



**TABLE OF CONTENTS (cont'd)**

	<b><u>Pages</u></b>
GLP COMPLIANCE STATEMENT.....	4
QUALITY ASSURANCE .....	5
1. SUMMARY .....	6
2. INTRODUCTION.....	8
2.1. PURPOSE	8
2.2. GUIDELINES	9
2.3. RESPONSIBLE PERSONNEL	9
2.4. SCHEDULE OF THE STUDY	9
3. TEST/ CONTROL ARTICLES INFORMATION .....	10
3.1. TEST ARTICLE	10
3.2. VEHICLE AND CONTROL ARTICLES	11
3.3. TEST ARTICLE PREPARATION	12
3.4. ANALYSES	13
4. METHODS AND EXPERIMENTAL DESIGN.....	13
4.1. TEST SYSTEM	13
4.2. CULTURE CONDITIONS	13
4.3. PRE-TREATMENT PROCEDURES	14
4.4. EXPERIMENTAL DESIGN	14
4.5. PRECIPITATE EVALUATION	15
4.6. INITIATION OF THE EXPRESSION AND THE SUSPENSION GROWTH	16
4.7. INITIATION OF THE SURVIVAL PLATES	16
4.8. MUTANT SELECTION	16
5. DATA SCORING .....	17
5.1. CYTOTOXICITY EVALUATION	17
5.2. SCORING OF MUTANTS	18
6. DATA EVALUATION.....	18
6.1. DATA PROCESSING	18
6.2. ACCEPTANCE CRITERIA	19
6.3. EVALUATION CRITERIA	19

**TABLE OF CONTENTS (cont'd)**

	<u>Pages</u>
7. ARCHIVES.....	19
8. PROTOCOL ADHERENCE.....	20
9. RESULTS.....	21
9.1. LONG TREATMENT WITHOUT METABOLIC ACTIVATION	21
9.2. SHORT TREATMENT WITHOUT METABOLIC ACTIVATION	22
9.3. SHORT TREATMENT WITH METABOLIC ACTIVATION	23
10. DISCUSSION.....	24
11. CONCLUSION.....	25
12. TABLES: Summary results.....	26
Table 1: Long treatment (24 hours) without metabolic activation (- S9)	27
Table 2: Short treatment (4 hours) without metabolic activation (- S9)	28
Table 3: Short treatment (4 hours) with metabolic activation (+ S9)	29
13. APPENDICES: Individual values and statistics.....	30
INDIVIDUAL VALUES	31
Appendix 1: Long treatment (24 hours) without metabolic activation (- S9)	32
Relative Survival after treatment	33
Suspension Growth (SG) and Relative Total Growth (RTG) during the expression period	34
Viability during the selection	35
Mutant frequencies and sizing for Small (S) and Large (L) colonies after the selection	36
Appendix 2: Short treatment (4 hours) without metabolic activation (- S9)	37
Relative Survival after treatment	38
Suspension Growth (SG) and Relative Total Growth (RTG) during the expression period	39
Viability during the selection	40
Mutant frequencies and sizing for Small (S) and Large (L) colonies after the selection	41
Appendix 3: Short treatment (4 hours) with metabolic activation (+ S9)	42
Relative Survival after treatment	43
Suspension Growth (SG) and Relative Total Growth (RTG) during the expression period	44
Viability during the selection	45
Mutant frequencies and sizing for Small (S) and Large (L) colonies after the selection	46
STATISTICS	47
Appendix 4: Long treatment (24 hours) without metabolic activation (- S9)	48
Appendix 5: Short treatment (4 hours) without metabolic activation (- S9)	50
Appendix 6: Short treatment (4 hours) with metabolic activation (+ S9)	52
14. ADDENDA.....	54
Addendum 1: Standard Schema of the Study Design	55
Addendum 2: Bibliography	57
Addendum 3: Approbation of the protocol	60
Addendum 4: Quality Control Certificate of S9 fraction	62
Addendum 5: Certificate of Analysis	64

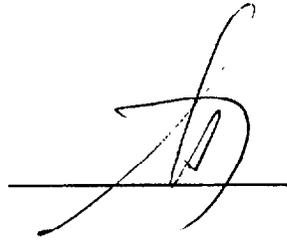
**GLP COMPLIANCE STATEMENT**

I, the undersigned, hereby declare that, unless otherwise stated, the work described in this report was performed in accordance with the following:

- "Good Laboratory Practice" described in the U.S. Federal Register (Food and Drug Administration) dated 22 December 1978 with any applicable amendments.
- "O.E.C.D. Principles of Good Laboratory Practice" concerning Mutual Acceptance of Data in the Assessment of Chemicals dated 26 November 1997 (C (97) 186 Final) , except that the formulation was not analysed for test article concentration.
- "M.H.W.: "Good Laboratory Practice Standards for Safety Studies on Drugs" described by the Japanese Ministry of Health and Welfare, dated 26 March 1997 (Ordinance n° 21).

This report is a true and accurate record of the results obtained.

Signature:



Name:

A. Forichon

Title:

Study Director

Date:

24 January 2001.

**QUALITY ASSURANCE**

**STUDY TITLE:** ING 911 - *In vitro* Mammalian Cell Gene Mutation Test on L5178Y Mouse Lymphoma Cells TK<sup>+/+</sup> (Microtitre method).

Inspection of the protocol was made in accordance with Standard Operating Procedure AQ PROT 1. Dates for inspection of any protocol amendments, in accordance with this SOP, are not quoted.

Dates (day - month - year)		
Inspection	Report to Study Director	Report to Management
10.07.2000	10.07.2000	10.07.2000

Inspection(s) of data generated on this type of study was made in accordance with Standard Operating Procedure AQ-AUD 1.

Dates (day - month - year)		
Inspection	Report to Study Director	Report to Management
26.12.2000	-	26.12.2000

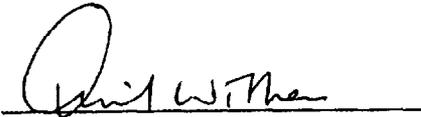
Inspection(s) of procedures on this type of study was made in accordance with Standard Operating Procedure AQ-INSP 1.

Dates (day - month - year)			
Inspected phase(s)	Inspection	Report to Study Director	Report to Management
Formulation	05.10.2000	-	05.10.2000
Administration	25.07.2000	-	25.07.2000
S9 Mix Preparation	05.10.2000	-	05.10.2000
Expression, numeration coulter	27.07.2000	-	27.07.2000
Cloning efficiency- Selection	04.08.2000	-	04.08.2000

Other routine procedures used in this type of study were inspected regularly and reports made in accordance with Standard Operating Procedure AQ-INSP 1.

This report has been reviewed by the Quality Assurance Department, employing methods detailed in Standard Operating Procedure AQ-RAP 1. The reported methods and procedures were found to describe those used, and the results constituted an accurate representation of recorded data. Any data supplied by or under the responsibility of the Sponsor were not subjected to review.

P. WITHERS, B. Sc.  
(Director of International Compliance)

  
25 January 2001

Date:

## 1. SUMMARY

The mutagenic potential of ING 911 was assessed in the Mouse Lymphoma Assay using L5178Y mouse lymphoma cells, clone -3.7.2C (ATCC number: CRL-9518, American Type Culture Collection, Virginia, USA). This mammalian cell line can detect induced forward mutations at the thymidine kinase locus ( $TK^{+/+} \rightarrow TK^{-/-}$ ).

### METHOD

The test has three principal phases, exposure, expression and determination of mutants.

#### **Exposure**

- Long treatment - approximately 24 hours -without metabolic activation.
- Short treatment- approximately 4 hours- either with or without metabolic activation.

The test article was prepared as milky suspension in water for injection at 250 mg/ml. The other dosing solutions were prepared by serial dilution from this stock suspension. Each treatment was performed at the following dose levels: 1.7, 5.4, 17, 52, 164, 512, 1600 and 5000  $\mu\text{g/ml}$ .

Duplicate cultures were performed for each experimental point.

Cytotoxicity was evaluated by the relative survival (relative to the negative control - RS) after the treatment period or relative total growth (relative to the negative control- RTG) during the expression period.

Methylmethanesulfonate (MMS) and Cyclophosphamide (CP) were used as positive control substances for experiments without and with S9, respectively, at the following dose levels:

MMS: 4  $\mu\text{g/ml}$  and 7.5  $\mu\text{g/ml}$

CP: 2.5  $\mu\text{g/ml}$  and 5  $\mu\text{g/ml}$

#### **Expression**

The cultures were washed and re-suspended in a standard medium and incubated for a further 2-days (expression period) after the end of the exposure period.

#### **Determination of mutants**

Cells were incubated for approximately 2 weeks in a selective media containing trifluorothymidine (TFT) and the number of colonies were counted. The mutation rate was calculated from the number of colonies, in the selective media, corrected by the number of colonies obtained in the standard medium to determine the cloning efficiency (Viability).

## RESULTS

The 3 Experiments (long or short treatments) were carried out over the same range of dose levels (1.7, 5.4, 17, 52, 164, 512, 1600 and 5000  $\mu\text{g/ml}$ ) and no precipitate was observed. The results did not show evidence of any cytotoxic effect. No dose-related decreases were noted in the Relative Survival rate or the Relative Total Growth. In the absence of precipitate or cytotoxicity, all the treated cultures were analysed for the mutant frequencies. No statistically or biologically significant increases in the frequency of mutants were noted at any of the dose level tested. All the values of treated groups were similar to the negative control values. In the presence or absence of metabolic activation, the test article ING 911 did not induce any increase in the mutant frequency when tested up to 5000  $\mu\text{g/ml}$  using long or short period of treatment.

## CONCLUSION

Under the experimental conditions and according to the criteria of the test protocol, it is concluded that when tested up to 5000  $\mu\text{g/ml}$ , **the test article ING 911 did not induce any mutagenic effect in the mammalian cell (L5178Y) Mouse Lymphoma Assay, either with or without metabolic activation.**

## **2. INTRODUCTION**

### **2.1. PURPOSE**

#### **2.1.1. AIM OF THE STUDY**

To evaluate the mutagenic potential of ING 911 by its effects on a mammalian cell line - the L5178Y Mouse Lymphoma - in the presence and absence of a metabolic activation system.

This test is used to screen for possible mammalian mutagens and carcinogens using the "microwell method" [27]. Many compounds that are positive in this test are mammalian carcinogens, however, there is not a perfect correlation between this test and carcinogenicity. Correlation is dependent on chemical class and there is increasing evidence that there are carcinogens that are not detected by this test because they appear to act through other non-genotoxic mechanisms [6].

#### **2.1.2. PRINCIPLE**

The principle of this test is to detect forward mutations which functionally mutate the thymidine kinase (TK) locus present in the cells. The mutant cells ( $TK^{+/+} \rightarrow TK^{-/-}$ ) are detected by their ability to grow in the presence of the pyrimidine analogue trifluorothymidine (TFT). The normal thymidine kinase proficient ( $TK^{+/+}$ ) cells are sensitive to TFT, which causes the inhibition of further cell division. By contrast the mutant cells ( $TK^{-/-}$ ) are able to proliferate in the presence of TFT.

Several chemicals are not directly mutagenic but are only seen to be mutagenic after metabolic activation (promutagenic). To investigate possible metabolic activation the test article is incubated in the presence of liver enzymes (S9 -metabolic activation system) [16, 18].

Cells suspensions are exposed to the test substance for 4 hours, either with or without metabolic activation (Short treatment) and for 24 hours without metabolic activation (Long treatment). After the appropriate exposure time, the cell suspensions are washed and subcultured in a standard growth medium to determine cytotoxicity and to allow phenotypic expression prior to mutant selection [11, 13].

Cytotoxicity is determined by measuring the relative survival (relative to the negative control -RS) or relative total growth (relative to the negative control - RTG) of the cultures after the treatment period. A cytotoxicity of greater than the range of 80 - 90% is considered as too great to allow interpretation of the results and such groups would be excluded from any analysis.

The treated cultures are subcultured for 2 days to allow near-optimal phenotypic expression of induced mutations.

Mutant frequency is then determined by seeding known numbers of cells in a medium containing the selective agent (TFT) to detect mutant cells, and in medium without selective agent to determine the cloning efficiency (Viability). After a further incubation period of approximately 2 weeks, the number of colonies is counted. The mutant frequency is derived from the number of mutant colonies in selective medium and the number of colonies in non-selective medium [9, 10, 11, 12, 13, 27].

## **2.2. GUIDELINES**

This study was adapted from OECD guideline 476, FDA Redbook II and EPA part 798, Sec. 798-5300.

In addition, this study complies fully with the guidances developed by the International Conference on Harmonisation (ICH): S2A and S2B.

## **2.3. RESPONSIBLE PERSONNEL**

- Study Director: A. Forichon, Maître ès Sciences, Doctorat 3<sup>ème</sup> cycle.
- Deputy Study Director: M. Aujoulat, D.U.T. de Biologie Appliquée.
- Quality Assurance: P. Withers, B.Sc.
- Study Monitor for the Study Sponsor: B. Demagny.

## **2.4. SCHEDULE OF THE STUDY**

- Study initiation date (protocol signed by Study Director): 4 July 2000.
- Study completion date (final report signed by Study Director): 24 January 2001.

The approbation of the protocol ("*SIGNATURE PAGE*") is presented in Addendum 3.

### **3. TEST/ CONTROL ARTICLES INFORMATION**

#### **3.1. TEST ARTICLE**

- Denomination: ING 911.
- Batch number: ING 15 Z 102
- Expiry date: 02/2002.
- Intended use: food ingredient.
- Appearance: powder.
- Purity: assumed to be 100% for the dose calculation.
- Storage: at refrigerator temperature, protected from light and humidity.
- Solubility information: the limit of solubility of the test article is approximately 10 % (w/v) at 40°C.
- Hazards: standard precautions.

The Study Sponsor is responsible for sending a certificate of conformity to the Study Director for each batch of test or control article supplied to the testing facility.

This certificate documents that appropriate checking procedures have been used to ensure that the test or control article conforms to established specifications and is that intended for use in the study.

### **3.2. VEHICLE AND CONTROL ARTICLES**

#### **3.2.1. VEHICLE**

- Denomination: water for injection.
- Rationale of choice: the limit of solubility of the test article is approximately 10% (w/v). Information supplied by the Study Sponsor.
- Supplier: Biosedra.
- Storage: at room temperature.

#### **3.2.2. NEGATIVE CONTROL**

The negative control article was the vehicle used for the formulation of the test article.

#### **3.2.3. POSITIVE CONTROL ARTICLES**

The following positive control articles were used:

<b>Metabolic Activation System (S9 Mix)</b>	<b>Chemical</b>	<b>Concentrations (µg/ml)</b>
Absent	Methylmethanesulfonate (MMS)	4.0 and 7.5
Present	Cyclophosphamide (CP)	2.5 and 5

Detailed information relating to the positive control articles used is maintained in the raw data of the study.

### 3.2.4. METABOLIC ACTIVATION SYSTEM: S9 MIX

In many cases a test article is not itself mutagenic, but possesses a mutagenic activity through one or several metabolic derivatives. To take this phenomenon into account the test article was placed in presence of liver enzymes to mimic the normal process of metabolism (metabolic activation system). This solution was used at the rate of 1 ml per 19 ml of culture, containing the test chemical or controls. For the plates tested without metabolic activation, this was replaced by a similar volume of phosphate buffer.

#### PREPARATION OF ENZYME S9 FRACTION:

Commercially available S9 fraction kept below  $-70^{\circ}\text{C}$ . Each batch is checked by the manufacturer for sterility, protein content and enzymatic activities. This induced S9 fraction was obtained from the liver of rats.

The Quality Control Certificate of S9 fraction is presented in Addendum 4.

#### PREPARATION OF S9 Mix:

The mixture of metabolic activation S9 Mix was prepared immediately prior to the test and kept on ice during the test. Its composition was as follows (for a 5 ml preparation):

- $\text{MgCl}_2$  (0.4 M) + KCl (1.65 M) \_\_\_\_\_ 0.1 ml
- Glucose 6 Phosphate (1 M) \_\_\_\_\_ 0.025 ml
- NADP (0.1 M) \_\_\_\_\_ 0.2 ml
- Phosphate buffer (pH 7.4 - 0.2 M) \_\_\_\_\_ 2.5 ml
- S9 fraction \_\_\_\_\_ 1.5 ml
- Water for injection \_\_\_\_\_ 0.675 ml

### 3.3. TEST ARTICLE PREPARATION

- Preparation: the test article was prepared as a solution in the vehicle at different concentrations.

Experimental design	Metabolic activation	Duration of exposure	Dosing concentrations (mg/ml)
<u>Long treatment</u>	-	~ 24 hours	0.085, 0.27, 0.85, 2.6, 8.2, 25.6, 80 and 250
<u>Short treatment</u>	-	~ 4 hours	0.085, 0.27, 0.85, 2.6, 8.2, 25.6, 80 and 250
	+	~ 4 hours	0.085, 0.27, 0.85, 2.6, 8.2, 25.6, 80 and 250

At the concentration of 250 mg/ml, the test article formulation was a milky suspension. No higher concentrated preparation could be performed. To reach the maximum dose level of 5000  $\mu\text{g/ml}$  in cell culture, the dose volume was changed from 200 $\mu\text{l}$  to 400 $\mu\text{l}$ .

- Frequency of preparation: the formulations were prepared on every treatment day.
- Storage: room temperature. The preparation were used within 2 hours of preparation.

### **3.4. ANALYSES**

The determination of the stability of the formulated test article is the responsibility of the Sponsor.

## **4. METHODS AND EXPERIMENTAL DESIGN**

### **4.1. TEST SYSTEM**

- Identification: L5178Y mouse lymphoma cells, clone -3.7.2C, designated L5178Y TK<sup>+/+</sup>.
- Origin: American Type Culture Collection (ATCC), Virginia, USA.  
ATCC number: CRL-9518.
- Storage: For long-term storage, aliquots of the L5178Y cells are maintained deep frozen in liquid nitrogen.

### **4.2. CULTURE CONDITIONS**

#### **4.2.1. CULTURE MEDIA**

- Stock cultures:  
The stock cultures were performed in an appropriate medium (F<sub>10</sub>) composed as follows:
  - Fischer's Medium for Leukemic Cells of Mice,
  - 10% (v/v) heat-inactivated horse serum,
  - Sodium pyruvate,
  - Antibiotics.
- Treatment cultures (see § 4.3, and 4.6):  
The treatments were performed using a mixture (1:1) of the F<sub>10</sub> medium (see above) and the same medium without serum (F<sub>0</sub>).

- Cultures for cloning and selection (see § 4.7 and 4.8):

The cultures in microplates were performed in the same medium, but containing 20% (v/v) of heat-inactivated horse serum (F<sub>20</sub>).

#### 4.2.2. INCUBATION CONDITIONS

All the cultures were incubated at  $37 \pm 1^\circ\text{C}$  in a humidified incubator containing  $5 \pm 1\%$  of carbon dioxide. The stock cultures were grown in suspension using 75-80 cm<sup>2</sup> flasks, and were counted and diluted every 2-3 days to maintain the cells in exponential growth.

#### 4.3. PRE-TREATMENT PROCEDURES

- Cell suspensions for treatment were prepared in F<sub>10</sub> medium (see § 4.2.1) at approximately 10<sup>6</sup> cells/ml then diluted in F<sub>0</sub> medium (see § 4.2.1) resulting in a medium with 5% (v/v) of heat-inactivated horse serum at a density of approximately  $5 \times 10^5$  cells/ml.

For each treated or control culture, two independent cultures were initiated in a 75-80 cm<sup>2</sup> flask using 18.6 ml (see § 4.4.4) of the above mentioned cell suspension.

- Identification of the culture: the culture flasks were labelled with the study number, the dose level code, the replicate number, the duration of treatment and the date of the culture initiation.

#### 4.4. EXPERIMENTAL DESIGN

(A standard schema of the study design is presented in Addendum 1)

##### 4.4.1. DOSE SELECTION

The dose level were selected over a range from 1.7 to 5000 µg/ml using half-log interval.

Experimental design	Metabolic activation	Duration of exposure	Dose levels (µg/ml)
<u>Long treatment</u>	-	~ 24 hours	1.7, 5.4, 17, 52, 164, 512, 1600 and 5000
<u>Short treatment</u>	-	~ 4 hours	1.7, 5.4, 17, 52, 164, 512, 1600 and 5000
	+	~ 4 hours	1.7, 5.4, 17, 52, 164, 512, 1600 and 5000

#### **4.4.2. EXPERIMENTAL CONTROLS**

Every time an experiment was conducted, the following test control cultures were carried out:

- Untreated control with and without metabolic activation (receiving culture medium instead of vehicle).
- Negative vehicle control (see § 3.2.2.) with and without metabolic activation.
- Positive controls (see § 3.2.3.) with and without metabolic activation.

#### **4.4.3. EXPOSURE PERIOD**

Different experiments were conducted using a long treatment period (~24 hours) without metabolic activation, or a short treatment period (~4 hours) with or without metabolic activation. All assays were performed using independent duplicate cultures (A and B) for each test article concentration, as well as positive and negative controls.

#### **4.4.4. TREATMENT**

After treatment, each culture contained:

- 18.6 ml of cell suspension at  $5 \times 10^5$  cells/ml
- 1 ml of sterile buffer (without metabolic activation)
- or
- 1 ml of S9 Mix (with metabolic activation)
- 400  $\mu$ l of the appropriate dosing concentration.

As the dose volume was increased (400 $\mu$ l instead of 200 $\mu$ l), the final volume of cell culture was adjusted to 20 ml using 18.6 ml of cell suspension instead of 18.8ml as stated in the protocol.

Following addition of the test and control articles, all the cultures were gently mixed and incubated under standard conditions for approximately 4 or 24 hours. After the appropriate treatment period, the cell cultures were washed twice by centrifugation and resuspended in F<sub>10</sub> culture medium (see § 4.2.1). After the treatment period, the cell suspensions were processed for the estimation of the relative survival and for the expression period.

#### **4.5. PRECIPITATE EVALUATION**

Any precipitate was evaluated with the naked eye in the final treatment medium described in § 4.4.4.

#### **4.6. INITIATION OF THE EXPRESSION AND THE SUSPENSION GROWTH**

After the appropriate treatment period, the cultures were counted<sup>(1)</sup> and diluted (if necessary) to a cell density of  $\sim 2 \times 10^5$  cells/ml in F<sub>10</sub> culture medium (see § 4.2.1), and incubated in a 75-80 cm<sup>2</sup> flask under standard conditions for an overnight period. The next day (Day 2), the cell cultures were counted and diluted (if necessary) to a cell density of  $\sim 2 \times 10^5$  cells/ml in F<sub>10</sub> culture medium (see § 4.2.1), and incubated under standard conditions for an additional overnight period. On day 3, the cultures were processed for mutant selection (see § 4.8.). This two-day growth period is necessary for expression of the TK<sup>-</sup> phenotype.

#### **4.7. INITIATION OF THE SURVIVAL PLATES**

After the appropriate treatment period (on Day 1), the cultures were adjusted by serial dilutions to the density of approximately 8 cells/ml in F<sub>20</sub> culture medium (see § 4.2.1) to determine cloning efficiency (Survival).

For each culture, 0.2 ml of the suspension at 8 cells/ml were distributed in each well of duplicate 96-well microtiter plates (i.e. mean  $\sim 1.6$  cells/well).

The plates were incubated under standard conditions for 10-13 days then scored as described in § 5.1.

#### **4.8. MUTANT SELECTION**

Following the two-day expression period (on Day 3), the cell cultures were counted, diluted and plated to select mutants and to determine cloning efficiency (Viable). All platings were performed in 96-well microtiter plates. Mutant selection plates were seeded (in quadruplicate) with 0.2 ml/well of a cell suspension at  $\sim 10^4$  cells/ml (i.e. mean  $\sim 2000$  cells/well) in F<sub>20</sub> culture medium (see § 4.2.1) containing 4.00  $\mu$ g/ml of trifluorothymidine (TFT). The plates were then incubated under standard conditions for  $\sim 2$  weeks (12-15 days).

Concurrently, cloning efficiency plates (Viable) were seeded in duplicate with 0.2 ml/well of a cell suspension at  $\sim 8$  cells/ml (i.e. mean  $\sim 1.6$  cells/well) in F<sub>20</sub> culture medium (see § 4.2.1) without trifluorothymidine (TFT). The plates were then incubated under standard conditions for  $\sim 2$  weeks (10-13 days).

---

<sup>(1)</sup> Counting was required only for the long treatment period ( $\sim 24$  hours) where significant cell divisions may occur. The short treatment period ( $\sim 4$  hours) did not allow cell divisions to occur to any extent and the cell density was considered to be  $\sim 5 \times 10^5$  cells/ml (see § 4.3) and diluted to  $\sim 2 \times 10^5$  cells/ml on this basis.

## 5. DATA SCORING

### 5.1. CYTOTOXICITY EVALUATION

The cytotoxicity was evaluated on the basis of the relative survival (RS) and/or the relative total growth (RTG).

#### 5.1.1. RELATIVE SURVIVAL (RS)

After incubation, the survival plates (see § 4.7.) were scored and the percentage of survival, relative to the concurrent negative control, calculated as follows:

$$\%RS = \frac{\%S \text{ of the dose level}}{\%S \text{ of the negative control}} \times 100$$

Where:

$$\bullet \ %S = \frac{-\ln P(0)}{\text{number of cells subcultured/well}} \times 100$$

$$\bullet \ P(0) = \frac{\text{number of wells with no colony}}{\text{total number of wells}}$$

#### 5.1.2. RELATIVE TOTAL GROWTH (RTG)

The cell counts obtained on day 2 and 3 during expression (see § 4.6.) and the cloning efficiency (Viable) were used to calculate the relative total growth for each dose level as follows:

$$RTG = \frac{SG \text{ of the dose level}}{SG \text{ of the negative control}} \times \frac{\%V \text{ of the dose level}}{\%V \text{ of the negative control}}$$

Where:

$$\bullet \ SG = \frac{\text{Day 2 cell count}}{\text{number of cells subcultured at Day 1}} \times \frac{\text{Day 3 cell count}}{\text{number of cells subcultured at Day 2}}$$

$$\bullet \ \%V = \frac{-\ln P(0)}{\text{number of cell subcultured/well}} \times 100$$

- $$P(0) = \frac{\text{number of wells with no colony}}{\text{total number of wells}}$$

## 5.2. SCORING OF MUTANTS

After incubation the selection and the cloning efficiency plates (see § 4.8.), were scored.

### Cloning efficiency (Viable):

The cloning efficiency plates (without TFT) was calculated as follows:

- $$CE = \frac{-\ln P(0)}{\text{number of cells subcultured/well}}$$

- $$P(0) = \frac{\text{number of empty wells}}{\text{total number of wells}}$$

### Mutant frequency (MF):

The selection plates (with TFT) were used to calculate the mutant frequency as follows:

$$MF = \frac{\text{CE in medium with TFT}}{\text{CE in medium without TFT}} \times 10^6$$

### Colony sizing:

In addition, the size of each mutant colony (large, small) was recorded for all cultures [23, 24, 25, 26].

The cut-off for the size is defined as ¼ or less of the well area for the small colonies (S) and more than ¼ of the well area for the large colonies (L).

## 6. DATA EVALUATION

### 6.1. DATA PROCESSING

Dunnett's test was used to compare the mutant frequency at each dose level to the concurrent negative control.

## **6.2. ACCEPTANCE CRITERIA**

The assay is considered valid if the following criteria are met:

1. The maximum of the mean negative control counts fall within or close to the range of 100-300 spontaneous mutants for  $10^6$  cells,
2. The positive control chemicals induce clear increases in mutant frequencies confirming the sensitivity of the test system and an active S9 Mix preparation,
3. No more than 5 % of the plates in the assay are lost through contamination or any other unforeseen event.

## **6.3. EVALUATION CRITERIA**

Biological relevance of the results was considered first. Statistical methods (Dunnett's test at  $p \leq 0.05$ ) may be used as an aid in evaluating the test results [6]. Statistical significance is not the only determination of a positive response.

A test article is considered positive if it meets both of the following criteria (using either short or long treatment times):

1. Statistically significant dose-response.
2. At least one concentration produces a statistically significant increase in average mutant frequency which is >2-fold the negative control value.

The above criteria were used as a guide in evaluating the test results. However, the Study Director may take other factors into consideration in evaluating the test results, since biological significance should be considered in evaluation of the assay results.

## **7. ARCHIVES**

All raw data supporting documents and materials will be maintained in the archives of the testing facility for 5 years as specified in the study protocol and any amendment(s).

## **8. PROTOCOL ADHERENCE**

The study was performed in accordance with the protocol no. 755/002-D with the following deviations:

- Mutant selection plates were in F<sub>20</sub> culture medium (see § 4.2.1) containing 4.00 µg/ml of trifluorothymidine (TFT), instead of 2.00 µg/ml as indicated in the protocol, to improve the selection pressure of mutants (change in the standard procedure of the testing facility).
- Following a change of ownership, effective from 28 September 2000, the name of the testing facility has changed from Phoenix International Preclinical Services Europe to MDS Pharma Services.
- The S<sub>9</sub> fraction was supplied frozen instead of freeze-dried (supplier out of stock).
- Due to the limit of solubility, the dose volume was increased (400 µl instead of 200 µl) and the final volume of cell culture was adjusted to 20 ml using 18.6 ml of cell suspension instead of 18.8 ml as stated in the protocol.

These deviations did not affect the quality or interpretation of the results.

## **9. RESULTS**

The results are presented in the following tables/appendices:

	Long treatment (-S9)	Short treatment (-S9)	Short treatment (+S9)
Summary of Results	Table 1	Table 2	Table 3
Individual values	Appendix 1	Appendix 2	Appendix 3
Statistical analysis	Appendix 4	Appendix 5	Appendix 6

### **9.1. LONG TREATMENT WITHOUT METABOLIC ACTIVATION**

This experiment was performed at the dose levels of 1.7, 5.4, 17, 52, 164, 512, 1600 and 5000 µg/ml (1/2-log intervals).

The negative and positive controls gave the expected responses.

No plates were lost through contamination or any other unforeseen event.

The experiment was considered valid and all data accepted.

#### ◇ Precipitate evaluation:

After incorporation of the dosing solution, no precipitate was observed in the test system mixture.

#### ◇ Cytotoxicity evaluation:

No signs of cytotoxicity were noted in the range of dose levels (1.7 to 5000 µg/ml). No dose-related decreases were noted in the Relative Survival rate or the Relative Total Growth (RTG).

#### ◇ Scoring of mutants:

In the absence of cytotoxicity, all the dose levels were included in the analysis of the mutant frequencies. When compared to negative (water) control, no statistically significant increases in the frequency of mutants were noted over the range of dose levels tested. All the values in treated groups were similar to the negative control values.

## **9.2. SHORT TREATMENT WITHOUT METABOLIC ACTIVATION**

This experiment was performed at the dose levels of 1.7, 5.4, 17, 52, 164, 512, 1600 and 5000  $\mu\text{g/ml}$  (1/2-log intervals).

The spontaneous mutant frequencies of the negative control (387.24) and untreated control (404.92) were above the maximum expected values indicated in § 6.2 (100 – 300 spontaneous mutants for  $10^6$  cells). These values were in the range of 80 – 600 spontaneous mutants for  $10^6$  cells accepted in the literature [27]. These negative control values obtained in this experiment were considered as being representative of a normal biological variation and were accepted. The positive and negative controls were therefore considered to have given the expected responses.

No plates were lost through contamination or any other unforeseen event.

The experiment was considered valid and all data accepted.

### ◇ Precipitate evaluation:

No precipitate was noted in the test system at any of the dose levels.

### ◇ Cytotoxicity evaluation:

No signs of cytotoxicity were noted in the range of dose levels (1.7 to 5000  $\mu\text{g/ml}$ ). No dose-related decreases were noted in the Relative Survival rate or the Relative Total Growth (RTG).

### ◇ Scoring of mutants:

All the dose levels tested were included in the analysis of the mutant frequencies. No statistically or biologically significant increases in the frequency of mutants were noted at any of the dose levels tested. All the values of treated groups were similar to the negative control values.

### **9.3. SHORT TREATMENT WITH METABOLIC ACTIVATION**

This experiment was performed at the dose levels of 1.7, 5.4, 17, 52, 164, 512, 1600 and 5000  $\mu\text{g/ml}$  (1/2-log intervals).

The positive and negative controls gave the expected responses.

No plates were lost through contamination or any other unforeseen event.

*The experiment was considered valid and all data accepted.*

◇ Precipitate evaluation:

No precipitate was noted in the test system at any of the dose levels.

◇ Cytotoxicity evaluation:

No signs of cytotoxicity were noted in the range of dose levels (1.7 to 5000  $\mu\text{g/ml}$ ). No dose-related decreases were noted in the Relative Survival rate or the Relative Total Growth (RTG).

◇ Scoring of mutants:

All the dose levels tested were included in the analysis of the mutant frequencies. No statistically or biologically significant increases in the frequency of mutants were noted at any of the dose levels tested. All the values of treated groups were similar to the negative control values.

## **10. DISCUSSION**

The test article ING 911 was evaluated for its mutagenic potential by the induction of forward mutations which functionally mutate the thymidine kinase locus ( $TK^{+/+} \rightarrow TK^{-/-}$ ) present in a mammalian cell line (L5178Y Mouse Lymphoma). The cell cultures were exposed to the test substance for approximately 24 hours without metabolic activation (Long treatment) and for approximately 4 hours, either with or without metabolic activation (Short treatment). The maximum dose level tested was 5000  $\mu\text{g/ml}$  using water for injection as the vehicle.

The 3 Experiments (long or short treatments) were carried out over the same range of dose levels (1.7, 5.4, 17, 52, 164, 512, 1600 and 5000  $\mu\text{g/ml}$ ) without precipitate in the test system. The results from each experiment did not show evidence of any cytotoxic effect. No dose-related decreases were noted in the Relative Survival rate or the Relative Total Growth. In the absence of precipitate or cytotoxicity, all the treated cultures were analysed for the mutant frequencies. No statistically or biologically significant increases in the frequency of mutants were noted at any of the dose levels tested. All the values of treated groups were similar to the negative control values. In the presence or absence of metabolic activation, the test article ING 911 did not induce any increase in the mutant frequency when tested up to 5000  $\mu\text{g/ml}$  using long or short period of treatment.

## **11. CONCLUSION**

Under the experimental conditions and according to the criteria of the test protocol, it is concluded that when tested up to 5000 µg/ml, **the test article ING 911 did not induce mutagenic effect in the mammalian cell (L5178Y) Mouse Lymphoma Assay, either with or without metabolic activation.**

**12. TABLES:**  
**Summary results**

**Table 1:**  
**Long treatment (24 hours) without metabolic activation (- S9)**

Dose µg/ml	Replicate culture	CYTOTOXICITY				MUTATION					
		% Relative Survival	Mean (S.D.)	RTG	Mean (S.D.)	% Absolute Viable	Mean (S.D.)	Mutant Frequency for 10 <sup>6</sup> cells	Mean (S.D.)	Ratio Small/Large colonies	Mean (S.D.)
0	A	100.00	<b>100.00</b>	1.00	<b>1.00</b>	81.65	<b>86.16</b>	125.17	<b>91.35</b>	0.48	<b>0.69</b>
	B	100.00	(0.00)	1.00	(0.00)	90.67	(6.38)	57.52	(47.84)	0.90	(0.30)
0 UT	A	106.78	<b>113.37</b>	1.00	<b>1.02</b>	81.65	<b>81.65</b>	100.24	<b>95.59</b>	0.57	<b>0.73</b>
	B	119.95	(9.31)	1.04	(0.03)	81.65	(0.00)	90.94	(6.58)	0.89	(0.23)
1.7	A	77.29	<b>89.39</b>	0.90	<b>1.01</b>	74.81	<b>84.16</b>	107.34	<b>89.37</b>	0.46	<b>0.38</b>
	B	101.48	(17.10)	1.12	(0.16)	93.51	(13.22)	71.39	(25.42)	0.30	(0.11)
5.4	A	96.87	<b>101.48</b>	0.89	<b>1.08</b>	68.67	<b>84.96</b>	125.97	<b>93.04</b>	0.45	<b>0.73</b>
	B	106.09	(6.52)	1.26	(0.26)	101.25	(23.04)	60.10	(46.58)	1.00	(0.39)
17	A	81.95	<b>91.72</b>	1.03	<b>1.12</b>	80.46	<b>92.55</b>	99.80	<b>80.39</b>	0.78	<b>0.57</b>
	B	101.48	(13.81)	1.21	(0.13)	104.63	(17.09)	60.98	(27.45)	0.35	(0.30)
52	A	95.33	<b>105.78</b>	1.18	<b>1.06</b>	98.05	<b>85.38</b>	86.59	<b>103.85</b>	0.82	<b>0.98</b>
	B	116.22	(14.77)	0.93	(0.18)	72.70	(17.93)	121.11	(24.41)	1.14	(0.23)
164	A	89.63	<b>94.08</b>	1.09	<b>1.14</b>	82.86	<b>89.69</b>	80.56	<b>77.20</b>	0.71	<b>0.65</b>
	B	98.52	(6.29)	1.18	(0.06)	96.51	(9.65)	73.83	(4.76)	0.59	(0.08)
512	A	120.79	<b>159.43</b>	1.34	<b>1.23</b>	102.91	<b>99.71</b>	84.05	<b>84.43</b>	0.56	<b>0.86</b>
	B	198.06	(54.64)	1.11	(0.16)	96.51	(4.53)	84.81	(0.54)	1.15	(0.42)
1600	A	93.86	<b>95.48</b>	1.02	<b>1.04</b>	108.19	<b>105.55</b>	85.73	<b>81.88</b>	0.81	<b>0.72</b>
	B	97.10	(2.29)	1.06	(0.03)	102.91	(3.73)	78.03	(5.44)	0.63	(0.13)
5000	A	93.86	<b>111.95</b>	1.13	<b>1.01</b>	113.96	<b>106.01</b>	109.82	<b>111.13</b>	0.63	<b>0.77</b>
	B	130.04	(25.58)	0.89	(0.17)	98.05	(11.25)	112.44	(1.85)	0.90	(0.19)
MMS 4	A	54.14	<b>48.01</b>	0.26	<b>0.22</b>	28.34	<b>26.12</b>	1177.51	<b>1046.30</b>	1.63	<b>2.02</b>
	B	41.87	(8.68)	0.17	(0.06)	23.89	(3.15)	915.09	(185.56)	2.40	(0.54)
MMS 7.5	A	16.33	<b>18.54</b>	0.06	<b>0.05</b>	8.72	<b>7.98</b>	1629.82	<b>1444.42</b>	1.64	<b>2.32</b>
	B	20.74	(3.12)	0.04	(0.01)	7.24	(1.05)	1259.02	(262.20)	3.00	(0.96)

**Abbreviations:**

UT: Untreated control

MMS: Positive control

S.D.: Standard deviation

**Table 2:**  
**Short treatment (4 hours) without metabolic activation (- S9)**

Dose µg/ml	Replicate culture	CYTOTOXICITY				MUTATION					
		% Relative Survival	Mean (S.D.)	RTG	Mean (S.D.)	% Absolute Viable	Mean (S.D.)	Mutant Frequency for 10 <sup>6</sup> cells	Mean (S.D.)	Ratio Small/Large colonies	Mean (S.D.)
0	A	100.00	100.00	1.00	1.00	101.25	106.62	405.33	387.24	0.11	0.10
	B	100.00	(0.00)	1.00	(0.00)	111.98	(7.59)	369.14	(25.59)	0.09	(0.01)
0 UT	A	91.75	98.80	0.91	0.97	86.64	100.30	406.03	404.92	0.10	0.12
	B	105.85	(9.97)	1.03	(0.08)	113.96	(19.32)	403.80	(1.58)	0.14	(0.03)
17	A	81.90	83.74	0.75	0.80	75.90	83.29	525.30	504.03	0.08	0.08
	B	85.57	(2.60)	0.85	(0.07)	90.67	(10.44)	482.75	(30.09)	0.07	(0.01)
54	A	87.92	96.89	0.89	1.00	86.64	101.32	418.26	379.74	0.07	0.08
	B	105.85	(12.68)	1.10	(0.15)	116.00	(20.76)	341.21	(54.48)	0.08	(0.01)
17	A	77.39	89.41	0.87	0.88	92.08	94.30	417.54	406.53	0.10	0.10
	B	101.43	(17.00)	0.88	(0.01)	96.51	(3.13)	395.52	(15.57)	0.09	(0.01)
52	A	76.31	85.40	1.09	1.23	116.00	133.78	301.03	322.95	0.14	0.11
	B	94.48	(12.85)	1.37	(0.20)	151.55	(25.14)	344.87	(31.00)	0.07	(0.05)
164	A	86.66	88.61	0.92	1.04	92.08	102.03	439.32	388.81	0.05	0.08
	B	90.55	(2.75)	1.15	(0.16)	111.98	(14.07)	338.29	(71.44)	0.10	(0.04)
512	A	81.90	90.95	1.06	0.95	122.61	104.63	359.51	408.16	0.04	0.07
	B	100.00	(12.80)	0.84	(0.16)	86.64	(25.43)	456.81	(68.80)	0.10	(0.04)
1600	A	97.16	108.01	1.23	1.32	104.63	109.30	348.96	365.17	0.04	0.06
	B	118.85	(15.34)	1.40	(0.12)	113.96	(6.60)	381.37	(22.92)	0.08	(0.03)
5000	A	98.57	93.93	1.06	1.07	99.63	101.27	448.80	429.60	0.09	0.09
	B	89.28	(6.57)	1.08	(0.01)	102.91	(2.32)	410.40	(27.15)	0.08	(0.01)
MMS 4	A	91.75	97.32	0.72	0.82	77.00	84.54	927.79	933.89	0.25	0.26
	B	102.88	(7.87)	0.91	(0.13)	92.08	(10.66)	939.99	(8.63)	0.27	(0.01)
MMS 7.5	A	73.10	89.48	0.61	0.49	72.70	61.09	960.59	1273.02	0.31	0.33
	B	105.85	(23.16)	0.37	(0.17)	49.48	(16.42)	1585.45	(441.84)	0.35	(0.03)

**Abbreviations:**

UT: Untreated control

MMS: Positive control

S.D.: Standard deviation

**Table 3:**  
**Short treatment (4 hours) with metabolic activation (+ S9)**

Dose µg/ml	Replicate culture	CYTOTOXICITY				MUTATION					
		% Relative Survival	Mean (S.D.)	RTG	Mean (S.D.)	% Absolute Viable	Mean (S.D.)	Mutant Frequency for 10 <sup>6</sup> cells	Mean (S.D.)	Ratio Small/Large colonies	Mean (S.D.)
0	A	100.00	<b>100.00</b>	1.00	<b>1.00</b>	96.51	<b>92.91</b>	54.04	<b>57.82</b>	0.52	<b>1.10</b>
	B	100.00	(0.00)	1.00	(0.00)	89.30	(5.10)	61.59	(5.34)	1.67	(0.81)
0 UT	A	97.17	<b>103.82</b>	1.04	<b>1.08</b>	86.64	<b>89.36</b>	65.15	<b>62.44</b>	0.95	<b>0.89</b>
	B	110.47	(9.40)	1.12	(0.06)	92.08	(3.85)	59.73	(3.83)	0.82	(0.09)
17	A	93.14	<b>96.57</b>	0.95	<b>0.92</b>	80.46	<b>78.18</b>	47.04	<b>67.49</b>	0.27	<b>0.41</b>
	B	100.00	(4.85)	0.89	(0.04)	75.90	(3.22)	87.94	(28.92)	0.55	(0.20)
54	A	88.02	<b>90.57</b>	1.08	<b>1.16</b>	101.25	<b>96.67</b>	38.77	<b>50.86</b>	0.61	<b>0.76</b>
	B	93.12	(3.61)	1.24	(0.11)	92.08	(6.48)	62.94	(17.09)	0.91	(0.21)
17	A	79.67	<b>99.12</b>	0.93	<b>1.00</b>	94.98	<b>91.47</b>	48.80	<b>51.55</b>	0.42	<b>0.63</b>
	B	118.56	(27.50)	1.06	(0.09)	87.96	(4.96)	54.29	(3.88)	0.84	(0.30)
52	A	71.01	<b>86.97</b>	0.99	<b>1.12</b>	93.51	<b>93.51</b>	55.77	<b>65.99</b>	0.73	<b>0.89</b>
	B	102.92	(22.56)	1.25	(0.18)	93.51	(0.00)	76.20	(14.45)	1.04	(0.22)
164	A	69.99	<b>85.00</b>	1.11	<b>1.09</b>	101.25	<b>88.58</b>	52.89	<b>67.52</b>	0.56	<b>0.76</b>
	B	100.00	(21.22)	1.07	(0.03)	75.90	(17.93)	82.15	(20.69)	0.96	(0.28)
512	A	93.14	<b>98.77</b>	1.07	<b>1.08</b>	104.63	<b>97.65</b>	51.18	<b>74.15</b>	0.86	<b>0.79</b>
	B	104.40	(7.96)	1.09	(0.01)	90.67	(9.87)	97.11	(32.48)	0.72	(0.10)
1600	A	113.78	<b>108.35</b>	1.18	<b>1.29</b>	111.98	<b>109.18</b>	38.85	<b>54.33</b>	0.45	<b>0.53</b>
	B	102.92	(7.68)	1.40	(0.16)	106.38	(3.96)	69.80	(21.88)	0.61	(0.11)
5000	A	105.87	<b>108.97</b>	1.17	<b>1.54</b>	87.96	<b>125.82</b>	60.88	<b>53.58</b>	1.60	<b>1.17</b>
	B	112.06	(4.38)	1.90	(0.52)	163.67	(53.54)	46.28	(10.32)	0.74	(0.61)
CP	A	65.01	<b>76.44</b>	0.52	<b>0.47</b>	63.06	<b>48.94</b>	509.42	<b>827.35</b>	1.94	<b>1.71</b>
25	B	87.87	(16.16)	0.41	(0.08)	34.81	(19.98)	1145.28	(449.62)	1.48	(0.33)
CP	A	14.90	<b>11.71</b>	0.05	<b>0.05</b>	6.88	<b>6.16</b>	2142.55	<b>2623.46</b>	1.88	<b>2.01</b>
5	B	8.52	(4.51)	0.04	(0.01)	5.44	(1.02)	3104.37	(680.11)	2.14	(0.18)

**Abbreviations:**

UT: Untreated control

CP: Positive control

S.D.: Standard deviation

**13. APPENDICES:**

**Individual values and statistics**

**INDIVIDUAL VALUES**

**Appendix 1:**  
**Long treatment (24 hours) without metabolic activation (- S9)**

**Relative Survival after treatment**  
**Long treatment (24 hours) without metabolic activation (- S9)**

Dose μg/ml	Replicate culture	Number of positive well		Number of wells seeded	Number of cells seeded per well	P(0)	-ln P(0)	% Cloning Efficiency	% Relative Survival
0	A	72	81	192	1.6	0.2031	1.5941	99.63	100.00
	B	66	73	192	1.6	0.2760	1.2874	80.46	100.00
0 UT	A	79	78	192	1.6	0.1823	1.7021	106.38	106.78
	B	72	79	192	1.6	0.2135	1.5441	96.51	119.95
1.7	A	66	70	192	1.6	0.2917	1.2320	77.00	77.29
	B	76	64	192	1.6	0.2708	1.3064	81.65	101.48
5.4	A	77	74	192	1.6	0.2135	1.5441	96.51	96.87
	B	70	73	192	1.6	0.2552	1.3657	85.36	106.09
17	A	70	70	192	1.6	0.2708	1.3064	81.65	81.95
	B	68	72	192	1.6	0.2708	1.3064	81.65	101.48
52	A	77	73	192	1.6	0.2188	1.5196	94.98	95.33
	B	77	72	192	1.6	0.2240	1.4961	93.51	116.22
164	A	72	74	192	1.6	0.2396	1.4288	89.30	89.63
	B	69	69	192	1.6	0.2813	1.2683	79.27	98.52
512	A	86	78	192	1.6	0.1458	1.9255	120.34	120.79
	B	88	89	192	1.6	0.0781	2.5498	159.36	198.06
1600	A	75	74	192	1.6	0.2240	1.4961	93.51	93.86
	B	68	69	192	1.6	0.2865	1.2500	78.13	97.10
5000	A	75	74	192	1.6	0.2240	1.4961	93.51	93.86
	B	78	78	192	1.6	0.1875	1.6740	104.63	130.04
MMS 4	A	58	53	192	1.6	0.4219	0.8630	53.94	54.14
	B	39	41	192	1.6	0.5833	0.5391	33.69	41.87
MMS 7.5	A	20	24	192	1.6	0.7708	0.2603	16.27	16.33
	B	25	20	192	1.6	0.7656	0.2671	16.69	20.74

Abbreviations:

UT: Untreated control

MMS: Positive control

**Appendix 1 (cont'd)**

**Suspension Growth (SG) and Relative Total Growth (RTG) during the expression period**  
**Long treatment (24 hours) without metabolic activation (- S9)**

Dose µg/ml	Replicate culture	Day 2		Day 3		SG	RTG
		Number of cells seeded ( x 10 <sup>5</sup> /ml)	Cell Counts ( x 10 <sup>5</sup> /ml)	Number of cells seeded ( x 10 <sup>5</sup> /ml)	Cell Counts ( x 10 <sup>5</sup> /ml)		
0	A	2.00	6.10	2.00	7.50	11.44	1.00
	B	2.00	8.50	2.00	5.10	10.84	1.00
0 UT	A	2.00	6.10	2.00	7.50	11.44	1.00
	B	2.00	8.10	2.00	6.20	12.56	1.04
1.7	A	2.00	6.10	2.00	7.40	11.29	0.90
	B	2.00	8.10	2.00	5.80	11.75	1.12
5.4	A	2.00	6.30	2.00	7.70	12.13	0.89
	B	2.00	7.90	2.00	6.20	12.25	1.26
17	A	2.00	6.40	2.00	7.50	12.00	1.03
	B	2.00	7.70	2.00	5.90	11.36	1.21
52	A	2.00	6.00	2.00	7.50	11.25	1.18
	B	2.00	8.20	2.00	6.10	12.51	0.93
164	A	2.00	5.80	2.00	8.50	12.33	1.09
	B	2.00	8.30	2.00	5.80	12.04	1.18
512	A	2.00	7.70	2.00	6.30	12.13	1.34
	B	2.00	9.20	2.00	4.90	11.27	1.11
1600	A	2.00	5.20	2.00	6.80	8.84	1.02
	B	2.00	8.80	2.00	4.60	10.12	1.06
5000	A	2.00	6.20	2.00	6.00	9.30	1.13
	B	2.00	6.60	2.00	5.40	8.91	0.89
MMS 4	A	2.00	6.30	2.00	5.50	8.66	0.26
	B	2.00	5.50	2.00	5.00	6.88	0.17
MMS 7.5	A	2.00	5.60	2.00	4.70	6.58	0.06
	B	2.00	6.00	2.00	3.90	5.85	0.04

**Abbreviations:**

UT: Untreated control

MMS: Positive control

**Appendix 1 (cont'd)**

**Viability during the selection**  
**Long treatment (24 hours) without metabolic activation (- S9)**

Dose µg/ml	Replicate culture	Number of positive well		Number of wells seeded	Cell seeded per well	P(0)	-ln P(0)	% Absolute Viable	% Relative Viability
0	A	77	63	192	1.6	0.2708	1.3064	81.65	100.00
	B	76	71	192	1.6	0.2344	1.4507	90.67	100.00
0 UT	A	70	70	192	1.6	0.2708	1.3064	81.65	100.00
	B	71	69	192	1.6	0.2708	1.3064	81.65	90.05
1.7	A	64	70	192	1.6	0.3021	1.1970	74.81	91.62
	B	74	75	192	1.6	0.2240	1.4961	93.51	103.13
5.4	A	63	65	192	1.6	0.3333	1.0987	68.67	84.10
	B	76	78	192	1.6	0.1979	1.6200	101.25	111.67
17	A	67	72	192	1.6	0.2760	1.2874	80.46	98.54
	B	82	74	192	1.6	0.1875	1.6740	104.63	115.40
52	A	78	74	192	1.6	0.2083	1.5688	98.05	120.09
	B	70	62	192	1.6	0.3125	1.1632	72.70	80.18
164	A	75	66	192	1.6	0.2656	1.3258	82.86	101.48
	B	78	73	192	1.6	0.2135	1.5441	96.51	106.44
512	A	74	81	192	1.6	0.1927	1.6466	102.91	126.04
	B	70	81	192	1.6	0.2135	1.5441	96.51	106.44
1600	A	75	83	192	1.6	0.1771	1.7310	108.19	132.50
	B	76	79	192	1.6	0.1927	1.6466	102.91	113.50
5000	A	79	82	192	1.6	0.1615	1.8233	113.96	139.57
	B	82	70	192	1.6	0.2083	1.5688	98.05	108.14
MMS 4	A	37	33	192	1.6	0.6354	0.4535	28.34	34.71
	B	31	30	192	1.6	0.6823	0.3823	23.89	26.35
MMS 7.5	A	10	15	192	1.6	0.8698	0.1395	8.72	10.68
	B	12	9	192	1.6	0.8906	0.1159	7.24	7.99

**Abbreviations:**

UT: Untreated control

MMS: Positive control

Appendix 1 (cont'd)

**Mutant frequencies and sizing for Small (S) and Large (L) colonies after the selection**  
**Long treatment (24 hours) without metabolic activation (- S9)**

Dose µg/ml	Replicate culture	Positive wells				Colony sizing												Number of wells seeded	Cell seeded per well	P(0)	-ln P(0)	Mutant Frequency for 10 <sup>6</sup> cells
		Individual values				Sum	S	L	S	L	S	L	S	L	S	L	Sum "S"					
0	A	19	17	15	20	71	9	10	4	13	5	10	5	15	23	48	384	2000	0.8151	0.2044	125.17	
	B	7	11	11	9	38	4	3	5	6	4	7	5	4	18	20	384	2000	0.9010	0.1043	57.52	
0 UT	A	15	16	14	13	58	7	8	4	12	3	11	7	6	21	37	384	2000	0.8490	0.1637	100.24	
	B	17	10	11	15	53	10	7	2	8	6	5	7	8	25	28	384	2000	0.8620	0.1485	90.94	
1.7	A	19	14	9	15	57	7	12	7	7	1	8	3	12	18	39	384	2000	0.8516	0.1606	107.34	
	B	11	15	12	10	48	4	7	3	12	2	10	2	8	11	37	384	2000	0.8750	0.1335	71.39	
5.4	A	18	14	15	14	61	7	11	4	10	3	12	5	9	19	42	384	2000	0.8411	0.1730	125.97	
	B	12	10	13	9	44	8	4	4	6	6	7	4	5	22	22	384	2000	0.8854	0.1217	60.10	
17	A	14	13	15	15	57	6	8	4	9	6	9	9	6	25	32	384	2000	0.8516	0.1606	99.80	
	B	9	11	11	15	46	1	8	2	9	3	8	6	9	12	34	384	2000	0.8802	0.1276	60.98	
52	A	16	12	20	12	60	8	8	5	7	10	10	4	8	27	33	384	2000	0.8438	0.1698	86.59	
	B	23	16	15	8	62	13	10	10	6	6	9	4	4	33	29	384	2000	0.8385	0.1761	121.11	
164	A	13	9	12	14	48	6	7	3	6	7	5	4	10	20	28	384	2000	0.8750	0.1335	80.56	
	B	13	16	10	12	51	5	8	5	11	2	8	7	5	19	32	384	2000	0.8672	0.1425	73.83	
512	A	15	20	12	14	61	6	9	9	11	5	7	2	12	22	39	384	2000	0.8411	0.1730	84.05	
	B	14	13	13	18	58	5	9	6	7	9	4	11	7	31	27	384	2000	0.8490	0.1637	84.81	
1600	A	16	16	15	18	65	9	7	6	10	9	6	5	13	29	36	384	2000	0.8307	0.1855	85.73	
	B	12	16	15	14	57	6	6	6	10	3	12	7	7	22	35	384	2000	0.8516	0.1606	78.03	
5000	A	26	26	22	11	85	12	14	8	18	10	12	3	8	33	52	384	2000	0.7786	0.2503	109.82	
	B	17	19	22	18	76	6	11	9	10	10	12	11	7	36	40	384	2000	0.8021	0.2205	112.44	
MMS 4	A	45	52	47	43	187	31	14	32	20	26	21	27	16	116	71	384	2000	0.5130	0.6675	1177.51	
	B	33	40	30	33	136	24	9	29	11	21	9	22	11	96	40	384	2000	0.6458	0.4373	915.09	
MMS 7.5	A	23	23	22	27	95	14	9	14	9	16	6	15	12	59	36	384	2000	0.7526	0.2842	1629.82	
	B	14	22	14	14	64	12	2	12	10	11	3	13	1	48	16	384	2000	0.8333	0.1824	1259.02	

Abbreviations:

UT: Untreated control

MMS: Positive control

**Appendix 2:**

**Short treatment (4 hours) without metabolic activation (- S9)**

**Appendix 2 (cont'd)**

**Relative Survival after treatment**  
**Short treatment (4 hours) without metabolic activation (- S9)**

Dose µg/ml	Replicate culture	Number of positive well		Number of wells seeded	Number of cells seeded per well	P(0)	-ln P(0)	% Cloning Efficiency	% Relative Survival
0	A	67	69	192	1.6	0.2917	1.2320	77.00	100.00
	B	56	71	192	1.6	0.3385	1.0832	67.70	100.00
0 UT	A	70	60	192	1.6	0.3229	1.1304	70.65	91.75
	B	61	70	192	1.6	0.3177	1.1466	71.66	105.85
1.7	A	62	60	192	1.6	0.3646	1.0090	63.06	81.90
	B	61	55	192	1.6	0.3958	0.9268	57.93	85.57
5.4	A	56	71	192	1.6	0.3385	1.0832	67.70	87.92
	B	62	69	192	1.6	0.3177	1.1466	71.66	105.85
17	A	58	60	192	1.6	0.3854	0.9535	59.59	77.39
	B	68	60	192	1.6	0.3333	1.0987	68.67	101.43
52	A	59	58	192	1.6	0.3906	0.9401	58.76	76.31
	B	64	59	192	1.6	0.3594	1.0233	63.96	94.48
164	A	67	59	192	1.6	0.3438	1.0677	66.73	86.66
	B	59	61	192	1.6	0.3750	0.9808	61.30	90.55
512	A	58	64	192	1.6	0.3646	1.0090	63.06	81.90
	B	64	63	192	1.6	0.3385	1.0832	67.70	100.00
1600	A	68	66	192	1.6	0.3021	1.1970	74.81	97.16
	B	72	67	192	1.6	0.2760	1.2874	80.46	118.85
5000	A	71	64	192	1.6	0.2969	1.2144	75.90	98.57
	B	65	54	192	1.6	0.3802	0.9671	60.44	89.28
MMS 4	A	70	60	192	1.6	0.3229	1.1304	70.65	91.75
	B	64	65	192	1.6	0.3281	1.1144	69.65	102.88
MMS 7.5	A	52	62	192	1.6	0.4063	0.9007	56.29	73.10
	B	69	62	192	1.6	0.3177	1.1466	71.66	105.85

**Abbreviations:**

UT: Untreated control

MMS: Positive control

**Appendix 2 (cont'd)**

**Suspension Growth (SG) and Relative Total Growth (RTG) during the expression period**  
**Short treatment (4 hours) without metabolic activation (- S9)**

Dose µg/ml	Replicate culture	Day 2		Day 3		SG	RTG
		Number of cells seeded ( x 10 <sup>5</sup> /ml)	Cell Counts ( x 10 <sup>5</sup> /ml)	Number of cells seeded ( x 10 <sup>5</sup> /ml)	Cell Counts ( x 10 <sup>5</sup> /ml)		
0	A	2.00	4.20	2.00	8.70	9.14	1.00
	B	2.00	4.30	2.00	9.40	10.11	1.00
0 UT	A	2.00	4.20	2.00	9.30	9.77	0.91
	B	2.00	4.50	2.00	9.10	10.24	1.03
1.7	A	2.00	4.10	2.00	8.90	9.12	0.75
	B	2.00	4.40	2.00	9.60	10.56	0.85
5.4	A	2.00	4.10	2.00	9.30	9.53	0.89
	B	2.00	4.50	2.00	9.50	10.69	1.10
17	A	2.00	4.10	2.00	8.50	8.71	0.87
	B	2.00	4.40	2.00	9.40	10.34	0.88
52	A	2.00	3.60	2.00	9.70	8.73	1.09
	B	2.00	4.60	2.00	8.90	10.24	1.37
164	A	2.00	4.50	2.00	8.20	9.23	0.92
	B	2.00	5.00	2.00	9.30	11.63	1.15
512	A	2.00	3.20	2.00	10.00	8.00	1.06
	B	2.00	4.20	2.00	10.50	11.03	0.84
1600	A	2.00	4.90	2.00	8.90	10.90	1.23
	B	2.00	5.50	2.00	10.10	13.89	1.40
5000	A	2.00	3.50	2.00	11.30	9.89	1.06
	B	2.00	4.80	2.00	9.90	11.88	1.08
MMS 4	A	2.00	4.00	2.00	8.70	8.70	0.72
	B	2.00	4.50	2.00	9.90	11.14	0.91
MMS 7.5	A	2.00	3.60	2.00	8.60	7.74	0.61
	B	2.00	3.60	2.00	9.40	8.46	0.37

**Abbreviations:**

UT: Untreated control

MMS: Positive control

Appendix 2 (cont'd)

**Viability during the selection**  
**Short treatment (4 hours) without metabolic activation (- S9)**

Dose $\mu\text{g/ml}$	Replicate culture	Number of positive well		Number of wells seeded	Cell seeded per well	P(0)	-ln P(0)	% Absolute Viable	% Relative Viability
0	A	79	75	192	1.6	0.1979	1.6200	101.25	100.00
	B	84	76	192	1.6	0.1667	1.7916	111.98	100.00
0 UT	A	72	72	192	1.6	0.2500	1.3863	86.64	85.57
	B	84	77	192	1.6	0.1615	1.8233	113.96	101.77
1.7	A	65	70	192	1.6	0.2969	1.2144	75.90	74.96
	B	74	73	192	1.6	0.2344	1.4507	90.67	80.97
5.4	A	71	73	192	1.6	0.2500	1.3863	86.64	85.57
	B	83	79	192	1.6	0.1563	1.8560	116.00	103.59
17	A	71	77	192	1.6	0.2292	1.4732	92.08	90.94
	B	77	74	192	1.6	0.2135	1.5441	96.51	86.19
52	A	82	80	192	1.6	0.1563	1.8560	116.00	114.57
	B	84	91	192	1.6	0.0885	2.4248	151.55	135.34
164	A	75	73	192	1.6	0.2292	1.4732	92.08	90.94
	B	79	81	192	1.6	0.1667	1.7916	111.98	100.00
512	A	81	84	192	1.6	0.1406	1.9618	122.61	121.10
	B	70	74	192	1.6	0.2500	1.3863	86.64	77.37
1600	A	81	75	192	1.6	0.1875	1.6740	104.63	103.34
	B	81	80	192	1.6	0.1615	1.8233	113.96	101.77
5000	A	74	79	192	1.6	0.2031	1.5941	99.63	98.40
	B	75	80	192	1.6	0.1927	1.6466	102.91	91.90
MMS 4	A	68	68	192	1.6	0.2917	1.2320	77.00	76.05
	B	70	78	192	1.6	0.2292	1.4732	92.08	82.23
MMS 7.5	A	68	64	192	1.6	0.3125	1.1632	72.70	71.80
	B	55	50	192	1.6	0.4531	0.7916	49.48	44.19

**Abbreviations:**

UT: Untreated control

MMS: Positive control

**Appendix 2 (cont'd)**

**Mutant frequencies and sizing for Small (S) and Large (L) colonies after the selection**  
**Short treatment (4 hours) without metabolic activation (- S9)**

Dose µg/ml	Replicate culture	Positive wells				Sum	Colony sizing								Sum "S"	Sum "L"	Number of wells seeded	Cell seeded per well	P(0)	-ln P(0)	Mutant Frequency for 10 <sup>6</sup> cells
		Individual values	S	L	S		L	S	L	S	L										
0	A	44	56	58	57	215	4	40	8	48	5	53	4	53	21	194	384	2000	0.4401	0.8208	405.33
	B	49	61	50	56	216	8	41	2	59	3	47	5	51	18	198	384	2000	0.4375	0.8267	369.14
0 UT	A	53	50	45	46	194	5	48	6	44	4	41	2	44	17	177	384	2000	0.4948	0.7036	406.03
	B	62	57	58	54	231	7	55	7	50	8	50	6	48	28	203	384	2000	0.3984	0.9203	403.80
17	A	54	51	56	50	211	7	47	3	48	1	55	4	46	15	196	384	2000	0.4505	0.7974	525.30
	B	47	63	56	58	224	3	44	1	62	5	51	6	52	15	209	384	2000	0.4167	0.8754	482.75
54	A	53	51	51	43	198	7	46	2	49	3	48	1	42	13	185	384	2000	0.4844	0.7248	418.26
	B	51	53	58	48	210	4	47	2	51	5	53	5	43	16	194	384	2000	0.4531	0.7916	341.21
17	A	47	59	49	51	206	5	42	1	58	6	43	6	45	18	188	384	2000	0.4635	0.7689	417.54
	B	56	44	53	52	205	8	48	2	42	3	50	4	48	17	188	384	2000	0.4661	0.7634	395.52
52	A	48	50	46	49	193	3	45	6	44	7	39	7	42	23	170	384	2000	0.4974	0.6984	301.03
	B	67	64	59	59	249	1	66	5	59	5	54	5	54	16	233	384	2000	0.3516	1.0453	344.87
164	A	53	53	47	60	213	1	52	2	51	5	42	2	58	10	203	384	2000	0.4453	0.8090	439.32
	B	50	53	55	46	204	2	48	4	49	10	45	2	44	18	186	384	2000	0.4688	0.7576	338.29
512	A	53	66	55	51	225	2	51	3	63	1	54	3	48	9	216	384	2000	0.4141	0.8816	359.51
	B	49	61	48	52	210	4	45	7	54	2	46	6	46	19	191	384	2000	0.4531	0.7916	456.81
1600	A	52	42	52	53	199	4	48	0	42	3	49	0	53	7	192	384	2000	0.4818	0.7302	348.96
	B	57	54	56	56	223	5	52	5	49	0	56	7	49	17	206	384	2000	0.4193	0.8692	381.37
5000	A	61	54	54	58	227	4	57	4	50	5	49	5	53	18	209	384	2000	0.4089	0.8943	448.80
	B	63	53	47	56	219	6	57	4	49	2	45	5	51	17	202	384	2000	0.4297	0.8447	410.40
MMS 4	A	76	73	75	68	292	18	58	14	59	14	61	13	55	59	233	384	2000	0.2396	1.4288	927.79
	B	79	74	77	86	316	16	63	21	53	12	65	19	67	68	248	384	2000	0.1771	1.7310	939.99
MMS 7.5	A	73	83	68	65	289	22	51	16	67	16	52	14	51	68	221	384	2000	0.2474	1.3967	960.59
	B	76	83	72	73	304	25	51	21	62	15	57	18	55	79	225	384	2000	0.2083	1.5688	1585.45

**Abbreviations:**

UT: Untreated control

MMS: Positive control

**Appendix 3:**  
**Short treatment (4 hours) with metabolic activation (+ S9)**

**Appendix 3 (cont'd)**

**Relative Survival after treatment**  
**Short treatment (4 hours) with metabolic activation (+ S9)**

Dose µg/ml	Replicate culture	Number of positive well		Number of wells seeded	Number of cells seeded per well	P(0)	-ln P(0)	% Cloning Efficiency	% Relative Survival
0	A	73	55	192	1.6	0.3333	1.0987	68.67	100.00
	B	54	59	192	1.6	0.4115	0.8879	55.49	100.00
0 UT	A	64	62	192	1.6	0.3438	1.0677	66.73	97.17
	B	57	63	192	1.6	0.3750	0.9808	61.30	110.47
1.7	A	64	59	192	1.6	0.3594	1.0233	63.96	93.14
	B	53	60	192	1.6	0.4115	0.8879	55.49	100.00
5.4	A	55	64	192	1.6	0.3802	0.9671	60.44	88.02
	B	57	51	192	1.6	0.4375	0.8267	51.67	93.12
17	A	55	57	192	1.6	0.4167	0.8754	54.71	79.67
	B	64	61	192	1.6	0.3490	1.0527	65.79	118.56
52	A	55	49	192	1.6	0.4583	0.7802	48.76	71.01
	B	55	60	192	1.6	0.4010	0.9138	57.11	102.92
164	A	47	56	192	1.6	0.4635	0.7689	48.06	69.99
	B	56	57	192	1.6	0.4115	0.8879	55.49	100.00
512	A	62	61	192	1.6	0.3594	1.0233	63.96	93.14
	B	60	56	192	1.6	0.3958	0.9268	57.93	104.40
1600	A	71	66	192	1.6	0.2865	1.2500	78.13	113.78
	B	56	59	192	1.6	0.4010	0.9138	57.11	102.92
5000	A	67	65	192	1.6	0.3125	1.1632	72.70	105.87
	B	58	63	192	1.6	0.3698	0.9948	62.18	112.06
CP 2.5	A	53	45	192	1.6	0.4896	0.7142	44.64	65.01
	B	48	56	192	1.6	0.4583	0.7802	48.76	87.87
CP 5	A	17	12	192	1.6	0.8490	0.1637	10.23	14.90
	B	5	9	192	1.6	0.9271	0.0757	4.73	8.52

**Abbreviations:**

UT: Untreated control

CP: Positive control

**Appendix 3 (cont'd)**

**Suspension Growth (SG) and Relative Total Growth (RTG) during the expression period**  
**Short treatment (4 hours) with metabolic activation (+ S9)**

Dose µg/ml	Replicate culture	Day 2		Day 3		SG	RTG
		Number of cells seeded ( x 10 <sup>5</sup> /ml)	Cell Counts ( x 10 <sup>5</sup> /ml)	Number of cells seeded ( x 10 <sup>5</sup> /ml)	Cell Counts ( x 10 <sup>5</sup> /ml)		
0	A	2.00	3.20	2.00	7.40	5.92	1.00
	B	2.00	3.40	2.00	7.20	6.12	1.00
0 UT	A	2.00	3.70	2.00	7.40	6.85	1.04
	B	2.00	3.40	2.00	7.80	6.63	1.12
1.7	A	2.00	3.50	2.00	7.70	6.74	0.95
	B	2.00	3.50	2.00	7.30	6.39	0.89
5.4	A	2.00	3.10	2.00	7.90	6.12	1.08
	B	2.00	3.60	2.00	8.20	7.38	1.24
17	A	2.00	3.30	2.00	6.80	5.61	0.93
	B	2.00	3.50	2.00	7.50	6.56	1.06
52	A	2.00	3.30	2.00	7.30	6.02	0.99
	B	2.00	3.40	2.00	8.60	7.31	1.25
164	A	2.00	3.30	2.00	7.60	6.27	1.11
	B	2.00	3.70	2.00	8.30	7.68	1.07
512	A	2.00	3.40	2.00	6.90	5.87	1.07
	B	2.00	3.10	2.00	8.50	6.59	1.09
1600	A	2.00	3.60	2.00	6.70	6.03	1.18
	B	2.00	3.60	2.00	8.00	7.20	1.40
5000	A	2.00	3.80	2.00	8.00	7.60	1.17
	B	2.00	3.90	2.00	6.50	6.34	1.90
CP 2.5	A	2.00	2.60	2.00	7.30	4.75	0.52
	B	2.00	3.10	2.00	8.40	6.51	0.41
CP 5	A	2.00	2.20	2.00	7.90	4.35	0.05
	B	2.00	2.20	2.00	7.50	4.13	0.04

**Abbreviations:**

UT: Untreated control

CP: Positive control

Appendix 3 (cont'd)Viability during the selection

Short treatment (4 hours) with metabolic activation (+ S9)

Dose µg/ml	Replicate culture	Number of positive well		Number of wells seeded	Cell seeded per well	P(0)	-ln P(0)	% Absolute Viable	% Relative Viability
0	A	77	74	192	1.6	0.2135	1.5441	<b>96.51</b>	<b>100.00</b>
	B	72	74	192	1.6	0.2396	1.4288	<b>89.30</b>	<b>100.00</b>
0 UT	A	70	74	192	1.6	0.2500	1.3863	<b>86.64</b>	<b>89.77</b>
	B	69	79	192	1.6	0.2292	1.4732	<b>92.08</b>	<b>103.11</b>
1.7	A	69	70	192	1.6	0.2760	1.2874	<b>80.46</b>	<b>83.37</b>
	B	73	62	192	1.6	0.2969	1.2144	<b>75.90</b>	<b>84.99</b>
5.4	A	81	73	192	1.6	0.1979	1.6200	<b>101.25</b>	<b>104.91</b>
	B	74	ns	96	1.6	0.2292	1.4732	<b>92.08</b>	<b>103.11</b>
17	A	72	78	192	1.6	0.2188	1.5196	<b>94.98</b>	<b>98.41</b>
	B	69	76	192	1.6	0.2448	1.4073	<b>87.96</b>	<b>98.50</b>
52	A	76	73	192	1.6	0.2240	1.4961	<b>93.51</b>	<b>96.89</b>
	B	78	71	192	1.6	0.2240	1.4961	<b>93.51</b>	<b>104.71</b>
164	A	74	80	192	1.6	0.1979	1.6200	<b>101.25</b>	<b>104.91</b>
	B	68	67	192	1.6	0.2969	1.2144	<b>75.90</b>	<b>84.99</b>
512	A	85	71	192	1.6	0.1875	1.6740	<b>104.63</b>	<b>108.41</b>
	B	74	73	192	1.6	0.2344	1.4507	<b>90.67</b>	<b>101.53</b>
1600	A	81	79	192	1.6	0.1667	1.7916	<b>111.98</b>	<b>116.03</b>
	B	79	78	192	1.6	0.1823	1.7021	<b>106.38</b>	<b>119.13</b>
5000	A	69	76	192	1.6	0.2448	1.4073	<b>87.96</b>	<b>91.14</b>
	B	88	90	192	1.6	0.0729	2.6187	<b>163.67</b>	<b>183.28</b>
CP 2.5	A	61	61	192	1.6	0.3646	1.0090	<b>63.06</b>	<b>65.34</b>
	B	39	43	192	1.6	0.5729	0.5570	<b>34.81</b>	<b>38.98</b>
CP 5	A	12	8	192	1.6	0.8958	0.1100	<b>6.88</b>	<b>7.13</b>
	B	7	9	192	1.6	0.9167	0.0870	<b>5.44</b>	<b>6.09</b>

Abbreviations:

UT: Untreated control

CP: Positive control

ns: not scored.

**Appendix 3 (cont'd)**

**Mutant frequencies and sizing for Small (S) and Large (L) colonies after the selection**  
**Short treatment (4 hours) with metabolic activation (+ S9)**

Dose µg/ml	Replicate culture	Positive wells				Colony sizing										Number of wells seeded	Cell seeded per well	P(0)	-ln P(0)	Mutant Frequency for 10 <sup>6</sup> cells	
		Individual values				Sum	S	L	S	L	S	L	S	L	S						L
0	A	11	17	3	7	38	4	7	6	11	1	2	2	5	13	25	384	2000	0.9010	0.1043	54.04
	B	7	10	12	11	40	3	4	5	5	9	3	8	3	25	15	384	2000	0.8958	0.1100	61.59
0 UT	A	11	15	7	8	41	5	6	10	5	2	5	3	5	20	21	384	2000	0.8932	0.1129	65.15
	B	10	9	16	5	40	3	7	3	6	8	8	4	1	18	22	384	2000	0.8958	0.1100	59.73
17	A	10	7	5	6	28	2	8	1	6	1	4	2	4	6	22	384	2000	0.9271	0.0757	47.04
	B	12	13	13	10	48	4	8	4	9	5	8	4	6	17	31	384	2000	0.8750	0.1335	87.94
54	A	12	6	5	6	29	5	7	2	4	1	4	3	3	11	18	384	2000	0.9245	0.0785	38.77
	B	11	8	11	12	42	7	4	4	4	6	5	3	9	20	22	384	2000	0.8906	0.1159	62.94
17	A	9	8	9	8	34	3	6	2	6	4	5	1	7	10	24	384	2000	0.9115	0.0927	48.80
	B	11	8	10	6	35	9	2	4	4	3	7	0	6	16	19	384	2000	0.9089	0.0955	54.29
52	A	5	12	11	10	38	2	3	3	9	6	5	5	5	16	22	384	2000	0.9010	0.1043	55.77
	B	11	11	8	21	51	3	8	7	4	3	5	13	8	26	25	384	2000	0.8672	0.1425	76.20
164	A	9	6	14	10	39	3	6	1	5	5	9	5	5	14	25	384	2000	0.8984	0.1071	52.89
	B	14	6	10	15	45	7	7	5	1	4	6	6	9	22	23	384	2000	0.8828	0.1247	82.15
512	A	7	10	8	14	39	3	4	5	5	6	2	4	10	18	21	384	2000	0.8984	0.1071	51.18
	B	11	19	15	17	62	7	4	7	12	8	7	4	13	26	36	384	2000	0.8385	0.1761	97.11
1600	A	8	8	4	12	32	1	7	2	6	2	2	5	7	10	22	384	2000	0.9167	0.0870	38.85
	B	14	16	10	13	53	5	9	5	11	2	8	8	5	20	33	384	2000	0.8620	0.1485	69.80
5000	A	9	11	10	9	39	5	4	8	3	5	5	6	3	24	15	384	2000	0.8984	0.1071	60.88
	B	17	14	11	12	54	8	9	5	9	3	8	7	5	23	31	384	2000	0.8594	0.1515	46.28
CP 2.5	A	48	45	47	42	182	30	18	31	14	34	13	25	17	120	62	384	2000	0.5260	0.6425	509.42
	B	52	48	52	59	211	33	19	30	18	30	22	33	26	126	85	384	2000	0.4505	0.7974	1145.28
CP 5	A	28	32	20	18	98	23	5	17	15	12	8	12	6	64	34	384	2000	0.7448	0.2946	2142.55
	B	23	33	31	23	110	14	9	24	9	27	4	10	13	75	35	384	2000	0.7135	0.3376	3104.37

**Abbreviations:**

UT: Untreated control

CP: Positive control

**STATISTICS**

**Appendix 4:**  
**Long treatment (24 hours) without metabolic activation (- S9)**

**Long treatment (24 hours) without metabolic activation (- S9)**

Dose level ( $\mu\text{g/ml}$ )	Mutant Frequency §	Dunnett's Significance	Ratio Small/Large colonies
0	91.35		0.69
1.7	89.37	NS	0.38
5.4	93.04	NS	0.73
17	80.39	NS	0.57
52	103.85	NS	0.98
164	77.20	NS	0.65
512	84.43	NS	0.86
1600	81.88	NS	0.72
5000	111.13	NS	0.77

§

Mutants/ $10^6$  viable cells 2 days after treatment

NS

Not significant

\*, \*\*, \*\*\*

Significant at 5%, 1% and 0.1% level respectively

**Appendix 5:**  
**Short treatment (4 hours) without metabolic activation (- S9)**

Short treatment (4 hours) without metabolic activation (- S9)

Dose level ( $\mu\text{g/ml}$ )	Mutant Frequency §	Dunnett's Significance	Ratio Small/Large colonies
0	387.24		0.10
1.7	504.03	NS	0.08
5.4	379.74	NS	0.08
17	406.53	NS	0.10
52	322.95	NS	0.11
164	388.81	NS	0.08
512	408.16	NS	0.07
1600	365.17	NS	0.06
5000	429.60	NS	0.09

§

NS

\*, \*\*, \*\*\*

Mutants/ $10^6$  viable cells 2 days after treatment

Not significant

Significant at 5%, 1% and 0.1% level respectively

**Appendix 6:**  
**Short treatment (4 hours) with metabolic activation (+ S9)**

**Short treatment (4 hours) with metabolic activation (+ S9)**

Dose level ( $\mu\text{g/ml}$ )	Mutant Frequency §	Dunnett's Significance	Ratio Small/Large colonies
0	57.82		1.10
1.7	67.49	NS	0.41
5.4	50.86	NS	0.76
17	51.55	NS	0.63
52	65.99	NS	0.89
164	67.52	NS	0.76
512	74.15	NS	0.79
1600	54.33	NS	0.53
5000	53.58	NS	1.17

§

Mutants/ $10^6$  viable cells 2 days after treatment

NS

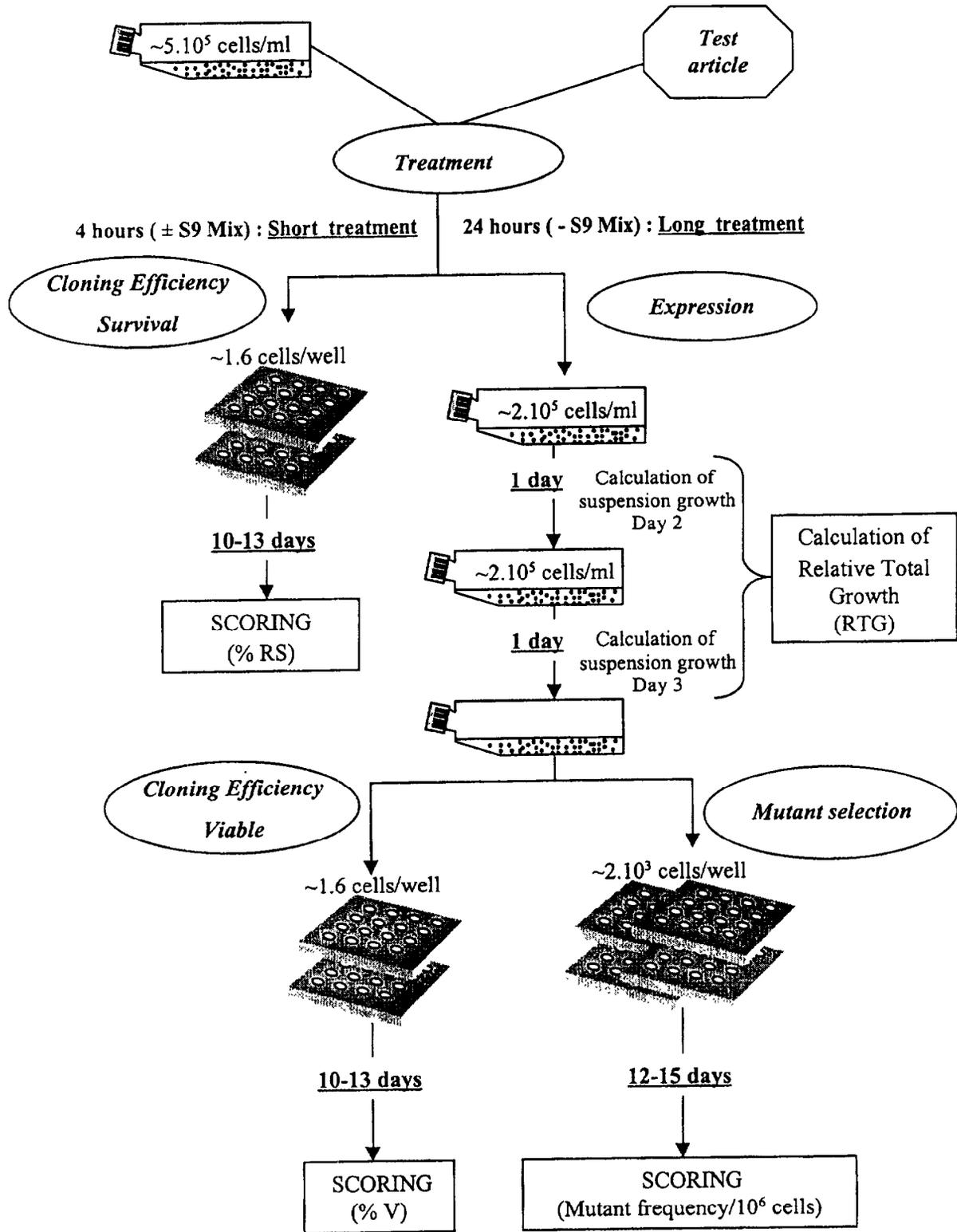
Not significant

\*, \*\*, \*\*\*

Significant at 5%, 1% and 0.1% level respectively

**14. ADDENDA**

**Addendum 1:**  
**Standard Schema of the Study Design**



**Addendum 2:**  
**Bibliography**

**Addendum 2 (cont'd)**

- [1] Moore, M.M., DeMarini, D.M., DeSerres, F.J. and Tindall, K.R. (Eds.) (1987). Banbury Report 28: Mammalian Cell Mutagenesis, Cold Spring Harbor Laboratory, New York.
- [2] Chu, E.H.Y. and Malling, H.V. (1968). Mammalian Cell Genetics. II. Chemical Induction of Specific Locus Mutations in Chinese Hamster Cells *In Vitro*, Proc. Natl. Acad. Sci., USA, 61, 1306-1312.
- [3] Liber, H.L. and Thilly, W.G. (1982). Mutation Assay at the Thymidine Kinase Locus in Diploid Human Lymphoblasts. *Mutation Res.*, 94, 467-485.
- [4] Moore, M.M., Harrington-Brock, K., Doerr, C.L. and Dearfield, K.L. (1989). Differential Mutant Quantitation at the Mouse Lymphoma TK and CHO HGPRT Loci. *Mutagenesis*, 4, 394-403.
- [5] Aaron, C.S. and Stankowski, Jr. L.F. (1989). Comparison of the AS52/XPRT and the CHO/HPRT Assays: Evaluation of Six Drug Candidates. *Mutation Res.*, 223, 121-128.
- [6] Aaron, C.S., Bolcsfoldi, G., Glatt, H.R., Moore, M., Nishi, Y., Stankowski, L., Theiss, J. and Thompson, E. (1994). Mammalian Cell Gene Mutation Assays Working Group Report. Report of the International Workshop on Standardisation of Genotoxicity Test Procedures. *Mutation Res.*, 312, 235-239.
- [7] Scott, D., Galloway, S.M., Marshall, R.R., Ishidate, M., Brusick, D., Ashby, J. and Myhr, B.C. (1991). Genotoxicity Under Extreme Culture Conditions. A report from ICPEMC Task Group 9. *Mutation Res.*, 257, 147-204.
- [8] Clive, D., McCuen, R., Spector, J.F.S., Piper, C. and Mavourmin, K.H. (1983). Specific Gene Mutations in L5178Y Cells in Culture. A Report of the U.S. Environmental Protection Agency Gene-Tox program. *Mutation Res.*, 115, 225-251.
- [9] Li, A.P., Gupta, R.S., Heflich, R.H. and Wasson, J. S. (1988). A Review and Analysis of the Chinese Hamster Ovary/Hypoxanthine Guanine Phosphoribosyl Transferase System to Determine the Mutagenicity of Chemical Agents: A Report of Phase III of the U.S. Environmental Protection Agency Gene-tox Program. *Mutation Res.*, 196, 17-36.
- [10] Li, A.P., Carver, J.H., Choy, W.N., Hsie, A.W., Gupta, R.S., Loveday, K.S., O'Neill, J.P., Riddle, J.C., Stankowski, L.F. Jr. and Yang, L.L. (1987). A Guide for the Performance of the Chinese Hamster Ovary Cell/Hypoxanthine-Guanine Phosphoribosyl Transferase Gene Mutation Assay. *Mutation Res.*, 189, 135-141.
- [11] Liber, H.L., Yandell, D.W. and Little, J.B. (1989). A Comparison of Mutation Induction at the tk and hpert Loci in Human Lymphoblastoid Cells; Quantitative Differences are Due to an Additional Class of Mutations at the Autosomal TK Locus. *Mutation Res.*, 216, 9-17.
- [12] Stankowski, L.F. Jr., Tindall, K.R. and Hsie, A.W. (1986). Quantitative and Molecular Analyses of Ethyl Methanesulfonate- and ICR 191-Induced Molecular Analyses of Ethyl Methanesulfonate- and ICR 191-Induced Mutation in AS52 Cells. *Mutation Res.* 160, 133-147.
- [13] Turner, N.T., Batson, A.G. and Clive, D. (1984). Procedures for the L5178Y/TK<sup>+/+</sup> - TK<sup>+/+</sup> Mouse Lymphoma Cell Mutagenicity Assay. In: Kilbey, B.J. et al. (eds.) *Handbook of Mutagenicity Test Procedures*, Elsevier Science Publishers, New York, pp. 239-268.

- [14] Arlett, C.F., Smith, D.M., Clarke, G.M., Green, M.H.L., Cole, J., McGregor, D.B. and Asquith, J.C. (1989). Mammalian Cell Gene Mutation Assays Based upon Colony Formation. In: Statistical Evaluation of Mutagenicity Test Data, Kirkland, D.J. Ed., Cambridge University Press, pp. 66-101.
- [15] Abbondandolo, A., Bonatti, S., Corti, G., Fiorio, R., Loprieno, N. and Mazzaccaro, A. (1977). Induction of 6-Thioguanine-Resistant Mutants in V79 Chinese Hamster Cells by Mouse-Liver Microsome-Activated Dimethylnitrosamine. *Mutation Res.*, 46, 365-373.
- [16] Ames, B.N., McCann, J. and Yamasaki, E. (1975). Methods for Detecting Carcinogens and Mutagens with the Salmonella/Mammalian-Microsome Mutagenicity Test. *Mutation Res.*, 31, 347-364.
- [17] Clive, D., Johnson, K.O., Spector, J.F.S., Batson, A.G. and Brown M.M.M. (1979). Validation and Characterization of the L5178Y/TK<sup>+</sup>/- Mouse Lymphoma Mutagen Assay System. *Mutat. Res.*, 59, 61-108.
- [18] Maron, D.M. and Ames, B.N. (1983). Revised Methods for the Salmonella Mutagenicity Test. *Mutation Res.*, 113, 173, 215.
- [19] Elliott, B.M., Combes, R.D., Elcombe, C.R., Gatehouse, D.G., Gibson, G.G., Mackay, J.M. and Wolf, R.C. (1992) Alternatives to Aroclor 1254-Induced S9 in *In Vitro* Genotoxicity Assays. *Mutagenesis*, 7, 175-177.
- [20] Matsushima, T., Sawamura, M., Hara, K. and Sugimura, T. (1976). A Safe Substitute for Polychlorinated Biphenyls as an Inducer of Metabolic Activation Systems. In: *In Vitro* Metabolic Activation in Mutagenesis Testing, de Serres, F.J., Fouts, J.R., Bend, J.R. and Philpot, R.M. (eds), Elsevier, North-Holland, pp. 85-88.
- [21] Krahn, D.F., Barsky, F.C., McCooey, K.T. (1982). CHO/HGPRT Mutation Assay: Evaluation of Gases and Volatile Liquids. In: Tice, R.R., Costa, D.L., Schaich, K.M. (eds.) *Genotoxic Effects of Airborne Agents*. New York, Plenum, pp. 91-103.
- [22] Zamora, P.O., Benson, J.M., Li, A.P. and Brooks, A.L. (1983). Evaluation of an Exposure System Using Cells Grown on Collagen Gels for Detecting Highly Volatile Mutagens in the CHO/HGPRT Mutation Assay. *Environmental Mutagenesis*, 5, 795-801.
- [23] Applegate, M.L., Moore, M.M., Broder, C.B., Burrell, A. and Hozier, J.C. (1990). Molecular Dissection of Mutations at the Heterozygous Thymidine Kinase Locus in Mouse Lymphoma Cells. *Proc. Natl. Acad. Sci. USA*, 87, 51-55.
- [24] Moore, M.M., Clive, D., Hozier, J.C., Howard, B.E., Batson, A.G., Turner, N.T. and Sawyer, J. (1985). Analysis of Trifluorothymidine-Resistant (TFT<sup>r</sup>) Mutants of L5178Y/TK<sup>+</sup>/- Mouse Lymphoma Cells. *Mutation Res.*, 151, 161-174.
- [25] Yandell, D.W., Dryja, T.P. and Little J.B. (1990). Molecular Genetic Analysis of Recessive Mutations at a Heterozygous Autosomal Locus in Human Cells. *Mutat. Res.*, 229, 89-102.
- [26] Moore, M.M. and Doerr, C.L. (1990). Comparison of Chromosome Aberration Frequency and Small-Colony TK-Deficient Mutant Frequency in L5178Y/TK<sup>+</sup>/-3.7.2C Mouse Lymphoma Cells. *Mutagenesis*, 5, 609-614.
- [27] Clay, P. and Cross, M.F. (1990). Microwell mutation assays: evaluation of ethylmethanesulfonate, benzo[a]pyrene and benzidine using the *tk* locus in L5178Y mouse lymphoma cells. *Mutagenesis*, 5 (supplement), 45-54.

**Addendum 3:**  
**Approbation of the protocol**

Addendum 3 (cont'd)

755/002-D 19 of 19  
ING 911 - *In vitro* Mammalian Cell Gene Mutation Test on L5178Y Mouse Lymphoma Cells TK<sup>+</sup>  
(Microtitre method)

---

PROTOCOL: 755/002-D.

PHOENIX INTERNATIONAL STUDY NUMBER: 755/002.

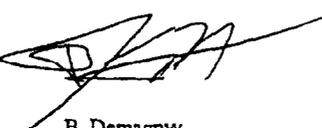
SIGNATURE PAGE

Approved by:

Phoenix International Preclinical Services Europe

STUDY SPONSOR

Signature:   
Name: A. Forichon

Signature:   
Name: B. Demagny

Title: Study Director

Title: Study Monitor

Date: 04 July 2000.

Date: July 22th 2000.

Signature:   
Name: REGNIER

Title: Management representative

Date: 15 December 2000

oubli de signature au  
moment de l'envoi du  
protocole

**Addendum 4:**  
**Quality Control Certificate of S9 fraction**

Addendum 4 (cont'd)

**MOLTOX™ POST MITOCHONDRIAL SUPERNATANT (S-9)  
QUALITY CONTROL & PRODUCTION CERTIFICATE**

LOT NO.: <u>1077</u>	SPECIES: <u>Rat</u>	PREPARATION DATE: <u>March 28, 2000</u>
PART NO.: <u>11-101</u>	STRAIN: <u>Sprague Dawley</u>	EXPIRATION DATE: <u>March 28, 2002</u>
VOLUME: <u>2.0 ml</u>	SEX: <u>Male</u>	BUFFER: <u>0.154 M KCl</u>
	TISSUE: <u>Liver</u>	INDUCING AGENT(s): <u>Aroclor 1254</u>
REFERENCE: <u>Maron, D &amp; Ames, B, <i>Mutat Res</i> 113:173, 1983</u>		<u>(Monsanto KL615), 500 mg/kg i.p.</u>

**BIOCHEMISTRY:**

## - PROTEIN

43.2 mg/ml

Assayed according to the method of Lowry et al., *JBC* 193:265, 1951 using bovine serum albumin as the standard.

## - ALKOXYRESORUFIN-0-DEALKYLASE ACTIVITIES

<u>Activity</u>	<u>P450</u>	<u>Fold - Induction</u>
EROD	IA1, IA2	144.1
PROD	2B1	31.2
BROD	2B1	41.6
MROD	IA2	69.9

Assays for ethoxyresorufin-0-deethylase (EROD), pentoxy-, benzyl- and methoxyresorufin-0-dealkylases (PROD, BROD, & MROD) were conducted using a modification of the methods of Burke, et al., *Biochem Pharm* 34:3337, 1985. Fold-inductions were calculated as the ratio of the sample vs. uninduced specific activities (SA's). Control SA's (pmoles/min/mg protein) were 3.9, 2.2, 8.3, & 2.7 for EROD, PROD, BROD and MROD, respectively.

**BIOASSAY:**

## - TEST FOR THE PRESENCE OF ADVENTITIOUS AGENTS

Samples of S-9 were assayed for the presence of contaminating microflora by plating 1.0 ml volumes on Nutrient Agar and Minimal Glucose (Vogel-Bonner E, supplemented with 0.05 mM L-histidine and D-biotin) media. Triplicate plates were read after 24 - 48 h incubation at 37°C. The tested samples met acceptance criteria.

## - PROMUTAGEN ACTIVATION

No. His+ Revertants	
EtBr/ CPA/	
<u>TA98</u> <u>TA1535</u>	
407.2 1114	

The ability of the sample to activate ethidium (EtBr) and cyclophosphamide (CPA) to intermediates mutagenic to TA98 and TA1535, respectively, was determined according to Lesca, et al., *Mutation Res* 129:299, 1984. Data were expressed as revertants per µg EtBr or per mg CPA.

Dilutions of the sample S9, ranging from 0.2 - 10% in S9 mix, were tested for their ability to activate benzo(a)pyrene (BP) and 2-aminoanthracene (2-AA) to intermediates mutagenic to TA100. Assays were conducted using duplicate plates as described by Maron & Ames, (*Mutat Res* 113:173, 1983).

µl S9 per plate/number his<sup>+</sup> revertants per plate

<u>Promutagen</u>	<u>0</u>	<u>1</u>	<u>5</u>	<u>10</u>	<u>20</u>	<u>50</u>
BP (5 µg)	120	171	287	330	469	777
2-AA (2.5 µg)	119	450	2055	2219	2085	1225

**MOLECULAR TOXICOLOGY, INC.**

157 Industrial Park Dr.

Boone, NC 28607

(828) 264-9099

**Addendum 5:**  
**Certificate of Analysis**

Unité par : 2123882      INGRECIA      11-204 18/07/00 16:271 Pg: 1/1

21238002



1 - JULI

Télécopie	
A: M...	FABRICATION
De: M. DEMAGNY	Nb de Pages: 2
Date: 18/07/00	

Certificat d'analyse

Le 13/07/00

ING911

Lot : ING 15 Z 102

Humidité : 2.5 %

MAT : 74 %

- Germes totaux : 1000 / g
- Coliformes : Abs/ g
- E. Coli : Abs/g
- Clostridium SR : 0/g
- Staphylococcus Aureus : Abs/g
- Salmonelles : Abs/25 g
- Levures - Moisissures : 0/g

