of the trypsin solution.

9.2.7 Add fresh growth medium to the flask and mix well to make a single cell suspension.

9.3 Counting the Number of CHO Cells in Single Cell Suspension

9.3.1 Mix 0.5 mL of cell suspension with 0.5 mL of 0.4% Trypan Blue solution.

Note: Care should be exercised when working with Trypan Blue, as it is toxic and may be carcinogenic.

9.3.2 Allow this mixture to stand for at least 5 minutes, but no more than 15 minutes.

- 9.3.3** Make sure that the hemocytometer is clean and dry prior to use. Keep the cover glass in place on the hemocytometer.

Note: Alternatively, one can use automated cell counting.

- 9.3.4** Transfer the cell/trypan blue suspension to the chamber of the hemocytometer with a capillary tube or other suitable device. Do not overfill or underfill the chambers.

- 9.3.5** Count the number of viable and non-viable cells from 4 corner squares and 1 central square. Non-viable cells will stain blue.

- 9.3.6** Calculate average viable cell count per square.

- 9.3.7** Calculate the number of viable cells per mL of cell suspension as follows:

Viable cells/mL = (Average cell count/square) x (Dilution factor) x 10^4 (Chamber conversion factor).

Example: (125 cells) x (2) x 10^4 = 2.5×10^6 cells/mL.

- 9.3.8** After determination of cell number, the cell suspension can be diluted with growth media and seeded into tissue culture flasks at a density of 1×10^5 cells/mL. Five mL of a 100,000 viable cell/mL culture is required per 25 cm² (50 mL) culture flask.

Note: Set up and use 2 flasks for each dose of the sample, negative (solvent) control and positive control.

10 COLLECTION OF TOTAL PARTICULATE MATTER OF THE SMOKE

- 10.1** Refer to Appendix 1: Collection of Total Particulate Matter on Glass Fibre Filter Disc.

11 PREPARATION OF TEST SAMPLE

- 11.1** Refer to Appendix 2: Preparation of Total Particulate Matter from Glass Fibre Filter Disc.

12 THE CLASTOGENICITY/GENOTOXICITY TEST (*In Vitro* MICRONUCLEUS ASSAY)

12.1 Preparation of Tissue Culture Flask

- 12.1.1** Dispense 5 mL of the cell suspension into each of the 25 cm² (50 mL) tissue culture flasks.

Note: If volumes other than 5 mL are to be used, then all other volumes must be adjusted accordingly.

- 12.1.2** Confirm presence or absence of CHO cells using an inverted microscope.

- 12.1.3** Incubate the flasks at $37 \pm 1^\circ\text{C}$ in a humidified, 5% CO₂ atmosphere for 24 ± 3 hours.

12.2 Preparing Desired Concentrations of Sample and Controls for the Test

- 12.2.1** Prepare the desired concentrations of the negative, TPM or positive control solution by mixing with the appropriate amount of growth medium.

Note 1: A range finder experiment may be required to ensure that the highest concentration of the test sample produces approximately 60% toxicity as compared to solvent control culture (see 12.4.3 for toxicity determination).

Note 2: Experience with typical Canadian flue-cured cigarettes shows that concentrations of 0, 75, 100, 150, and 200 µg TPM/mL will generally give a satisfactory response with short-term treatment.

Note 3: Solvent used to prepare dilutions shall correspond to solvent used in preparation of test sample (e.g. DMSO).

12.3 Short and Long-Term Exposure of CHO Cells

12.3.1 Short-term Treatment in Absence of Metabolic Activation Followed by Recovery – Schedule (i)

12.3.1.1 Remove the culture medium from each flask.

12.3.1.2 Treat the flasks by adding 5 mL of growth medium containing either negative control/positive control/differing concentrations of TPM to each flask.

Note: Suggested TPM concentration range: 0, 75, 100, 150 and 200 µg TPM/mL growth medium. (This typical range is likely to include the TPM concentration that can produce approximately 60% cytotoxicity as compared to solvent/negative control.)

12.3.1.3 Incubate the flask(s) at $37 \pm 1^\circ\text{C}$ in a humidified, 5% CO_2 atmosphere for desired time (e.g. 3 hours)

12.3.1.4 Remove the medium with solvent, positive control or different concentrations of tobacco smoke sample from the flask(s).

12.3.1.5 Rinse the flask(s) with CMF-PBS.

12.3.1.6 Add 5 mL of fresh growth medium only to each flask.

12.3.1.7 Incubate the flask(s) at $37 \pm 1^\circ\text{C}$ in a humidified, 5% CO_2 atmosphere for approximately a period of 2 cell cycles after beginning of the treatment (e.g. 27 hours).

12.3.2 Short-term Treatment in Presence of Metabolic Activation Followed by Recovery - Schedule (ii)

12.3.2.1 Remove the culture medium from each flask.

12.3.2.2 Treat the flasks by adding 4.625 mL of nutrient mixture F-12 Ham without serum, plus 0.1 mL either negative control/positive control/differing concentrations of TPM and 0.275 mL of S9 mixture [as per section 8.5] to each flask.

Note: Suggested TPM concentration range: 0, 75, 100, 150 and 200 µg TPM/mL growth medium. (This typical range is likely to include the TPM concentration that can produce approximately 60% cytotoxicity as compared to solvent/negative control.)

12.3.2.3 Incubate the flask(s) at $37 \pm 1^\circ\text{C}$ in a humidified, 5% CO_2 atmosphere for desired time (e.g. 3 hours)

12.3.2.4 Remove the medium with solvent, positive control or different concentrations of TPM sample from the flask(s).

12.3.2.5 Rinse the flask(s) with CMF-PBS.

12.3.2.6 Add 5 mL of fresh growth medium only (nutrient mixture F-12 Ham with serum) to each flask.

12.3.2.7 Incubate the flask(s) at $37 \pm 1^\circ\text{C}$ in a humidified, 5% CO_2 atmosphere for approximately a period of 2 cell cycles after beginning of the treatment (e.g. 27 hours).

12.3.3 Long-term Continuous Treatment in Absence of Metabolic Activation – Schedule (iii)

12.3.3.1 Remove the culture medium from each flask.

12.3.3.2 Treat the flasks by adding 5 mL of growth medium containing either negative control/positive control/differing concentrations of TPM to each flask.

Note: Suggested TPM concentration range: 0, 75, 100, 150 and 200 μg TPM/mL growth medium. (This typical range is likely to include the TPM concentration that can produce approximately 60% cytotoxicity as compared to solvent/negative control.)

12.3.3.3 Incubate the flask(s) at $37 \pm 1^\circ\text{C}$ in a humidified, 5% CO_2 atmosphere for approximately a period of 2 cell cycles after beginning of the treatment (e.g. 30 hours).

12.4 Harvesting and Counting of Cells

12.4.1 Cells may be detached from the flask surface by trypsinization process as per Section 9.2.

12.4.2 Count the total number of cells from both negative (solvent) control as well as treated cultures as per Section 9.3.

12.4.3 Cell proliferation and toxicity may be determined by comparing cell counts at seeding or at start of treatment to cell counts at the time of harvest for both treated and control cultures. Refer to Sections 9.3 and 14.

12.5 Micronucleus Staining

12.5.1 Fix the cells with fixative (e.g. 1:3 v/v glacial acetic acid:methanol) and prepare the slides by using a cytospin centrifuge. Alternatively, the cells may be dropped, spread, or smeared on slides.

12.5.2 Perform staining of cells with Acridine Orange, using the following method if desired:

12.5.2.1 Immerse the slide in working solution of Acridine Orange [section 8.8.2].

12.5.2.2 Stain for maximum of 5 minutes.

12.5.2.3 Rinse the slide immediately by immersing in sterile deionised water.

12.5.2.4 Cover cells using a micro cover glass and mounting medium.

12.6 Scoring for the Presence of Micronuclei

12.6.1 Scan a minimum of randomly selected 1000 cells for the presence of micronucleus from each culture flask. A total of 2000 cells per concentration (1000 cells per culture; 2 cultures per concentration) will be scored for the presence of micronuclei. Micronuclei not exceeding 1/3 of the main nucleus diameter, not overlapping with the main nucleus and with distinct borders, will be included in the scoring.

13 QUALITY CONTROL AND DOCUMENTATION

13.1 Chemicals and Media

13.1.1 Verify and record the sterility of media, reagents and solutions as per good laboratory practice for tissue culture laboratories. Verify the performance characteristics of the control solutions.

13.2 Cell Culture Maintenance

13.2.1 The cell culture should be examined daily under an inverted microscope and any changes in morphology or adhesive properties noted.

13.2.2 Cells should be checked regularly for the absence of mycoplasma contamination and only used if none is found.

13.2.3 The background frequency of micronuclei for the cell line should be lower than 25/1000 cells.

13.3 Laboratory Controls

13.3.1 To assess the overall performance of the analysis, a Kentucky Reference 2R4F control cigarette must be included in the sample. (The results of the control cigarette may be compared, using appropriate statistical techniques, to “expected values” generated by the laboratory or, if none exist, to values found in literature. This will provide information to the laboratory on test accuracy and precision.)

13.3.2 There should be at least 90% increase in the number of cells at the time of harvesting in solvent (negative) control cultures as per 12.4.3.

13.3.3 The average relative %MNC and %MN of the positive control must pass appropriate acceptance criteria defined by the individual laboratory.

13.3.4 Coding of slides must be performed prior to analysis to minimize potential operator bias.

13.4 Toxicity

13.4.1 The highest concentration of the test article should exhibit less than or equal to approximately 60% toxicity.

14 REPORTING OF ASSAY RESULTS

14.1 Reports of results must include the following elements (see examples in Appendix 3):

- Sample ID (for reference to cigarette brand)
- Smoking data (smoking machine identity, smoking date, puff count, number of test samples smoked, total particulate matter)
- Raw micronucleus assay cell counts (normal cells per 1000 observed cells, micronucleated cells per 1000 observed cells, total micronuclei per 1000 observed cells and number of viable cells per mL of cell suspension) for all positive control and TPM treatment doses.
- Number of viable cells per mL of cell suspension at the time of seeding as well as at the time of harvesting.

15 REFERENCES

- 15.1 Fenech, M. (2000) The *in vitro* micronucleus technique. *Mutation Research* 455:81-95.
- 15.2 Garriott, M.L., Barry Phelps, J., and Hoffman, W.P. (2002) A protocol for the *in vitro* micronucleus test I. Contributions to the development of a protocol suitable for regulatory submissions from an examination of 16 chemicals with different mechanisms of action and different levels of activity. *Mutation Research* 517:123-134.
- 15.3 Kirsch-Volders, M., Sofuni, T., Aardema, M., Albertini, S., Eastmond, D., Fenech, M., Ishidate, M. Jr., Kirchner, S., Lorge E., Morita, T., Norppa, H., Surralles, J., Vanhauwaert, A., and Wakata, A. (2003) Report from the *in vitro* micronucleus working group. *Mutation Research* 540:153-163.
- 15.4 Matshushima, T., Hayashi, M., Matsuoka, A., Ishidate, M. Jr., Miura, K.F., Shimizu, H., Suzuki, Y., Morimoto, K., Ogura, H., Mure, K., Koshi, K., and Sofuni, T. (1999) Validation study of the *in vitro* micronucleus test in Chinese hamster lung cell line (CHL/IU). *Mutagenesis* 14(6):569-580.
- 15.5 Massey, E., Aufderheide, M., Koch, W., Lodding, H., Pohlmann, G., Windt, H., Jarck, P., and Knebel, J.W. (1998) Micronucleus induction in V79 cells after direct exposure to whole cigarette smoke. *Mutagenesis* 13(2):145-149.

APPENDIX 1

COLLECTION OF TOTAL PARTICULATE MATTER (TPM) ON GLASS FIBRE FILTER DISC

1 SUMMARY

- 1.1 Cigarettes (e.g. 20 cigarettes), are smoked on a rotary smoking machine under modified (intense) ISO conditions. TPM is trapped onto either a 44-mm or 92-mm diameter glass fibre filter disc.

Note: Smoke a sufficient amount of cigarettes such that breakthrough of TPM does not occur, and the limits of TPM, defined in ISO 4387, are not exceeded. The number of cigarettes samples may also need to be adjusted to provide a minimum of 180 mg TPM per 92-mm collection pad, or 100 mg TPM per 44-mm collection pad.

2 APPARATUS AND EQUIPMENT

- 2.1 Equipment needed to perform conditioning as specified in Health Canada Official Method T-115.
- 2.2 Equipment needed to perform marking for butt length as specified in Health Canada Official Method T-115.
- 2.3 Equipment needed to perform smoking of cigarettes as specified in Health Canada Official Method T-115.

3 REAGENTS AND SUPPLIES

Note: Wherever possible, reagents are identified by their Chemical Abstract Service [CAS] registry numbers in square brackets. All reagents shall be at least analytical grade.

- 3.1 Ethanol [67-17-5], 70% (v/v)
- 3.2 Reagents and supplies as specified in Health Canada Official Method T-115.

4 PREPARATION OF GLASSWARE

- 4.1 Clean and dry glassware in a manner to ensure that contamination from residues on glassware does not occur.
- 4.2 Sterilize all lab ware by autoclaving at 121°C for 30 minutes at 15 pounds per square inch (psi).

5 SAMPLING

- 5.1 The sampling of cigarette for the purpose of testing shall be as specified in Health Canada Official Method T-115.

6 CIGARETTE PREPARATION

- 6.1 Mark cigarettes for butt length as specified in Health Canada Official Method T-115.
- 6.2 Prepare cigarettes to be smoked as specified in Health Canada Official Method T-115.
- 6.3 Condition cigarettes as specified in Health Canada Official Method T-115.

7 SMOKING MACHINE PREPARATION

- 7.1** The ambient conditions for smoking shall be as specified in Health Canada Official Method T-115.
- 7.2** Use only non-UV lighting in the rooms in which the sample generation and sample analyses are conducted.
- 7.3** The machine conditions for a rotary machine shall be as specified in Health Canada Official Method T-115, noting the following:
- 7.3.1** To reduce bacterial contamination, all neoprene washers and labyrinth seals are to be cleaned with 70% ethanol.
- 7.4** To reduce bacterial contamination, various smoking machine parts are cleaned with a 70% ethanol solution prior to smoking. These parts are as follows:
- 7.4.1** Ash plate
- 7.4.2** Ports
- 7.4.3** Pad holders
- 7.4.4** All work surfaces, including the exterior of extraction flasks and stoppers
- 7.4.5** Any other items, such as gloves, that may come in contact with the sample or cleaned work surfaces

8 TPM GENERATION

- 8.1** Smoke the cigarettes and collect the TPM as specified in Health Canada Official method T-115 with the following modifications:
- The smoking conditions are modified in the following manner:
 - puff volume is increased from 35 mL to 55 mL,
 - puff interval is decreased from 60 s to 30 s, and
 - all ventilation holes are blocked by placing over them a strip of Mylar adhesive tape, Scotch Brand product no. 600 Transparent Tape, and the tape must be cut so that it covers the circumference and is tightly secured from the end of the filter to the tipping over-wrap seam, or by another method of equivalent efficiency.
 - After smoking the required number of test samples, perform three clearing puffs and remove the pad holder from the smoking machine.
- 8.2** Determine the TPM as specified in Health Canada Official method T-115.
- 8.3** Upon completion of TPM determination, the pad is to be removed from the pad holder, folded into quarters (TPM side in), and the pad holder wiped with the folded pad.
- 8.4** Perform sample extraction as per Appendix 2.

APPENDIX 2

PREPARATION OF TOTAL PARTICULATE MATTER (TPM) FROM GLASS FIBRE FILTER DISC

1 SUMMARY

- 1.1 TPM trapped on a glass fibre filter disc is extracted with DMSO to achieve a target concentration of 10 mg TPM/mL of DMSO.

2 APPARATUS AND EQUIPMENT

- 2.1 Centrifuge
- 2.2 Pipettors and sterile tips (various sizes)
- 2.3 Freezer, -20°C to -86°C
- 2.4 Fume hood, class II, type B
- 2.5 Rotary shaker at 200 rpm
- 2.6 Wrist action shaker
- 2.7 Thermometer
- 2.8 Timers
- 2.9 Incubator

3 REAGENTS AND SUPPLIES

Note: Wherever possible, reagents are identified by their Chemical Abstract Service [CAS] registry numbers in square brackets. All reagents shall be at least analytical grade.

- 3.1 Glass fibre filter disc and holder
- 3.2 Polymethylpentene (PMP) Erlenmeyer flask (125 mL) or equivalent
- 3.3 Dimethylsulphoxide (DMSO) [67-68-5]
- 3.4 Sterile 25 mL amber bottles
- 3.5 Sterile amber vials
- 3.6 Sterile cheesecloth
- 3.7 Sterile disposable graduated pipettes (5 mL and 10 mL)
- 3.8 Sterile disposable plastic conical tubes (50 mL capacity)
- 3.9 Sterile forceps
- 3.10 Sterile funnels
- 3.11 Sterile graduated centrifuge tubes (15 mL and 50 mL)
- 3.12 Sterile nylon mesh (e.g. Tissue specimen bags from Thermo Shandon)

4 PREPARATION OF GLASSWARE

- 4.1 Sterilize all lab ware to be used by autoclaving at 121°C at 15 psi until sterility is achieved.

5 SAMPLE PREPARATION

Note 1: All procedures are to be performed such that background contamination is minimized by sterilizing/disinfecting equipment and work surfaces.

Note 2: If the glass fibre filter disc prepared as in Appendix 1 is not extracted immediately, it can be stored in an airtight flask at -70°C or below. The pads must be allowed to come to room temperature before extraction with DMSO.

- 5.1 Transfer the glass fibre filter disc prepared as in Appendix 1 to a sterile 125 mL PMP Erlenmeyer flask.

- 5.2** Pipette the appropriate amount of DMSO to the flask to achieve a target concentration of 10 mg TPM/mL DMSO. The volume of DMSO required to prepare a 10mg/mL solution of TPM is determined by the following calculation:

$$\text{Volume (mL)} = \text{Total Weight TPM}/10$$

Note: The volume of DMSO to be added is to be determined to two decimal places.

- 5.3** Shake the PMP Erlenmeyer flask for 20 minutes on a wrist action shaker.
- 5.4** Filter the DMSO extract into a 50 mL sterile centrifuge tube through sterile cheesecloth to remove the filter disc material.

Note: If TPM was collected on a 44-mm glass fibre filter disc, removal of the filter disc material may also be performed as follows:

- Shake the filter disc with the appropriate amount of DMSO in a 25-mL amber bottle on a rotary shaker.
 - Centrifuge at 1500 rpm for 5 minutes using a sterilized mesh bag placed in a conical centrifugation tube.
- 5.5** Dispense an aliquot/aliquots of DMSO extract into appropriately pre-labelled sterile amber vial(s). This extract is the TPM stock sample solution.
- 5.6** Check the sterility of the TPM stock sample solution by plating onto and incubating a nutrient agar plate at $37 \pm 1^\circ\text{C}$ for 48 hours.
- 5.7** Store all solutions at -70°C or below until used.

APPENDIX 3

EXAMPLES OF SAMPLE REPORTING FORMATS FOR *IN VITRO* MICRONUCLEUS ASSAY DATA

1 SAMPLE ID

Laboratory Sample ID	Sample Description
030001	Kentucky Reference 2R4F
030002	Brand X regular full flavour
030003	Brand Y King size Medium

2 SMOKING DATA

Set Number	Run Number	Sample ID	Smoking Date	Cigarettes Smoked	Puff Count (per cig)	Weight of TPM (mg) ¹	Smoking Machine
1	2	030003	31-Dec-02	20	9.1	225	Borgwaldt rotary
1	3	030003	31-Dec-02	20	9.0	215	Borgwaldt rotary

1. Samples extracted in appropriate solvent control to give a final concentration of 10.0 mg/mL

3 POSITIVE CONTROL DATA AT CELL HARVEST

Control Substance	Assay Date	Treatment Time (h)	Metabolic Activation	Concentration (µg/ml)	Slide Number 1			Slide Number 2				
					Normal Cells	MN Cells	No. of MN	Cells (x10 ⁵)	Normal Cells	MN Cells	No. of MN	Cells (x10 ⁵)
Positive Control (+)												
Mitomycin C	18-Feb-03	3	-S9	0.05	983	17	18	5.48	985	15	15	4.98
	18-Feb-03	3	-S9	0.05	985	15	16	6.58	986	14	14	6.50
	20-Feb-03	3	-S9	0.05	985	15	17	4.94	982	18	18	5.02
	20-Feb-03	3	-S9	0.05	987	13	14	7.22	984	16	18	7.34

4 CELL COUNTS PRIOR TO CELL TREATMENT

Assay Date	Cells (x10 ⁵) per mL
18-Feb-03	2.52
20-Feb-03	2.88

5 RAW MICRONUCLEUS DATA AT CELL HARVEST (Observations per slide)

Set No.	Run No.	Sample ID	TPM (µg/ml)	Treatment Time (h)	Metabolic Activation	Cell Counts (Slide 1)				Cell Counts (Slide 2)			
						Normal Cells	MN Cells	No. of MN	Cells (x10 ⁵)	Normal Cells	MN Cells	No. of MN	Cells (x10 ⁵)
1	2	030003	0	3	-S9	995	5	5	7.02	996	4	4	7.36
1	2	030003	50	3	-S9	994	6	6	7.22	997	3	3	6.28
1	2	030003	75	3	-S9	996	4	4	5.74	997	3	3	4.92
1	2	030003	100	3	-S9	994	6	6	5.04	992	8	9	4.40
1	2	030003	150	3	-S9	986	14	15	4.70	990	10	11	4.12
1	2	030003	200	3	-S9	981	19	21	2.78	983	17	17	2.76